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MOLECULAR DIVERSITY TECHNIQUES IN THE STUDY OF ALZHEIMER'S DISEASE

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Abbreviations used in the test

aa	amino acids
Aß	β-amyloid peptide
AD	Alzheimer's disease
Amp	ampicillin, working concentration of 100 µg/ml
AP	alkaline phosphatase
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
bp	base pair
cDNA	complementary DNA
cfu	colony forming units
DMSO	dimethylsulfoxide
dNTP	deoxynucleotide tri phosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
HCV	hepatitis C virus
HDL	high density lipoprotein

HRP	horseradish peroxidase
HSV	herpex simplex virus
IPTG	isopropyl-thio-β-D-galactoside
IsoAA	isoamyl alcohol
Kan	kanamycin , working concentration 25 μg/ml
Kb	kilobase
KD	kiloDalton
LB*	Luria-Bertani media
LDL	low density lipoprotein
MAP	microtubules associated protein
MPBS	dryed skim milk in PBS (2-5% w/v depending upon the experiment)
MPBST	dryed skim milk in PBST (2-5% w/v depending upon the experiment)
MOI	multiplicity of infection
MTBS	dryed skim milk in TBS (5% w/v)
MTBST	dryed skim milk in TBST (5% w/v)
MRI	magnetic resonance image
NFTs	neurofibrillary tangles
ON	over night
ORF	open reading frame
PAGE	polyacrilamide gel electrophoresis
PBS*	Dulbecco's phosphate buffer saline
PBST	PBS + Tween 20 (0.05-0.1% v/v depending upon the experiment)
PEG	polyethylene glycol 8000

pfu	plaque forming units
PHFs	paired helical filaments
rcf	relative centrifugal force
rpm	round per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SLB*	SDS-PAGE loading buffer
TE*	tris-edta
TBS*	tris buffered saline
TBST	TBS + tween 20 usually at 0.05%-0.1% v/v
TMB	3,3', 5,5'-Tetramethylbenzidine (SIGMA)
Tet	Tetracycline, working concentration 15 ug/ml

*See Materials and Methods for exact composition.

Abstract

In this thesis, I have applied a number of molecular diversity techniques, to the study of Alzheimer's disease (AD). In particular, I have investigated the nature of the epitope of an antibody, MN423, which is highly specific for the intracelullar tangles found in AD, and have attempted to derive antibodies specific for the different isoforms of tau, a protein important in microtubule organization and AD.

MN423 recognizes a truncated form of Tau protein that is structurally constrained in Paired Helical Filaments (PHFs) from Alzheimer brain. In this thesis I attempted to identify the epitopes recognized by MN423 using a number of different techniques. First, by screening two cDNA expression libraries derived from AD, clones with sequence similarity to the C terminus of truncated tau were identified. No other clones which may explain the exquisite sensitivity of this antibody for AD brain were found. On the basis of this, and previous work, which identified the truncated tau epitope as being C terminal, the attempt was made to create a C terminal peptide library based on filamentous phage. This was unsuccessful for reasons described in the thesis, and may explain why a commonly cited cDNA display system (pJuFo) has not been widely used.

Shortly after this work was carried out, a novel phage display vector based on lambda phage which has a random peptide library displayed at the C terminus of pD, was described. This library was used to identify a motif, consistent with the truncated tau sequence, and also confirmed in small diversity expression libraries prepared in the

context of truncated tau. Both methods come to similar conclusions, showing an absolute requirement for a glycine at position –3 and preference for an alanine at position –2. However, further analysis of some of the identified peptides showed that selected peptides may have very different affinities.

Bearing in mind the importance of tau in AD, and the fact that it has six different isoforms created by alternative splicing, I also attempted to derive isoform specific phage antibodies, with a final goal of precisely determining the ratio between different isoforms of Tau in AD versus normal brain. This was attempted in two different ways. One was to design peptides specific for regions which were isoform specific. While the results of this approach were not conclusive, polyclonal phage antibodies recognizing some of these peptides and their corresponding tau proteins were derived. The other approach was to utilize schemes of subtractive selections or depletive selections using two or more Tau isoforms. Eventually, after many such attempts, one single chain was isolated that recognizes one of the exons, an amino terminal region of 29 aa, and so was specific for Tau (441) and Tau (410) but not the others.

The AD brain immunoreactivity to the antibody MN423 is the basis for the idea that tau is proteolytically processed, perhaps as a result of early apoptotic processes, during the AD neurodegenerative process. This is consistent with studies which have shown that tau can be processed by apoptotic proteases. With the aim of clarifying this possibility, within the context of a broader pool of observations, a number of tau mutants were created which identified the exact point at which the molecule is effectively processed by a defined apoptotic protease.

1.1 Introduction

Alzheimer's disease: an overview

Alzheimer's disease (AD) is a degenerative debilitating pathology of the human nervous system associated with distinctive neuropathological features leading to progressive and irreversible cognitive decline during mid to late adult life. It is considered, together with vascular dementia (VaD) the most widespread cause of dementia (Fratiglioni, De Ronchi et al. 1999).

In North America and Europe, the proportion of elderly people, relative to the general population is increasing rapidly. AD is thought to affect between 2 and 10% of North Americans and Europeans over 65. The prevalence doubles after this age every 5.1 years, and it was calculated that the number of sufferers will rise to between 5 and 9 million in the US over the next 40 years, reflecting the increase associated with an aging population (Fratiglioni, De Ronchi et al. 1999).

The condition is progressive, with a life expectancy of 3 to 15 years from the time of diagnosis.

Characterising features of Alzheimer's disease include its gradual onset, progressive decline in cognitive but not motor and sensory function, difficulty in learning and retaining new information, and finally aphasia, disorientation, visuospatial dysfunction and impaired judgement.

The economic cost of treating and providing care for those with Alzheimer's was estimated to be between $US 24 and $US 48 billion (K. Alloul and S. Novosel e 1998).

This, of course, takes no account of the emotional cost to the sufferers of this disease, striking the very intimate essence of a human being, and their families, which is beyond any estimation.

The disease took its name from Aloïs Alzheimer, who in 1907, was the first to observe that two types of brain lesions were present in the post-mortem brains of patients suffering from what is now called Alzheimer's disease. These two characteristic lesions, labeled with a silver stain, consisted of abnormal fibrous material in degenerating nerve cells (neurofibrillary tangles), and the presence of extra cellular deposits defined as plaques (senile plaques). These brain lesions are mainly found in the grey matter of cortical brain areas. Alzheimer's report described the case of a patient named Auguste D, who died at the age of 53.

It is possible to divide AD patients into two categories: early and late onset. Early onset AD usually starts when the patient is less that 60 y.o. It comprises different familial forms, has defined genetic causes and accounts for less than 10 percent of all reported cases. Late onset or sporadic AD comprises the majority of cases, has no clearly defined causes and usually appears at ages >60.

General course of the disease

The onset is insidious. Problems of memory, particularly short-term memory, are common early in the course of the disease. This is followed by loss of intellectual

abilities, and forgetfulness, severe enough to disturb occupational or social functioning. Also mild personality changes, such as less spontaneity, apathy and a tendency to withdraw from social interactions, may occur early in the illness. As the disease progresses, problems in abstract thinking or in intellectual functioning develop. The worsening of symptoms is slow but progressive and irreversible.

The individual may begin to have trouble when working on bills, with understanding what is being read, or with organising the day's work. Further disturbances in behaviour and appearance may also be seen at this point, such as agitation, irritability and diminishing ability to dress appropriately.

Later in the course of the disorder serious confusion and disorientation appear, with social disinhibition causing socially inappropriate behaviour, and in the final stage patients become totally incapable of caring for themselves. After an average of 6-8 (min 3, max 15) years from beginning of the disease, death occurs, often from pneumonia or other similar disorders mainly due to the severely deteriorated states of health.

Diagnosis

The most widespread criteria for the diagnosis of probable Alzheimer's disease have been proposed by the National Institute of Neurological an Communicative Disorders and Stroke / Alzheimer's Disease and Related Disorder Association (NINCDS-ADRDA) (Tierney, Fisher et al. 1988). These are based on the following main points:

1) evaluation of dementia based on a neuropsychological test (Blessed dementia score)

2) deficit in two or more areas of cognition

3) progressive decline of memory and other cognitive functions.

4) onset in late adult life

5) absence of other systemic or brain diseases that could explain the cognitive impairment (diagnosis by exclusion).

A family history with AD cases and some laboratory analysis that exploit techniques for brain visualization such as MRI or CT (shrunken brain) can support the diagnosis.

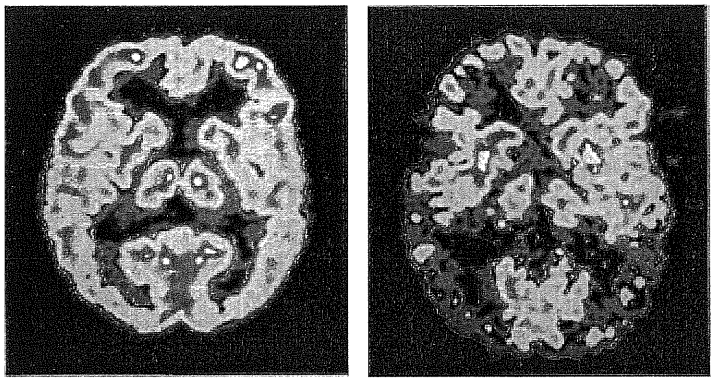
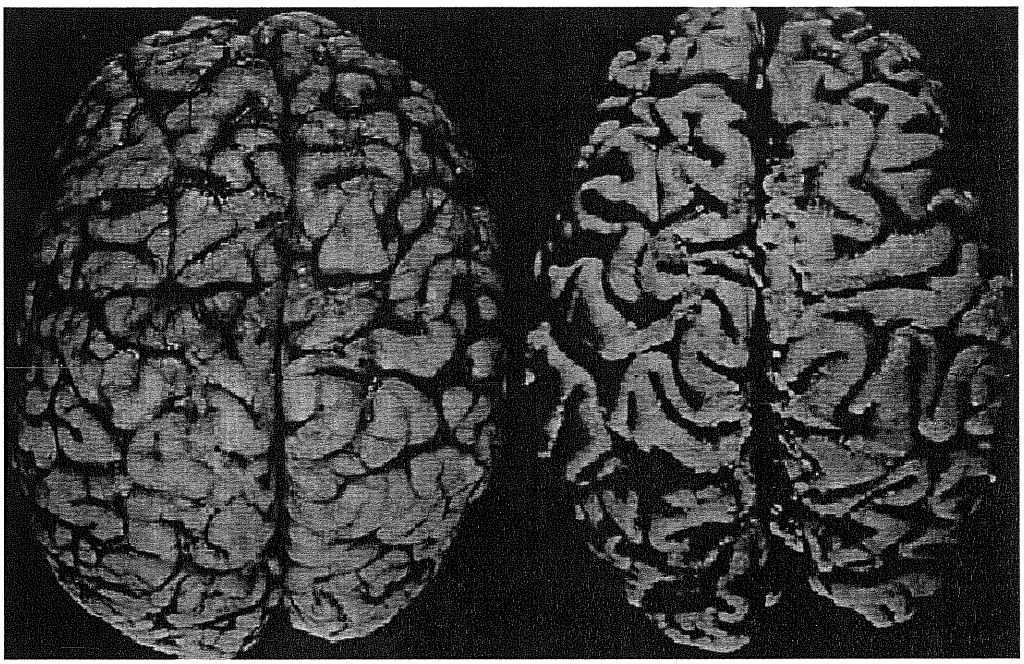


Fig. 1. Brains from AD patients (right in both figures) show reduced metabolic activity (left-shown using MRI) and macroscopic (below - post mortem) differences including brain atrophy and loss of brain substance when compared to a healthy age matched control. (http://library.thinkquest.org /10120/cyber) (Excellent brain images can also be found at http://www.med.harvard.edu/AANLIB/cases/case3/mr1/040.html)



Nevertheless, even following these criteria, Alzheimer's disease remains a heterogeneous clinico-pathological entity and the diagnosis remains probable and not definitive until post-mortem (Braak and Braak 1997).

Moreover, even though big efforts are being made worldwide to find a detectable biochemical marker of the disease, so far no reliable clinical test is available.

Neuropathological diagnosis

A post mortem neuropathological diagnosis is based on the presence of both neuritic plaques and neurofibrillary tangles in the neocortex and/or limbic regions (Braak and Braak 1997). AD related changes involve predominantly the functionally superior areas of the brain.

The formation of Alzheimer-related lesions, occurs in a progressive predictable fashion, starting in specific sites of the brain. Three stages have been identified in amyloid plaque deposition and six for the appearance of neurofibrillary lesions in aged non-selected brains (Braak and Braak 1997). The first regions to be involved in the formation of plaques are the entorhinal and perirhinal cortex, with lesions then spreading to the hippocampus and neocortex. In the final stages, deposits are found in all areas of the cortex as well as in myelinated areas, which usually have a lower lesion burden. Neurofibrillary tangles, start in the entorhinal cortex and spread in a predictable fashion across the cerebral cortex. Poorly myleniated areas show more neurofibrillary changes than those rich in myelin.

Senile Plaques

Masses of fine filaments form in the extra cellular space within the brains of AD patients, the major component of which is a 39-43 (~4kDa) aa long peptide, the β-amyloid peptide, which is derived from the larger β-amyloid precursor protein (APP) (Gandyab and Petanceskaa 2000). In AD, these insoluble formations occur diffusely in the parenchyma as well in more compact and spherical structures known as plaques, some of which have a dense inner core. The complexity of these lesions is not yet completely clarified. Together with the main component, in fact, many other molecules, proteinaceous and non, have been found to be present in these lesions. The plaques can be associated with non-neuronal cells and abnormal, dystrophic neuritic processes. The latter are referred to as neuritic plaques.

Neurofibrillary tangles

In AD profound cytoskeletal changes appear in neurons and their processes. Abnormal, highly insoluble filamentous structures, known as paired helical filaments (PHFs), and straight filaments participate in the formation of tangle like structures inside the cell bodies of neurons as well as of neuritic structures near the tangle bearing neurons. These structure are so insoluble and resistant to the action of proteases, that so called "ghost" neurofibrillary tangles remain intact in the extra cellular space, following the complete degeneration and disappearance of the affected neuron.

Fig. 2. Immunostaining from an AD patient postmortem brain using MN423 antibody. The two hallmarks of the disease, neurofibrillary tangles – the flame like and thread like structures,and plaques – the round diffuse staining structures, are heavily stained with this antibody. (from Michal Novak D.Sc. thesis)

Aetiology

Genetic causes of AD

So far, several genes have been identified that together appear to cause most of the early onset familial forms of the disease, whereas the ε4 allele of the apolipoprotein E (apoE) gene has been shown to be a significant risk factor for the late onset forms of AD. In total, there are four genetic loci predisposing to the disease: the Amyloid Protein Precursor gene (APP) on chromosome 21, the apolipoprotein E gene on chromosome 19 and the Presenilin 1 and 2 (PS-1 and PS-2) genes on chromosome 14 and 1 respectively.

About 120 families with the familial form of AD (FAD) have been carefully studied, and of these, the PS-1 gene accounts for 70% of the disease-causing mutations (about 10% of all AD cases).

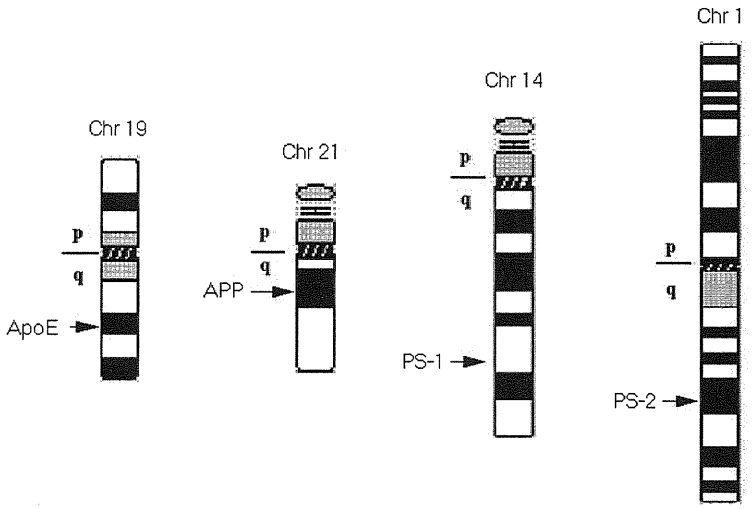


Fig. 3. Chromosomic location of four genetic loci known to be involved in Alzheimer's disease.

Amyloid precursor protein

Mutation in the APP gene on chromosome 21 affect about 25 known families world wide.

It is known that Down's syndrome (chromosome 21 trisomy) patients, approaching middle age, begin to show pathological changes - plaques and tangles - similar to Alzheimer's disease (Silverman, Popovitch et al. 1993).

Mutations in the gene encoding the amyloid precursor protein cause autosomal dominant early onset AD (Rossor, Newman et al. 1993; Mullan, Bennett et al. 1995). Cleavage of APP by β and γ secretase generate the amyloid β peptide (Citron, Oltersdorf et al. 1992), the main component of plaques. The disease causing mutations are found near the secretase cleavage site in APP and facilitate this processing (Tamaoka, Odaka et al. 1994; Tanzi, Kovacs et al. 1996; Selkoe 1998; St George-Hyslop 2000), resulting in either increases in the levels of the ßAP peptide, or increases in the ratio of the longer (42/3 amino acid) form with respect to the shorter (40 amino acid) form (Yang, Knauer et al. 1995). It is the longer form of the ßAP peptide which is thought to give problems related with AD, since it has a greater tendency to form plaques. (Morelli, Prat et al. 1999)

There is one gene for APP which can give rise to six different proteins by mean of alternative splicing of three exons, these isoforms being 770, 751, 714, 695, 563 and 365 aa long (Sandbrink, Masters et al. 1994; Sandbrink, Masters et al. 1996).

A schematic view of APP and secretases cleavage sites is shown below

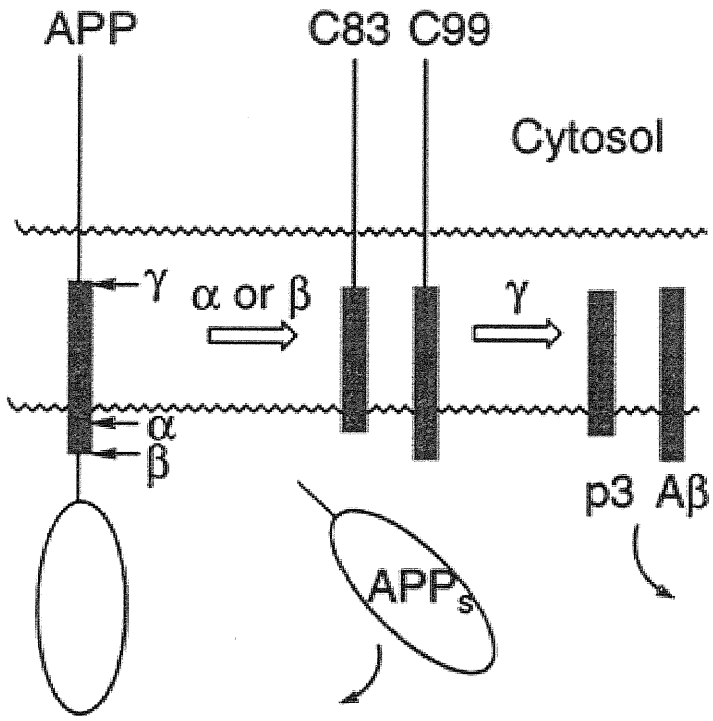


Fig. 4. Scheme of APP proteolitic processing. Redrawn from (Wolfe, Xia et al. 1999).

The heterogeneity of the APP proteins is further increased by various post-translational modifications such as N- and O- glycosylation, phosphorylation and proteolytic cleavage (Godfroid and Octave 1990; Pahlsson, Shakin-Eshleman et al. 1992; Tomita, Kirino et al. 1998). APP is a trans-membrane protein with a large extra-cellular domain and short cytoplasmic tail. The function of APP proteins and derived fragments is not known, but a role in synaptogenesis during brain development as well as cell growth modulators has been suggested (Clarris, Key et al. 1995; Apelt, Schliebs et al. 1997; Chen, Patel et al. 1997; Mattson 1997). They can form complexes with extracellular proteases which are internalised via the ApoE receptor LRP (low density lipoprotein receptor-related protein) (Haas, Cazorla et al. 1997; Pillot, Goethals et al. 1999; Hyman,

Strickland et al. 2000). APP knock out mice have been produced by homologous recombination. Mice are fertile and do not show overt abnormalities up to 12 weeks of age. Studies of the brain did not reveal significant differences in the knockout mice as compared to the wild-type controls. These results show that APP is not essential at least in mouse embryonic and early neuronal development (Zheng, Jiang et al. 1996). APP is processed in the same way as the Notch family receptor (Levitan and Greenwald 1995; Wolfe, De Los Angeles et al. 1999). It is first cut by β-secretase, producing an extracellular soluble domain. Then γ-sectretase catalyzes an intramembranous cleavage of this membrane associated fragment, producing the Aß peptide. It is not clear whether Aß has a biological function or is just a by-product.

It is produced during normal cellular metabolism and can stimulate inflammatory response from microglia. Moreover the Aß peptide inhibits neurite outgrowth, is neurotoxic at high concentrations (Galdzicki, Fukuyama et al. 1994; Abe and Kimura 1996; Hertel, Hauser et al. 1996) and activates protein phosphorylation of MAPs, including tau (Greenberg and Kosik 1995).

The Presenilin proteins 1 and 2

Mutations in genes encoding presenilin 1 and presenilin 2 account for the majority of cases of early-onset familial Alzheimer's disease (Lehmann, Chiesa et al. 1997). Presenilins, the proteins encoded by these genes, are homologous integral membrane proteins, with multiple membrane spanning domains. The homologous sel-12 in C. Elegans is a facilitator of Notch signalling during the determination of cell fate in development (Levitan and Greenwald 1995).

A scheme of presenilin-1 is shown below.

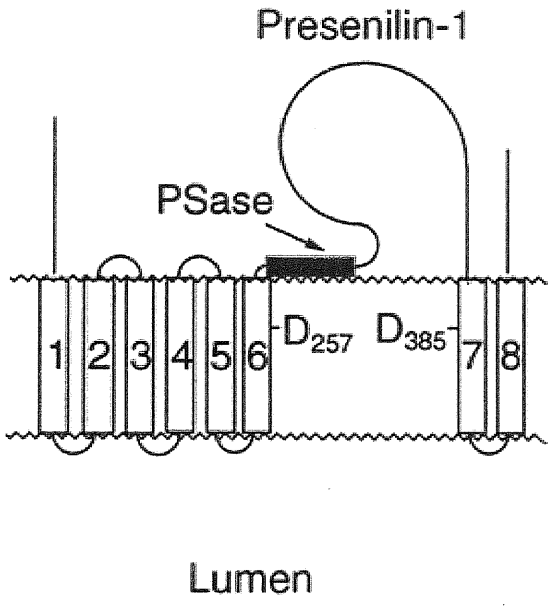


Fig. 5. The Presenilin-1 with its eight transmembrane domains. Redrawn from (Wolfe, Xia et al. 1999).

On the basis of transfection experiments with mutated presenilin genes, it has been proposed that such mutations cause an increase in the production of the longest amyloidogenic (42/3 amino acids long) form of Aß (Tomita, Maruyama et al. 1997), but it is not known by which mechanisms presenilins contribute to this increase.

Three possible roles have been hypothesized for presenilins: 1) they regulate the interaction between γ-secretase and APP, 2) constitute the γ-secretase activity themselves or 3) control the γ-secretase activity. (Hardy and Israel 1999; Steiner, Duff et al. 1999)

Presenilins have been found to be located in endoplamic reticulum (ER) and in the Golgi apparatus(De Strooper, Beullens et al. 1997; Rohan de Silva and Patel 1997; Thinakaran 1999)

A genetic predisposing factor: Apolipoprotein E (Bullido and Valdivieso 2000; Drouet, Pincon-Raymond et al. 2000; Horsburgh, McCarron et al. 2000)

Apo E is a serum protein involved in lipid and lipoprotein metabolism. Three different alleles, ε2, ε3 and ε4, code for three isoforms, E2, E3 and E4. These have an important role in plasma cholesterol homeostasis. ApoE is a lipid transport molecule that is a constituent of very low density lipoproteins, a subclass of proteins involved in cellular uptake of lipids complexes. The ApoE4 allele has normal LDL receptor binding but is associated with elevated plasma cholesterol and LDL levels (Saundersa, Trowersb et al. 2000). In fact ε4 allele bearers have higher plasma levels of LDL-cholesterol when compared to ε3 and ε2 allele bearers, the last showing lower levels. It has been shown that myocardial infarction risk is increased in ε4 bearers. For AD the risk in ε4 bearers has been calculated to be 4 fold increased, while 50% of sporadic late onset Alzheimer's disease patients and more than 65% of familial forms patients carry the ε4 allele in comparison with the 20% present in normal population. Nevertheless, not all ε4 allele bearers get AD (Mortimer, Redgrave et al. 1994; Tanzi, Kovacs et al. 1996; Bullido and Valdivieso 2000). An apoE4 transgenic mice has been generated and tested with different type of diets (Mortimer, Redgrave et al. 1994). Mice with apoE4 had three- to sixfold increases in total plasma apoE, associated primarily with the non-high-density lipoprotein (HDL) fractions of plasma lipoproteins. In response to a high-fat atherogenic

diet the transgenic mice developed hypercholesterolemia similar to that in nontransgenic mice but did not experience the decrease in HDL cholesterol normally observed in control mice. There is increasing evidence that suggests that the association between AD and apoE4 may be linked to the ability of ApoE to interact with the amyloid-beta (Aß) peptide and influence its concentration and structure (Holtzman, Bales et al. 2000; Holtzman, Fagan et al. 2000).

Other factors and hypotheses

Today it is possible to view late onset AD as a multifactorial syndrome rather than a single disease. The clearest clue to this is that more than one genetic defect has been found to cause the disease, and many diverse factors, such as inflammation, head injury and aluminium exposure (see below) are thought to play a role. The second observation is that one of the most important risk factors is ageing. Of course one cannot consider ageing as a unique definite factor, but more likely as a combined cluster of metabolic failures.

Several epidemiological studies have proposed a number of potential risk factors, including biological and environmental (aging, oxidative stress, head trauma, herpes virus, exposure to heavy metals such as aluminium, hyperthyroidism, late maternal age), sociological (depression, emotional stress and level of education) and family history (Down's syndrome, Alzheimer's disease). During the past years, it has become evident that the clinical and histopathological phenotypes of this disease can be caused by a variable superimposition of genetic factors with heterogeneous environmental factors.

Nerve Growth Factor (NGF) in Alzheimer's disease.

A lack of NGF has been suspected to play a role in AD and other neurodegenerative disorders for several reasons: NGF deprivation can be used as way to induce apoptosis (Fraser, McCarthy et al. 1996); NGF plays a protective role on on the basal forebrain cholinergic neurons (afffected in AD) in a surgical model for the loss of axons innervating the hippocampus, (Hefti 1986; Hefti and Weiner 1986); NGF expression mirrors those brain areas affected by AD. All these observations suggest that a decrease in NGF levels (or its receptor) in the aging human brain, (perhaps promoted by other upstream events) could be an important factor in Alzheimer's disease aetiology.

NGF promotes both differentiation and survival of neuronal cells (Levi-Montalcini 1952). In the brain, high levels of NGF are present in regions innervated by cholinergic neurons of the basal forebrain (neocortex, hippocampus, olfactory bulb)(Korsching, Auburger et al. 1985; Korsching and Thoenen 1985; Korsching and Thoenen 1985; Thoenen, Korsching et al. 1985). The highest level of NGF is found in hippocampus and cortex. These are the same regions that are severely affected by the hallmarks of AD, plaques and tangles (Braak and Braak 1991), suggesting that this protein may play a role in the disease.

Recently a transgenic mouse has been reported in which the expression of a neutralising antibody (αD11) against NGF late in brain development, induces a neuropathological picture very similar to AD (Capsoni, Ugolini et al. 2000; Ruberti, Capsoni et al. 2000). This antibody expression has been obtained by crossing two mice strains each bearing complete light or heavy chain immunoglobulin genes under the control of the CMV promoter. One of the resulting mice lines expressed the antibody at very high levels

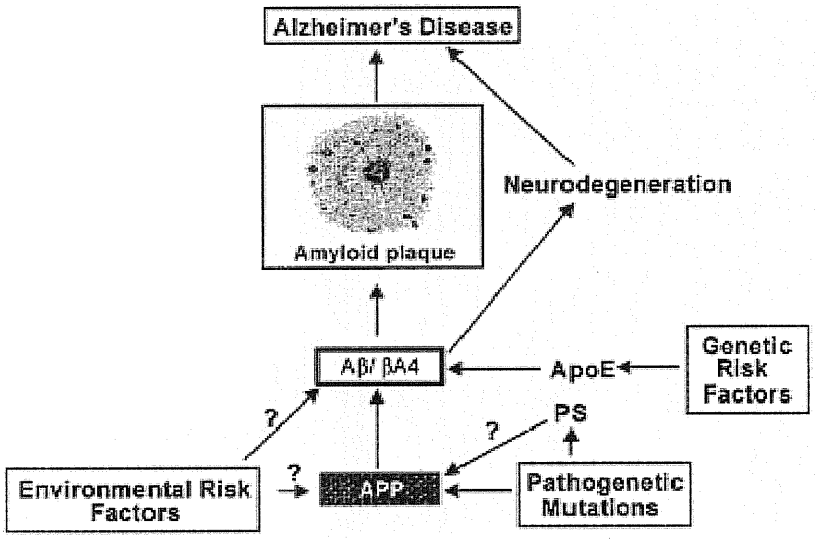
after the second month of birth. As NGF knock out mice die soon after birth (Crowley, Spencer et al. 1994), these αD11 antibody expressing mice are able to develop correctly during this early phase, with NGF activity only being affected after the second postnatal month, simulating in this way an age related decrease of NGF activity. These mice show amyloid plaques, neurofibrillary degeneration, apoptosis, neuronal loss, behavioural deficits and also an inflammatory muscle disorder called inclusion body myositis sometimes linked to AD and presenting muscular fibrillary deposits that share several charactheristics with AD brain lesions (Golabek, Soto et al. 1996; Vogel 1998)

The Amyloid Hypothesis

The amyloid hypothesis imputes increased levels of the longer form of Aß (42-43) as the prime problem in AD. Its accumulation in the brain being thought to cause brain degeneration, plaques, tau phosphorylation, neurofibrillary tangles and all the other hallmarks of the disease.

Low concentrations of Aβ have been reported to be neurotrophic (Storey and Cappai 1999), while high concentrations are toxic (Galdzicki, Fukuyama et al. 1994; Abe and Kimura 1996; Hertel, Hauser et al. 1996). This could suggest that dysfunction in Aβ synthesis during AD may replace a trophic activity with a toxic activity.

Despite the evidence of heterogeneity in AD, it has been suggested that many factors may work through a common pathway by triggering the deposition of Aβ in the brain, which is ultimately responsible for the neuronal degeneration of AD. The hypothesis is depicted in the following scheme (Cappai and White 1999).



The amyloid cascade showing the central role Aβ occupies in AD pathogenesis. The main candidates that could modulate Aβ are shown and they all represent key drug targets. The environmental factors, which are presumably responsible for sporadic AD and hence the majority of all AD cases, represent the biggest unknown.

Fig. 6. The Amyloid hypothesis (from (Cappai and White 1999))

This hypothesis presumes that different gene defects or environmental factors can lead, directly or indirectly, to altered APP expression or proteolytic processing or to changes in Aß stability or aggregation. These result in a chronic imbalance between Aß production and clearance. Gradual accumulation of aggregated Aß initiates a complex, multistep cascade that includes gliosis, inflammatory changes, neuritic/synaptic change, loss of synaptic contacts and formation of neurofibrillary tangles. Several different mutations have been found in the Amyloid Precursor Protein (APP) gene.

The mutations identified in the APP gene together with others found in the presenilin1 1 and 2 genes seem to alter APP metabolism such that more of the insoluble form of Aß

peptide (42/43) is produced. This form aggregates more readily in fibrils than the prevalent form (Aß40). In particular, mutations found in the APP gene occur near secretase cutting sites, thus affecting Aß processing (Tamaoka, Odaka et al. 1994; Lichtenthaler, Wang et al. 1999; Storey and Cappai 1999).

The Amyloid cascade however is considered a controversial theory, primarily because there is a poor correlation between the concentrations and distribution of amyloid depositions in the brain and several parameters of AD pathology, including degree of dementia, loss of synapses, loss of neurons and abnormalities of the cytoskeleton, while the correlation between these parameters and neurofibrillary pathology is far better. And there are also other frequently used counter-arguments. For example, initial NFT changes can occur frequently without the presence of Aß deposits, and on average, they seem to predate plaque formation (Braak and Braak 1991; Braak and Braak 1997). An atypical AD family exists in which very few plaques are found in the brains of patients. Transgenic mice over expressing APP develop almost no neurodegeneration and they show behavioral changes before the formation of plaques (Price, Tanzi et al. 1998; Sturchler-Pierrat and Sommer 1999; Emilien, Maloteaux et al. 2000; Gurney 2000).

Tau microtubule associated protein in the Alzheimer's Disease

In the Amyloid Hypotesis, alterations in tau and the appearance of PHFs and NFTs are considered to be downstream events. In this thesis we will analyze in detail some aspects of the role of the tau protein in Alzheimer's disease that we consider extremely interesting for a possible central role of tau in neuronal degeneration (see chapter 1.2).

Others possible risk factors in AD

Oxidative stress (Behl 1999)

Aging has been described has one of the most important risk factor for AD and radical oxidative species (ROS) are essential in aging processes. ROS are permanently generated to some extent with the reduction of molecular oxygen to water during the normal cell respiration and metabolism. ROS can damage cells via their reaction with macromolecules (Behl 1999).

Compared to other tissues, the brain can be particularly subject to oxidative stress due to the following reasons:

1) normal adult brain has a high glucose metabolism and respiratory turnover;
2) neuronal membranes consist of high concentrations of polyunsaturated fatty acids which are potential substrate for peroxidation by hydroxyl radicals (Halliwell 1989)
3) the brain has lower levels of antioxidant enzymes when compared to other tissues (Halliwell 1989).

Moreover, it has been hypothesized that Aβ can induce the accumulation of hydrogen peroxide in neurons in one or more different possible ways (reviewed in (Behl 1999)).

Therefore, it seems that oxidative stress can be considered, together with inflammatory reactions, an important component of AD pathogenesis.

Aluminium

A possible role of aluminium in AD was suggested by the finding that it was present as a component of plaques (Dukett and Galle 1976; Edwardson, Oakley et al. 1990). Since then, many studies, both toxicological and epidemiological, have indicated that this metal as a possible risk factor for AD. Recently it has been proposed that aluminium, after chronic exposure, e.g. by intake in drinking water, could act on both the homeostasis of brain interstitial fluid as well as alter the permeability of blood brain barrier (Exley 1996; Exley, Burgess et al. 1996).

It has been proposed that aluminum could induce a potentiation of the activities of ATP receptors in the brain. In this hypothesis a cascade of neurotoxic events is started that impairs synaptic plasticity and results in neuronal loss. One possible mechanism is reviewed in (Exley 1999)

Even if the question of the relevance of aluminium to the etiology of AD cannot yet be satisfactorily answered, aluminium is currently regarded as a putative risk factor (Strunecka and Patocka 1999).

Herpes virus

Using PCR based techniques Herpes Simplex Virus (HSV) has been detected in AD brains with a frequency of 74% compared with the 63% found in age matched controls. The presence of this virus has been defined as a strong risk factor when present in Apo-E4 carriers. (Itzhaki and Lin 1998; Itzhaki, Lin et al. 1998; Dobson and Itzhaki 1999) Nevertheless the relation between these two factors has not been fully clarified. A

mechanism has been proposed in which ApoE genotype could influence the adsorption of HSV to lipoproteins reducing or increasing the particles of virus that can infect cells. Nevertheless the full meaning of this relation and the importance to AD is not yet clarified.

Alzheimer and autoimmunity (Singh 1997; Popovic, Caballero-Bleda et al. 1998; Stoll and Jander 1999)

The importance of the contribution of autoimmune processes or inflammatory components in the aetiology and pathogenesis of Alzheimer's disease (AD) has been suspected for many years. Antigen-presenting, HLA-DR-positive and other immunoregulatory cells, components of complement, inflammatory cytokines and acute phase reactants have all been found in tissue of AD pathology (Singh 1997; Popovic, Caballero-Bleda et al. 1998; Stoll and Jander 1999). Although these data do not confirm the immune response as a primary cause of AD, they indicate the possible involvement of immune processes at least at a secondary or tertiary stage in the progression of the disease. The importance of inflammatory processes in AD is bolstered by the finding that anti-inflammatory drugs administered for the treatment of some chronic inflammatory diseases have been shown to reduce the risk of AD in these patients (Delagarza 1998; Behl 1999; McGeer and McGeer 1999). Therefore, it seems that anti-inflammatory drugs and other substances which can control the activity of immunocompetent cells and the level of endogenous immune response may be valuable in the treatment of AD patients (Burke and Morgenlander 1999; Yamada, Ren et al.

1999).

Animal models for AD

Several attempts have been made to try to develop mutant animals that reproduce totally or partially the complexity of the AD syndrome (Higgins 1999).

Even if some functional, molecular and cellular marker of AD have been reproduced, the complete whole complex AD phenotype as observed in humans has not been reproduced. However, even a partial imitation of some aspect of the disease may be useful to begin developing and testing the validity of new drugs that target specific aspects of the disease, especially if they provide some insight into the pathogenetic mechanisms involved in the disease.

The accumulation of amyloid with age has been observed in mice over-expressing mutant APP (Howland, Savage et al. 1995; Moechars, Lorent et al. 1996), while mice with mutant presenilins show an increased production of Aβ42 and form deposits when mutant presenilins are over-expressed (Guenette and Tanzi 1999).

Mice expressing the three different human alleles for ApoE have been produced. Expression of human apoE3, but not of apoE4, protected against neuronal damage. ApoE3, but not apoE4, also protected against the age- dependent neurodegeneration seen in Apoe-/- mice. These differences in the effects of apoE isoforms on neuronal integrity may relate to the increased risk of Alzheimer's disease and to the poor outcome after head trauma and stroke associated with apoE4 in humans. (Buttini, Orth et al. 1999).

A new model for AD has been recently reported (Capsoni, Ugolini et al. 2000) in which transgenic mice expressing a neutralising anti-NGF antibody after the second month of life show a phenotype that strongly resembles AD. In these mice all the Alzheimer's disease neuropathological features are present, including plaque formation, neurofibrillary degeneration as well as behavioural deficits. This model seems to be one of the most similar to the actual disease condition. In fact, while other models reproduce a pathological condition by over-expressing wild type or mutant forms of proteins involved in the disease and reproduce only part of the complex AD phenotype, in this case all the features seem to be reproduced.

Therapy of AD

Many different drugs have been tried for AD with different results. Some of them targets different aspect of the disease, and for some the mechanism of action is not fully understood.

Anti-inflammatory drugs

These types of drugs are directed to slowing down or stopping the inflammatory components of AD since the inflammatory response has proven to be a an important factor during neurodegeneration in AD (Floyd 1999; Akiyama, Barger et al. 2000).
Drugs of this kind, Ibuprofen and Naproxen for example, have been proven to be of some utility in patients at early to middle stage for the disease (Pratico and Trojanowski 2000).

Acetylcholinesterase inhibitors

The neurons primarily affected during the progression of AD are cholinergic neurons. For this reason, drugs that inhibit the catabolism of acetylcholine have been extensively evaluated in AD patients, and some, such as tacrine or donepezil, have proven to be of some effect on patients (Shintani and Uchida 1997; Evans, Ellis et al. 2000)

Neurotrophic factors and related drugs. (Wyman, Rohrer et al. 1999)

A role for NGF in AD has been suggested by the fact that NGF has been shown to prevent degeneration of cholinergic neurons, which are profoundly affected in Alzheimer's disease (Kromer 1987; Koliatsos, Clatterbuck et al. 1991; Davies and Beardsall 1992), and the transgenic mouse model in which anti-NGF antibodies are produced (Capsoni, Ugolini et al. 2000). However, attempts to use NGF by intraventricular injection (not the most suitable of administration routes) has not shown any improvement in AD patients (Olson 1993). Other drugs, like propentofylline (Mielke, Moller et al. 1998) stimulate the endogenous production of NGF.

Estrogens

Several epidemiological studies (Slooter, Bronzova et al. 1999; Mulnard 2000) suggests the use of estrogen in postmenopausal women may delay the onset and/or lower the risk of AD. However, the role is not clearly known yet. It is thought to enhance the growth of neurons in the basal forebrain, and may have a direct effect on Aß since it reduces the

expression of APP (Jaffe, Toran-Allerand et al. 1994; Bonnefont, Munoz et al. 1998; Shi, Panickar et al. 1998). Estrogen may also improve lipoproteins/lipids metabolism (Birge and Mortel 1997) and has a role in neuronal survival during development (Beyer 1999).

A vaccine for AD?

Recently it has been shown that immunization using Aß prevents the formation of plaques in PDAPP mice (mice bearing a mutated human amyloid precursor protein (hAPP717V-->F) minigene under the control of the platelet-derived growth factor promoter, the PDAPP minigene)(Masliah, Sisk et al. 1996). Also it has been shown that in older animals the treatment can reduce the extent and progression of these AD lesions, and in some cases even reverse apparent cognitive decline (Barinaga 1999; Blass 1999; Schenk, Barbour et al. 1999). These observations open the possibility for a vaccine based treatment of AD.

The use of molecular diversity techniques in AD

We believe that phage display technology is a powerful tool for molecular biology. We have used phage display to understand some aspects of Alzheimer's disease, in particular in the study of tau protein. In one of the next chapter, phage display technology and its general issues will be described. Our main objective was to better understand the binding characteristics of an AD specific monoclonal antibody MN423,

which is able to recognize tau derived from PHFs, and to generate monoclonal antibodies using phage display libraries which were specific for different tau isoforms.

1.2 Tau and its relation to AD

Tau is a microtubule associated protein involved in microtubule assembly and stabilisation (Weingarten, Lockwood et al. 1975; Cleveland, Hwo et al. 1977). It has a microtubule-binding and tubulin polymerising activity and is thought to make short bridges between axonal microtubules. In fibroblasts transfected with tau microtubules bundles are formed. In addition, tau-transfected non-neuronal cells extend long axon-like processes in which microtubule bundles resembling those in axons are formed (Kanai, Takemura et al. 1989; Kanai, Chen et al. 1992). It has also been shown that tau antisense oligonucleotides can suppress axonal elongation, in cultured neurons (Caceres, Potrebic et al. 1991; Paglini, Peris et al. 2000). These observations lead to the conclusion that tau function is related to microtubule organization and turnover, cell shape and axonal transport. Tau is found in cells of the central nervous system (CNS) with six well known short isoforms, as well as in the peripheral nervous system (PNS) with higher molecular weight isoforms, due to the presence of an additional large exon (4a) of 254 aa (Georgieff, Liem et al. 1991; Couchie, Mavilia et al. 1992; Goedert, Spillantini et al. 1992).

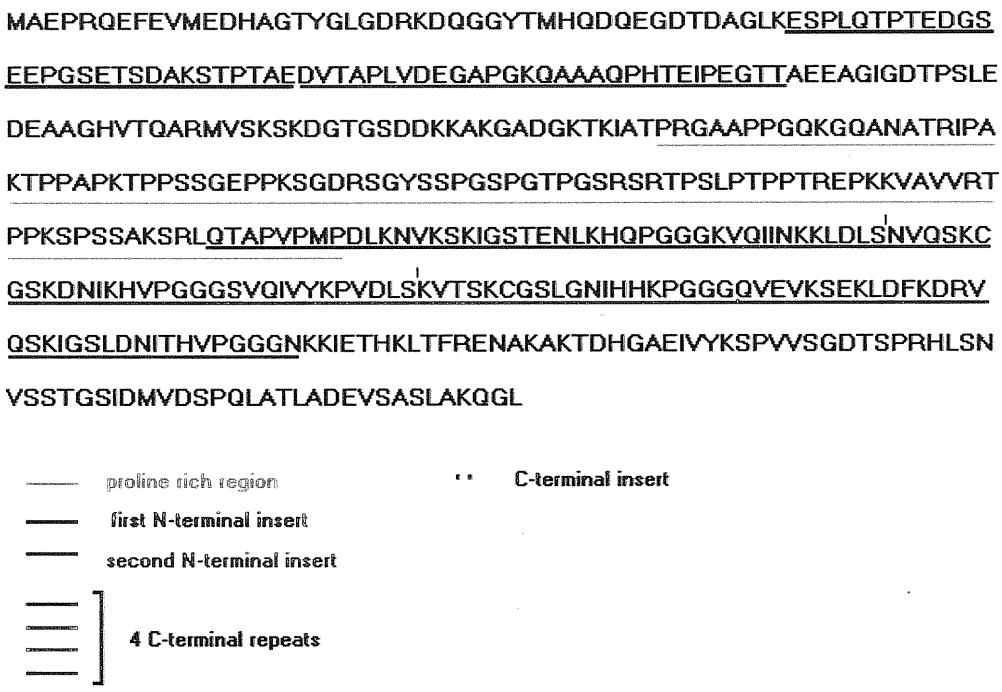


Fig. 7. The amino acid sequence of the longest Tau isoform is shown together with some defined characteristics. The protein has two N-terminal and one C-terminal insert alternatively spliced indicated with blue, turquoise and brackets respectively. A proline rich region is underscored in green. In the C-terminal half four imperfect repeats are found. These correspond to tubulin binding domains. One alternatively spliced exon is present surrounding the second repeat. Tau has not been crystalized.
Phosphorylation sites of tau in phf are: T181, S198, S199, S202, T205, S208, S210, T212, S214, T217, T231, S235, S262, S396, S400, T403, S404, S409, S412, S413, S422.

In adult human brain, six tau isoforms are produced from a single gene located on the long arm of chromosome 17, by alternative mRNA splicing. The isoforms differ from one another by the presence or absence of 29- or 58- amino-acid inserts located in the amino-terminal half and a 31-amino acid repeat located in the C-terminal region (figure 8.). There are four such repeats, shown to comprise the microtubule-binding region of tau (Aizawa, Kawasaki et al. 1988; Goedert, Wischik et al. 1988; Lee, Neve et al. 1989).

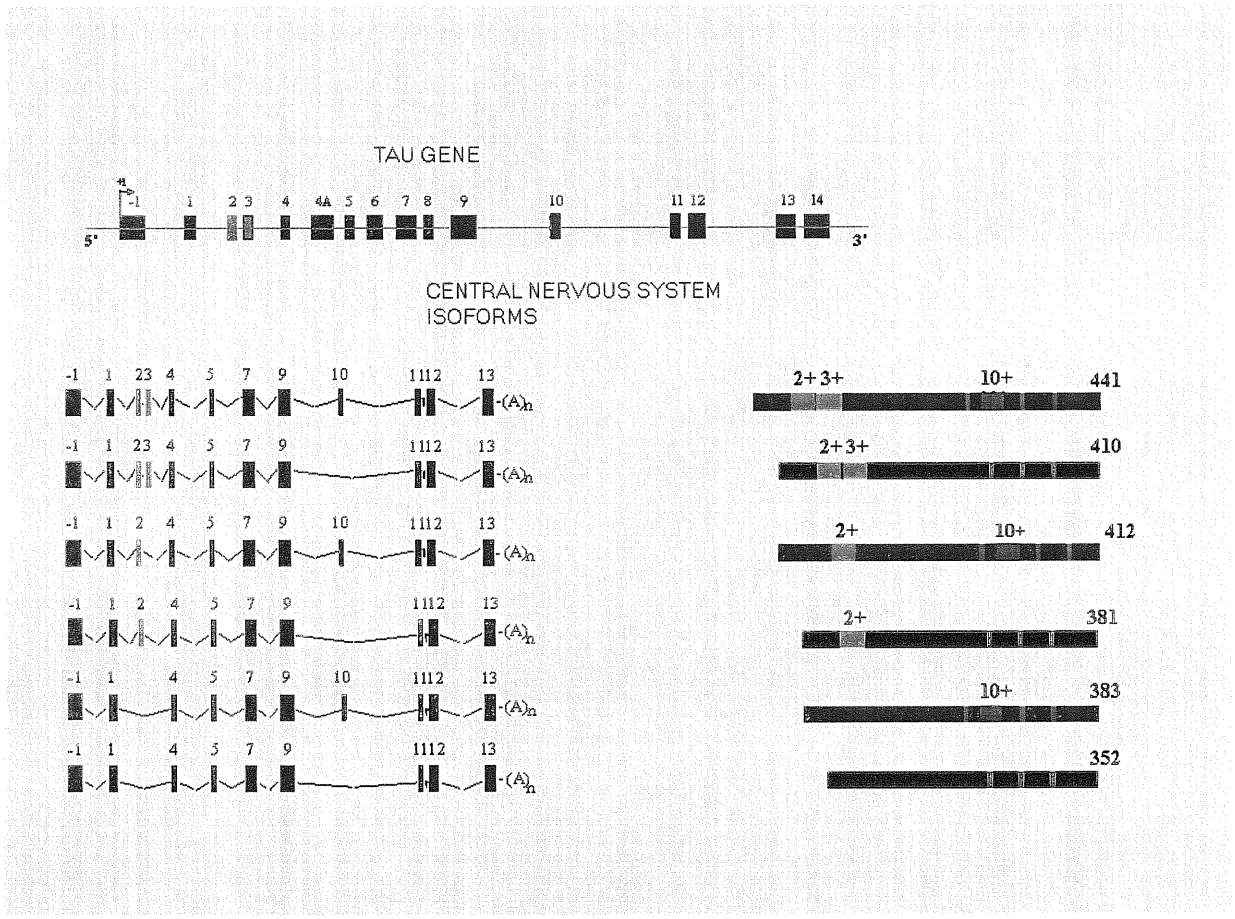


Fig. 8. The tau gene together with mRNAs and proteins expressed in CNS (Goedert, Spillantini et al. 1989; Goedert and Jakes 1990; Andreadis, Brown et al. 1992). Exons 2, 3 and 10 are alternatively spliced giving rise to 6 different isoforms. On the right is shown the length in aa of the various isoforms.

Interest in tau has especially increased after the discovery that one of the two major histopathological hallmarks of Alzheimer's disease, neurofibrillary degeneration and tangles consists of intraneuronal deposits of paired helical filaments made of hyper phosphorylated tau (Ihara, Nukina et al. 1986). Abnormal deposition of tau is not only present in AD but has also been observed in a variety of other neurodegenerative disorders including frontotemporal dementia, progressive supranuclear palsy (PSP), corticobasal ganglionic degeneration (CBD), Down's syndrome and dementia

pugilistica. Variability in the tau gene has been shown to be a risk factor for PSP (Conrad, Andreadis et al. 1997; Spillantini and Goedert 1998). Recent studies have shown that mutations in the coding and non-coding regions of tau are directly associated with the development of familiar fronto temporal dementia (Clark, Poorkaj et al. 1998; Grover, Houlden et al. 1999; Varani, Hasegawa et al. 1999), as well as other brain diseases, summarized in tab. 1-2 (The updated table can be found at http://www.alzforum.org/members /research/tau/tau_mutations.html). These are cues, together with the observation that tau aggregates in the neocortex are always associated with cognitive impairment, that gave a good reason to focus on this protein as a possible important factor for neurodegeneration in AD.

Mutation & Exon	Effect on Exon 10 splicing & Biochemistry	Clinical	Neuropathology	References
K257T	N/a	n/a	Pick bodies present.	http://www.alzforum.org/members/research/tau/tau_mutations.html
I260V	N/a	n/a	n/a	http://www.alzforum.org/members/research/tau/tau_mutations.html
G272V exon 9	All isoforms.	HFTD2; "familial Pick's disease." Onset 46.5 years; death 54.7 years; duration 4-16 years. Presenting symptoms: disinhibition, incl. Aggression, and/or obsessional behavior; later hyperorality, roaming, restlessness, speech loss.	Frontotemporal lobe atrophy; neuronal loss in hippocampus and caudate nucleus."Ballooned cells"in cortex and basal ganglia. Tau+ inclusions in multiple cortical and subcortical areas; granule cells in the dentate gyrus with unique 'Pick-like' inclusions	Hutton, et al. Heutink, et al. Spillantini, et al. Schenk VWD, Ann Hum Genet 1959, 23:325-333.
N279K exon 10	All isoforms, inclusions are mainly 4-repeat. Increased splicing of exon 10	Pallido-ponto-nigral degeneration (PPND). Onset 43 (32-52); duration 9 (5-19); death at 52 (46-63). Present with parkinsonism or parkinsonism with personality change, progressing in 2 years to dementia and supranuclear gaze disorder.	Mild F-T atrophy. Depigmentation of substantia nigra and changes in globus pallidus and caudate. Widespread neuronal and glial fibrillary tangles, composed of ptau in twisted and straight filaments. Tau isolated from the insoluble fraction is primarily 4 repeat.	Clark, et al. D'Souza, et al. Reed, et al. Hasegawa, et al. Hong, et al.

delK280 exon 10	3-repeat tau only? Reduces splicing in exon trapping assays. Strong effect on both microtubule binding and tau aggregation, hence mechanism is uncertain.	Mutation seen in a single Dutch FTD patient.	n/a	Rizzu, et al. D'Souza, et al.
L284L exon 10	Increases 4-repeat tau. Increases splicing	"LKL pedigree." Onset 51.8±4.8; death range 59-71 years. "Variant FTD", word-finding and visual- spatial difficulty; abnormal behaviour.	Bilateral frontal and some temporal lobe atrophy. Amyloid as well as tau deposits.	D'Souza, et al.
P301L exon 10	All isoforms	HFTD1-Dutch family. Onset 50.4; death 59; duration 4-16 years. Presenting symptoms similar to HFTD2, see G272V mutation.	Massive F-T atrophy, balloon cells, degeneration of substantia nigra; no amyloid. Tau+ inclusions: glial and neuronal tangles, irregularly twisted ribbons composed of 4 repeat tau.	Hutton, et al. Heutink, et al. Spillantini, et al. 1996 Hasegawa, et al. 1998 Nacharaju, et al.
P301S exon 10	All isoforms. Markedly reduces ability to promote microtubule assembly (similar to P301L)	"Rapidly progressing disease" in 3rd decade. Two patients in single family, one with FTD and the other with CBD	Extensive filamentous pathology of hyperphosphorylated tau	Bugiani, et al. Goedert, et al. 1999a
S305N (= Ex10-2) exon 10	All isoforms. Enhanced splicing of exon 10, rather than reduced microtubule assembly	Very early onset presenile dementia. Onset 35 (29-38); 2 patients died at 35, 41 years (3 patients described). Presenting symptoms: personality change, impaired cognition, memory loss, minimal parkinsonism.	F-T atrophy (autopsy 1 patient). Glial and neuronal; many unusual ring shaped NFT's composed of straight tubules.	D'Souza, et al. Iijima, et al. Hasegawa, et al.
V337M exon12	All isoforms. Variable region, 4th microtubule repeat.	"Seattle family A." Onset 53 (42-68); death 68 (55-78); duration 12.7 years. "Presenile dementia," antisocial psychotic or belligerent behavior, relatively long disease duration.	F-T atrophy. Neuronal NFT's, "indistinguishable from AD NFTs." All six tau isoforms present in filaments	Spillantini, et al. Poorkaj, et al. Sumi, et al. Hasegawa, et al. 1998
G389R	n/a	n/a	Pick bodies present.	http://www.alzforum.org/members/research/tau/tau_mutations.html
R406W exon 13	All isoforms	"Iowa family" autosomal dominant dementia with widespread NFTs. Onset 55 (45-75); generally long-lived. Insidious onset of dementia with memory loss, personality change. A few with parkinsonism and gaze disturbance.	Mild symmetric F-T atrophy; severe atrophy of hippocampus with abundant NFT's and pallor of substantia nigra with dense NFT's. Amyloid absent. Tau+ inclusions: neuronal, PHFs. Note: Pathology meets criteria for PSP.	Hutton, et al. Reed, et al., 1997 Hasegawa, et al. 1998
3'Ex10+3, GtoA intron 3' of exon 10	4-repeat tau; mutation enhances exon 10 splicing	Onset 49; duration 10 years. Dementia, generalized bradykinesia and rigidity, superior gaze palsy. Similarities to PS and CBD.	Diffuse atrophy of cortical, subcortical and brain stem nuclei. Neural and glial tau pathology composed of twisted ribbonlike filaments of 4-repeat tau	Spillantini, et al., 1997 Spillantini et al., 1998
3'Ex10+16 intron 3' of exon 10	4-repeat tau; mutation enhances exon 10 splicing	Onset 53 (39-66); duration 4-15 years. FTD "Australian" pedigree (2) and others (15); PSG (10). Early personality change, loss of executive function, progressive dementia, loss of speech.	FT atrophy. Neuronal and glial tau pathology composed of wide, twisted ribbonlike filaments of 4-repeat tau	Baker, et al. Goedert, et al. 1999b Hutton, et al.
3'Ex10+14 intron 3' of exon 10	4-repeat tau; mutation enhances exon 10 splicing	Onset 45; duration 13 years. Personality change, disinhibition progressing to withdrawn behavior and parkinsonism; amyotrophy in a few.	FT atrophy and depigmentation of substantia nigra. Ballooned neurons with tau staining.	Hutton, et al. Lynch, et al.
3'Ex10+13	4-repeat tau deposited in tangles. Enhances exon 10 splicing	n/a	n/a	Hutton, et al.
3'Ex10 - 2, G to A (aka S305N)	See S305N.	See S305N.	See S305N.	See S305N.

Tab. 9. Mutations found in Tau gene. Modified from (http://www.alzforum.org/members /research/tau/tau mutations.html)

Even if recent studies (Roks, Dermaut et al. 1999) exclude correlation between mutations and polymorphisms in the tau gene and AD, there are reasons to think that at least at some stage of AD pathogenesis, tau has an early role in the neurodegenerative processes. For example, some connection seems to exist between tau phosphorylation and the toxicity of beta amyloid. In a recent study it has been shown that inhibition of the protein kinase cdk5, a member of the tau protein kinase II system (TPK II), known to phosphorylate Tau protein, prevents beta-amyloid induced neuronal death (Alvarez, Toro et al. 1999). Moreover, we will see later how several observations relate tau to neuronal apoptotic events.

Tau is present mainly in nerve cell axons and is thought to have a role in neuronal cell morphogenesis and especially in axonal elongation and maintenance. However, gene knock out experiments show that tau does not appear to be an essential protein, at least in mice, since the nervous system of tau-deficient mice appears to be normal immuno histologically and axonal elongation is not affected in cultured neurons. Only in some small-calibre axons, microtubule stability is decreased and microtubules organisation is changed (Harada, Oguchi et al. 1994).

Tau expression is developmentally regulated since only the shortest isoform is present in foetal brain while all six isoforms are expressed in adult human brain (Goedert, Spillantini et al. 1989; Kosik, Orecchio et al. 1989).

Postranslational modifications of tau play an important role in the function and eventually malfunction of this protein. Tau can be phosphorylated, glycosylated,

glycated, ubiquitinated and truncated. The exact function of all these modifications in the formation of PHFs remains to be fully clarified.

Tau phosphorylation.

Protein phosphorylation is one of the major mechanisms for the regulation of cellular function. At least 20 phosphorylation sites are present in tau. Different kinases and functionally acting in the opposite direction, phosphatases, have been reported to have tau as substrate (Goedert 1993; Gong, Singh et al. 1993; Hall and Yao 2000).

Nowadays, it seems clear that tau phosphorylation is related to its functional state. One of the first observations made on PHF tau was that it was hyperphosphorylated. But another tau fraction in AD brain, whose molar tau/phosphate ratio is equal to that of PHFs, is in a non polymerised state (Grundke-Iqbal and Iqbal 1999). These would lead one to believe that phosphorylation alone is not enough to produce PHFs. Moreover the PHF aggregated state of tau protein seems to be biologically inert, i.e. unable to induce polymerisation of tubulin (Grundke-Iqbal and Iqbal 1999). On the other hand, the phosphorylated tau found in AD is toxic because it can compete with tubulin for binding to normal tau and other MAPs. The sequestration of these proteins results in inhibition of microtubule assembly. It has also been shown that when AD phospho-tau is added to already formed microtubules they are disrupted, a process which can be inhibited by dephosphorylation (Alonso, Grundke-Iqbal et al. 1996; Alonso, Grundke-Iqbal et al. 1997). These results clearly implicate phospho tau in AD, although at which level remains unclear.

Glycation

Glycation is a non enzymatic reaction between the amino group of a side chain of an amino acid and the CHO group of a sugar. Subsequent oxidative processes lead to the production of advanced glycation end products (AGEs). Accumulating AGEs have been related to normal aging and to AD.

AGE's have been shown to be present in NFTs by immunolocalization, suggesting that protein glycation plays a role in AD neurofibrillary degeneration. It has also been demonstrated that glycation can occur in the tau microtubule binding domain and that glycated tau is more prone to form aggregates. (Munch, Thome et al. 1997; Ledesma, Perez et al. 1998; Sasaki, Fukatsu et al. 1998).

Glycosylation

Studies in which protein glycosylation is revealed with immunostaining show that glycosylation colocalizes with both the histological markers of the disease, plaques and neurofibrillary tangles (Wang, Grundke-Iqbal et al. 1996; Takahashi, Tsujioka et al. 1999). As opposed to non enzymatic glycation, glycosylation is an enzymatic process. Whether the glycosylation process is important in the pathogenesis or is a secondary effect of the disease is not known.

Tau truncation

The possibility that tau can undergo specific proteolytic cleavage has been entertained for a number of reasons. MN423, an antibody raised against the PHF core, recognises a truncated form of tau that is a structural core constituent of PHFs (Novak, Wischik et al.

1989; Novak, Jakes et al. 1991). Whether this kind of tau truncation has a physiologic and/or pathologic role, or if this is just a final end product of non specific diffuse proteolytic events in AD post mortem brains has not yet been fully clarified. One possibility is that within the AD brain, or when PHFs are treated with pronase, the proteases are unable to digest tau beyond a point at which the protein structure is constrained in the core in way to expose the GAE ending in position 391. (Novak, Kabat et al. 1993). An alternative view, based on the fact that this proteolytic cleavage of tau is associated with the expression of markers for neuronal death in AD neurons (Ugolini, Cattaneo et al. 1997), is that tau cleavage in AD is an active process mediated by the apoptotic machinery. Although apoptosis in cerebellar granule cells induces tau cleavage (Canu, Dus et al. 1998), there is no indication that cleavage occurs at 391E, and indeed the sequence at this site does not resemble any of the known apoptotic protease cleavage sites. Although cleavage of upstram sites (see chapter 2.2) may predispose tau to further proteolysis.

When PHFs are treated with pronase they retain the characteristic overall morphology. This structure has been termed the protease resistant PHF core. Tau fragments, mapping to the tau repeat region, have been isolated from this core (Novak, Kabat et al. 1993, Novak, 1991 #1483). The antibody MN423, derived by immunizing mice with purified PHF core, has the ability to decorate PHFs in electron microscopy and to give specific immunostaining on AD brain slices which is not found on normal age matched controls (Harrington, Mukaetova-Ladinska et al. 1991; Mena, Edwards et al. 1996). Based on the protein sequences of the 12kDa tau fragments purified from PHF, recombinant tau analogues were constructed. Some of these were recognized by MN423. Each of them

is derived from full length tau by truncation of those isoforms with both three and four repeats. As a result, these truncated products have different N termini, and some terminate at the same C terminal sequence: 387-DHGAE-391 (Novak, Kabat et al. 1993). That this C terminal sequence is the site of recognition was suggested by a number of experiments in which the addition or removal of a single amino acid abolished recognition, and the transplant of the three C terminal amino acids (GAE) to full length tau was sufficient to confer recognition to a form of tau not usually recognized (Novak, unpublished).

Tau protein truncated at position 391 has shown a strongly reduced microtubule polymerizing activity when compared to full length tau (Novak, Kabat et al. 1993). Also one of the tau fragments that is recognised by this antibody, when produced and purified as recombinant fragment has shown to have a strong tendency to aggregate and form polymers, even in the presence of SDS (Novak, Kabat et al. 1993).

Together these observations have lead to the hypothesis that tau could be abnormally truncated endogenously, undergoing conformational changes, and that these conformational changes could be the basis for the formation of tau aggregates and subsequent neurodegeneration. The term 'tauons' (M. Novak) has also been used to describe the protease resistant units formed by tau, obviously referring to possible prion-like spreading mechanisms of the conformational abnormal truncated tau.

Other observations that support this hypothesis are the following:

1) expression of dGAE fragment in cos cells induces apoptosis (Fasulo 1996; Novak 1999) and the fragments form intracellular aggregates, even if not properly tangles.

2) A study has been done in which 423 immunoreactivity has been related to DNA apoptotic fragmentation (Ugolini, Cattaneo et al. 1997), this suggesting that truncation is an early event in the process of neuro-degeneration.

Tau and apoptosis

Tau has been related to apoptotic processes in previous studies (Lesort, Blanchard et al. 1997; Ugolini, Cattaneo et al. 1997; Arendt, Holzer et al. 1998; Canu, Dus et al. 1998; Esclaire, Terro et al. 1998).

Apoptosis is characterised by DNA condensation, fragmentation, cell shrinkage and the formation of apoptotic bodies. Programmed cell death is the physiological way in which organisms get rid of unwanted cells during development (Wang 2000). As opposed to necrosis, that occurs when cells are stressed beyond repair by extreme physical or chemical condition, apoptosis is a well defined pattern of cell "suicide". One key point of this pattern is the activation of specific proteases that are present in the cell in an inactive state and are activated in a cascade of events leading to apoptosis. Caspases are a family of proteases whose first member was discovered in C. Elegans as a protein required for apoptosis. Of these proteases, caspase-3 targets cytoskeletal proteins like gelsolin (Kothakota, Azuma et al. 1997; Geng, Azuma et al. 1998), actin (Kothakota, Azuma et al. 1997), fodrin (Janicke, Ng et al. 1998) and GAS2 (Brancolini, Benedetti et al. 1995; Lee, Tang et al. 1999; Sgorbissa, Benetti et al. 1999) in neuronal apoptosis. Caspase 3 is present as a proenzyme in an inactive state in many cell types, including neurons. It is converted to an active heterodimeric form by two other Caspases, 8 or 9 (Nicholson 1999) (Nicholson and Thornberry 1997). There is evidence that tau is

processed during apoptosis occuring in primary cerebellar neurons treated with low potassium and serum deprivation in vitro (Canu, Dus et al. 1998; Fasulo, Ugolini et al. 2000). In summary tau clearly plays a role in AD. It forms an essential component of NFTs and its posttranslational modifications can strongly affect its function.

1.3 The display technologies

Peptide libraries can be generated in two methodologically different ways: using chemical synthesis or exploiting biological systems. Via chemical synthesis, libraries have been produced on beads, pins or in solution. Biological systems include libraries on bacteria or phage surfaces, on DNA binding proteins or on ribosomes (Hanes and Plückthun 1997; Hanes and Plückthun 1997; Hanes, Jermutus et al. 1998; Hanes, Jermutus et al. 1999). This introduction will deal with the latter.

Phage display technology

Bacterial viruses have played a very important role as molecular biology tools since the beginning of this technology.

Viruses such as λ and M13 have been used in different modified versions as cloning vectors and expression vectors. Bacteriophage λ has provided an excellent cloning system for remarkably long fragments of DNA up to 47 kb (Collins and Hohn 1978; Williams and Blattner 1979; Hohn and Collins 1980; Rimm, Horness et al. 1980; Hohn, Koukolikova-Nicola et al. 1988; Collins and Hohn 1992), together with a vast array of expression vectors extensively used in cDNA library construction and screening. (Short, Fernandez et al. 1988; Sieg, Kun et al. 1989; Palazzolo, Hamilton et al. 1990; Brunelli and Pall 1993; Santi, Capone et al. 2000), while M13 has had extensive use as a single stranded DNA vector for sequencing (Sanger, Nicklen et al. 1977).

Today phage display represents one of the most advanced tools in molecular genetics. Phage display techniques are based on the use of phage particles to expose whole proteins, peptides or antibody fragments in a format suitable for allowing protein-protein interactions, selection and enrichment schemes. (Cabilly 1999; Forrer, Jung et al. 1999; Wittrup 1999; Yip and Ward 1999).

In the most common format, a DNA sequence coding for a polypeptide is genetically fused to one of the M13 phage coat proteins, creating a recombinant coat protein. Expression of the fusion protein and its incorporation into the mature phage results in the peptide being presented on the phage surface.

The key advantage of this technology is that one can deal with a protein or peptide whose coding information is physically bound to it. In other words, there is physical coupling between genotype and phenotype. As the information coding for the recombinant coat protein is packaged within the phage, selection of the phage on the basis of binding activity of the polypeptide displayed on the chimeric coat protein, results in the direct isolation of the DNA sequence coding for that polypeptide. This feature is crucial when dealing with high molecular diversity problems.

Another important advantage is that phages are very small and they can be grown, harvested and manipulated in very large numbers. DNA encoding for millions of variants can be batch cloned as part of one of the phage coat proteins resulting in a very large population of phages, each expressing one variant. The numbers of different phages used in one experiment are typically in the order of millions to billions. These can be selected against a target molecule in a way to isolate that polypeptide which binds the target molecule.

A third advantage is that M13 bacteriophages are relatively tough biological entities. One can perform selection experiments on a broad phage populations exposing them to relatively harsh chemical condition (e. g. phages can survive pHs between 2-12), without losing infectivity. These three factors allow one to perform different selection schemes as well as repeat the selection cycle in order to isolate a relatively pure population of binding phages. Figure10 shows the principle of a phage display selection cycle.

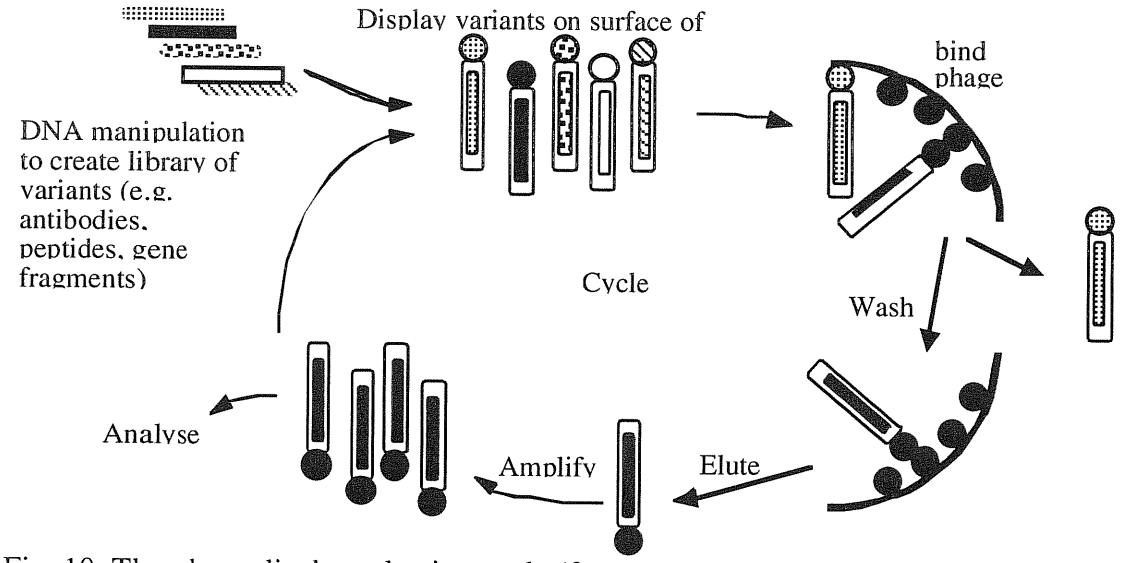


Fig. 10. The phage display selection cycle (from Andrew Bradbury)

Phage display vectors

Several different phage types have been used for phage display, but the most widespread for this purpose is the Ff filamentous phage family. Ff filamentous phages are single-stranded DNA phages that infect bacteria via the F pilus. This family includes M13, f1 and fd phages. The phage genome encodes eleven proteins of which five are coat proteins. The infection occurs utilizing the F pilus as carrier to enter bacteria (Bauer and Smith 1988; Bross, Bussmann et al. 1988; Stengele, Bross et al. 1990). A minor coat protein, g3p, located at the

end of the phage in 3 to 5 copies is involved in the binding to the F pilus and in the infection process (Holliger, Prospero et al. 1993; Riechmann and Holliger 1997; Lubkowski, Hennecke et al. 1998). After binding, the infecting single-stranded DNA is carried into the bacteria, converted into ds DNA and subsequent events, driven by both phage and host proteins, lead to the production of viral genomes and proteins that are carried to the membrane and assembled into rod like viral particles, which are secreted through the bacterial membrane, into the media. Since the viral particles are formed in a dynamic way, assembling g8p around the viral DNA exiting the bacteria, the virion length can vary according to the amount of DNA it contains. At the end of the process filamentous phage appear as a rod about 1 μ long and 6 nm in diameter (Marvin and Hohn 1969; Marvin and Wachtel 1975; Marvin and Wachtel 1976).

In typical filamentous phage display systems the protein or peptide is fused downstream of the leader of either g3p or g8p (Smith 1991; Smith and Scott 1993). The resulting recombinant coat proteins will have exogenous protein/peptide fused to the N-terminus part of the molecule. In addition to which protein is used as the display platform, the vector type (phage or phagemid) is the other main variable. Phage vectors involve modification of the phage genome itself, while phagemid vectors contain a filamentous phage origin of replication (to allow incorporation into particles) as well as the display protein gene (g3 or g8).

The display of a polypeptide on a phage coat protein can be obtained in different ways.

In the so called type 3 or type 8 phage display vectors, g3 or g8 are modified in the phage genome giving rise to phage in which all g3p or g8p bear the exogenous polypeptide. This system can suffer from problems related both to the transfection rate of phage DNA in bacteria, that is rather low, and to the stability of the genome, because phage vectors tend to eliminate

exogenous DNA and so deletions are often observed. Nevertheless, has been successfully applied to peptide display.

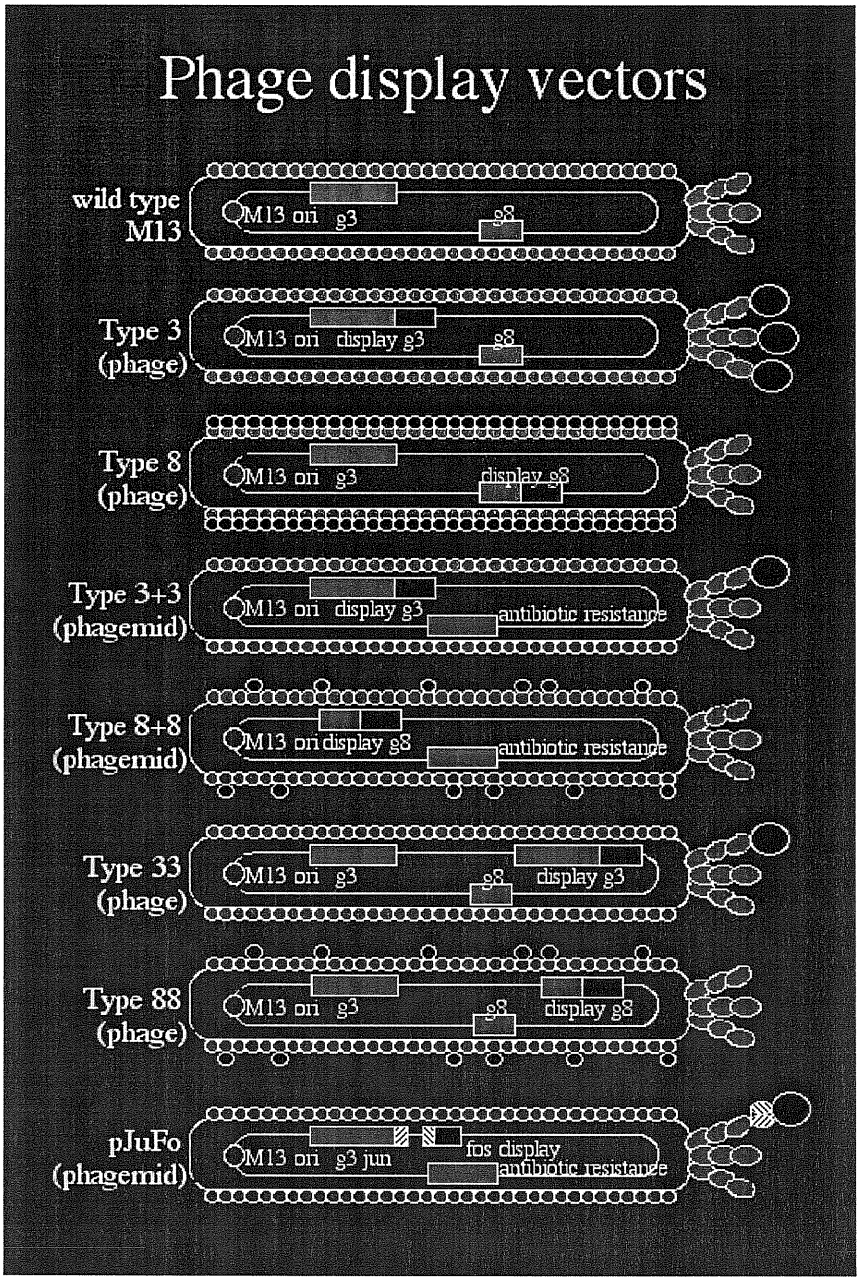


Fig. 11. Different types of filamentous phage display systems (from Andrew Bradbury).

Another possibility is to provide both the recombinant display protein with a wild type coat protein in the same phage vector. These kinds of vectors are called 33 or 88 and reduce problems due to massive expression of sterically bulky fusion proteins on the phage coat, allowing a mix of recombinant + wild type coat protein to be present on the phage surface. Although 88 vectors have been described for peptide display (Zwick, Shen et al. 1998, Craig, 1998 #2913), 33 vectors have not been developed. Finally, the 3+3 or 8+8 systems are phagemid systems in which both recombinant coat proteins and wild coat proteins are provided but form different sources. Under normal circumstances phagemid vectors can be grown as plasmids within the bacteria and no phage particles are produced. However, when bacteria bearing such phagemid vectors are infected with helper phage, all the other proteins required for phage replication and production (including wild type g3p) are produced, allowing the production of phagemid particles as well as phage particles. This creates a very heterogenous population of particles containing two different genomes (phage and phagemid) as well as two different forms of the display protein (recombinant and not). The helper phage usually has a defective origin of replication. As a result, the phagemid genome is packaged in preference to the helper phage one. The ratio of recombinant/wild type g3p gives an indication of how good the display level is. Generally the phagemid format allows a broader diversity of displayed peptides, even though the expression level can also vary significantly.

The expression level of a library is generally considered a crucial factor. For example, in the case of an antibody library the expression levels will influence the result of selection experiments and the subsequent purification of the isolated binders.

A typical example of the first widely used phagemid vector is shown in figure12. In this case, the phagemid bears a sequence coding for a ScFv.

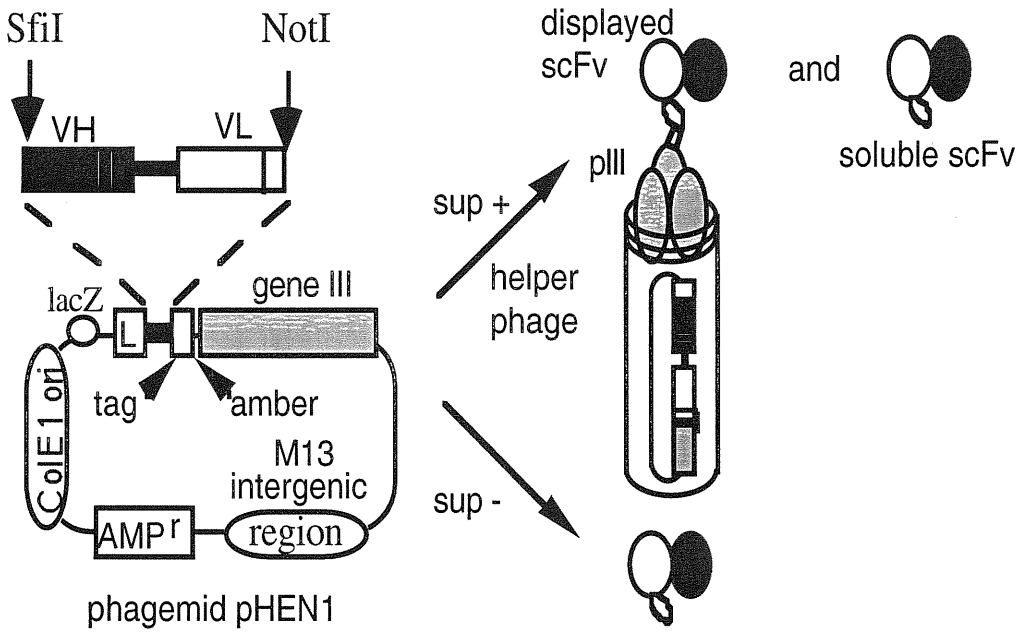


Fig. 12. Phagemid pHEN1 (from Greg Winter)

As a display protein, g3p is generally more permissive when compared to g8p, allowing fusion to whole proteins, while g8p allows the better expression of small peptides.

This difference is probably due to the presence of a max of 5 copies of g3p, compared with about 2700 g8p copies on the phage coat. This high number of copies is likely to impose a sterical constraint to the MW of peptide fused to g8p (Iannolo, Minenkova et al. 1995).

The use of phage display

Whole proteins displayed on phages can be correctly folded retaining their biological activity and their binding properties. Many different kinds of protein have been successfully displayed on phages, including enzymes, antibody fragments, hormones, toxins, growth factors, protein domains and random peptides (See table 13).

Protein displayed	vector type	Reference
CDNA libraries (i.e. random clones)	PJuFo	(Crameri and Suter, 1993; Crameri et al., 1994)
Genomic libraries (Staphylococcus and Streptococcus genomes)	3+3, 8+8	(Jacobsson and Frykberg, 1995; Jacobsson and Frykberg, 1996; Jacobsson and Frykberg, 1998)
Protein fragments		
ß galactosidase	3	(Parmley and Smith, 1988)
Bluetongue virus VP5 and NS1 proteins	3	(Du Plessis et al., 1995; Wang et al., 1995)
p53	3	(Petersen et al., 1995)
RNA polymerase II	3	(Petersen et al., 1995)
Cytokeratin 19	3	(Petersen et al., 1995)
PM/Scl	3	(Bluthner et al., 1996)
ZAG and MAG from streptococci	8+8	(Jacobsson et al., 1997)
Myb	3+3	(Kiewitz and Wolfes, 1997)
PAI-1	3+3	(van Zonneveld et al., 1995; van Meijer M et al., 1996)
Small constrained peptide domains		
Hybrid rop protein constrained peptide library	3	(Santiago Vispo et al., 1993)
Cytochrome b562		(Ku and Schultz, 1995)
Tendamistat constrained peptide library	33	(McConnell and Hoess, 1995)
Protein A (E, D, A and B domains or B domain alone)	3+3	(Djojonegoro et al., 1994; Kushwaha et al., 1994; Nord et al., 1995; Nord et al., 1997)
Knottins	3+3	(Smith et al., 1998)
Protease inhibitors		

Alzheimer's amyloid ß-protein precursor Kunitz domain	3+3	(Dennis and Lazarus, 1994; Dennis and Lazarus, 1994; Dennis et al., 1995)
Human plasminogen-activator inhibitor 1 (PAI-1)	3+3	(Pannekoek et al., 1993)
Kunitz domain libraries, BPTI	3, 88	(Markland et al., 1991; Roberts et al., 1992; Roberts et al., 1992)
Ecotin	3+3	(Wang et al., 1995)
Cystatin	3+3	(Tanaka et al., 1995)
Proteases		
Prostate specific antigen	3+3	(Eerola et al., 1994)
Trypsin	3+3, 33, 8+8, 88	(Corey et al., 1993; Wang et al., 1996)
Enzymes		
Glutathione transferase A1-1	3+3	(Widersten and Mannervik, 1995; Hansson et al., 1997)
Staphylococcal nuclease	3, 3+3	(Ku and Schultz, 1994; Light and Lerner, 1995)
Alkaline phosphatase	pJuFo, 3, 3+3	(McCafferty et al., 1991; Crameri and Suter, 1993; Maenaka et al., 1996)
ß lactamase	3	(Soumillion et al., 1994; Vanwetswinkel et al., 1995)
Lysozyme	3+3	(Maenaka et al., 1996)
Inactive phospholipase A2	PJuFo	(Crameri and Suter, 1993)
Cell surface receptor fragments		
CD4, e.c. and individual domains	3, 3+3	(Chiswell and McCafferty, 1992; Abrol et al., 1994; Krykbaev et al., 1997)
FceR1 a-chain, e.c. and individual domains	3, 3+3	(Robertson, 1993; Scarselli et al., 1993)
PDGF receptor, e.c domain	3	(Chiswell and McCafferty, 1992)
Hormones, interleukins and bioactive peptides		
Human growth hormone	3+3	(Bass et al., 1990; Lowman and Wells, 1993)
C5a	PJuFo	(Hennecke et al., 1997)
Thymosin ß4		(Rossenu et al., 1997)
Transforming growth factor alpha	3	(Tang et al., 1997)
Tumour necrosis factor	3+3	(Clackson and Wells, 1994)
Interleukin-2 (IL-2)	3+3	(Buchli et al., 1997; Vispo et al., 1997)
Interleukin-3 (IL-3)	3, 3+3	(Gram et al., 1993; Merlin et al., 1997)
Interleukin-6 (IL-6)	3+3	(Cabibbo et al., 1995)

Interleukin-8 (IL-8)	3+3	(Clackson and Wells, 1994)
Insulin like growth factor (IGF) binding protein	3+3	(Lucic et al., 1998)
Heregulin beta domain	3+3	(Ballinger et al., 1998)
Epidermal growth factor (EGF)	3	(Souriau et al., 1997)
Ciliary neurotrophic factor	3+3	(Saggio et al., 1995)
Atrial natriuretic peptide and derivatives	3+3	(Cunningham et al., 1994; Li et al., 1995; Jin et al., 1996)
Bone morphogenetic protein (2A)		(Liu et al., 1996)
Antibodies and derivatives		
Minibody (61aa, 4 beta sheets)	3, 3+3	(Pessi et al., 1993; Martin et al., 1996)
Fab	3, 3+3, 8+8	(Hoogenboom et al., 1991; Kang et al., 1991; Huse et al., 1992; Orum et al., 1993; Geoffroy et al., 1994; Griffiths et al., 1994)
ScFv	3, 3+3, 8+8	(McCafferty et al., 1990; Clackson et al., 1991; Marks et al., 1991; Marks et al., 1992; Griffiths et al., 1993; de Kruif et al., 1995; Kretzschmar and Geiser, 1995; Vaughan et al., 1996)
CH3	3+3	(Atwell et al., 1997)
VH domains	3+3	(Davies and Riechmann, 1996)
Dromedary heavy chain antibodies	3+3	(Lauwereys et al., 1998)
Toxins		
Ricin B-chain, complete or domain 2	3	(Swimmer et al., 1992; Lehar et al., 1994)
Fungal ribotoxin reAsp f I/a	3+3	(Crameri and Suter, 1993)
B. thuringiensis CryIA(a) toxin	3+3	(Marzari et al., 1997)
Nucleic acid binding proteins		
HIV tat	3+3	(Hoffmann and Willbold, 1997)
U1A protein	3+3	(Laird-Offringa and Belasco, 1996)
Zinc finger proteins, domains and libraries	3, 3+3	(Choo and Klug, 1994; Choo and Klug, 1994; Jamieson et al., 1994; Rebar and Pabo, 1994; Choo and Klug, 1995; Wu et al., 1995)
Miscellaneous		
T cell receptor alpha chain	3	(Onda et al., 1995)
Peptostreptococcal protein L		(Gu et al., 1995)
Pseudomonas aeruginosa protein F	3	(Kermani et al., 1995)

Table 13. Proteins displayed on filamenous phage (modified from Andrew Bradbury)

Phage display was first carried out using peptide libraries (Smith 1985), with the goal of identifying monoclonal antibody epitopes, but since then, the number of proteins displayed on phage has expanded enormously (see table 13) and its present application can be essentially reduced to three general categories:

1) peptide display: used to identify epitopes binding to antibodies or other proteins;

2) antibody display: used to isolate antibodies recognising different antigens or study an immune response;

3) protein display: used to modify or analyse a protein's properties.

Combinatorial peptide libraries on phage

These are libraries in which virtually all the possible amino acidic combinations are used to form a vast number of diverse peptides (Smith 1985; Burritt, Bond et al. 1996; Smith and Petrenko 1997). The complexity of such libraries grows exponentially with the length of the peptide (in a 20x20x20 ..... fashion, see below). Often the library does not contains all the theoretical variants, in part because the library is not large enough, and also because some peptides are discriminated against during bacterial growth. These types of libraries have been extensively used to map antibody epitopes, as well as binding surfaces for cell surface receptors, enzyme and intracellular proteins (Blond-Elguindi, Cwiria et al. 1993) (Gram, Schmitz et al. 1997) (Songyang 1994) (Cheadle, Ivashchenko et al. 1994) (Rickles, Botfield et al. 1994) (Rickles, Botfield et al. 1995) (Salcini, Confalonieri et al. 1997). These libraries can

be screened on targets to find peptides that mimic the properties of the ligand (peptidomimetic) (Smith 1991; Hoess 1993; Cortese, Felici et al. 1994).

Combinatorial peptide libraries are produced in phages using degenerate oligonucleotides, which are produced chemically adding mixtures of nucleotides, rather than single nucleotides, to a growing nucleotide chain. In this way a combination of random amino acids can be fused to a phage coat protein.

Phage peptide libraries have been largely used on the basis of their affinity to a target. This is the case of epitope mapping of monoclonal antibodies or ligand mimetic for a receptor, in which the antibody or the receptor is fixed to a solid matrix and phages are selected on the matrix. The system has been successfully used for a number of binder characterizations. This has also been successful for "mimotopes" that are linear peptides that mimic the 3D arrangement of amino acids on the protein surface(Felici, Luzzago et al. 1993). An example where this has shown to be the case is the linear sequence of the epitope selected by a monoclonal antibody against H Ferritin. This could not be found in the primary sequence of H Ferritin, but an examination of the crystal structure showed that amino acids far apart in the primary sequence (corresponding to those found in the linear peptide) were adjacent to one another on the surface of H Ferritin (Luzzago, Felici et al. 1993).

Peptide phage libraries can also be selected on a different basis. For example, a protein domain with high affinity for a receptor has been fused upstream of the random peptide on g3p. In this way, the whole library can be bound to the receptor adsorbed to a solid matrix. If a specific protease is added to the matrix, only the phages that bear the peptide acting as substrate for that protease, can be eluted, amplified and characterised to determine the protease specificity (Matthews and Wells 1993; Matthews, Goodman et al. 1994; Wang, Yang et al. 1996).

One of the problems connected with the use of peptide libraries is that usually peptides have a minor degree of three dimensional folded structure of a protein or of a protein domain. For this reason constrained peptide libraries have also been produced. The most common way to do this is to introduce cysteine residues fixed in certain positions in a random amino acidic sequence. These should allow the formation of intra-peptide disulfide bonds (McConnell, Kendall et al. 1994; Giebel, Cass et al. 1995; Bonnycastle, Mehroke et al. 1996).

Epitope discovery and vaccines

Peptide libraries can be selected against sera from patients with infectious diseases (Cortese, Felici et al. 1994). In this way it is possible to find epitopes specific for that particular infectious disease, i.e. epitopes that are recognised by the antibodies produced in response to that disease. Peptides found in this way would be useful either as diagnostic marker or as potential synthetic vaccines (Meola, Delmastro et al. 1995).

These peptides can mimic both linear and discontinuous epitopes, and can be selected using purified antibodies or preparations of polyclonal serum. For instance, using sera from hepatitis C virus (HCV)-infected patients and controls, peptides have been selected which specifically react with sera from infected patients. These phage peptides were shown to mimic different HCV epitopes and have been shown to be detected by antibodies in a large panel of patients' sera demonstrating the feasibility to use them as diagnostic markers. Phage-displayed HCV mimics have been used in mice to elicit an immunoresponse against the HCV itself. These experiments are the beginning of the development of a mimotope-based vaccine against viral infection. (Prezzi, Nuzzo et al. 1996; Tafi, Bandi et al. 1997).

The same kind of approach has also been tried for multiple sclerosis (Cortese, Tafi et al. 1996; Cortese, Capone et al. 1998) but with without finding disease specific peptides.

Phage antibodies libraries

So far, one of the best applications of phage display technology has been the expression of antibody fragment libraries and the selection of specific binders against various kind of protein targets (McCafferty, Griffiths et al. 1990; Barbas, Kang et al. 1991; Marks, Hoogenboom et al. 1991; Vaughan, Williams et al. 1996; Sblattero and Bradbury 2000)

Antibody derived protein fragments have been displayed in many different formats on phages, as minibodies, as VH, as ScFv, as Fab and as dromedary heavy-chain antibodies (Cabilly 1989; Hoogenboom, Griffiths et al. 1991; Martin, Toniatti et al. 1994; Davies and Riechmann 1996; Arbabi Ghahroudi, Desmyter et al. 1997; Dimasi, Martin et al. 1997). Even though many different antibody libraries have been done so far, the construction of a good phage antibody library remains a tricky step.

The most important feature of a library is the diversity that is mainly determined by the source of variability. It has been shown both theoretically (Perelson and Oster 1979; Perelson 1989) and practically (Griffiths, Williams et al. 1994) that the more diverse a library is, the greater the affinity of the antibodies which can be isolated.

Libraries can be organised into three different categories, on the basis of the source of DNA encoding the antibody genes.

Immunised libraries are made using lymphocytes from immunised animals and blood or tissue lymphocytes from immunised donors (e.g. infectious or auto-immune diseases patients). These kinds of libraries are enriched with high affinity antibody fragments against the antigen used to

immunise the animal, or the auto or infectious antigen and therefore provide high affinity antibodies against the immunising antigen, although they have also been used to select antibodies against other antigens.

Naïve libraries are made starting with peripheral blood lymphocytes, spleen or bone marrow from non immunised human donors or animals. Usually these contain a larger repertoire of specificities, even if the average affinity of binders are usually lower than those to found in an immune library.

Finally, synthetic libraries are built starting from one or more V genes (usually human) which provide a suitable protein scaffold, usually selected for properties such as good expression, and increasing diversity artificially in regions directly involved in antigen binding (i.e. CDRs) using oligonucleotides. CDR3 of both VH and VL have been targeted in this way. In some systems (Griffiths, Williams et al. 1994; Knappik, Ge et al. 2000) diversity is introduced randomly, while in others selectively (Tomlinson, unpublished).

Libraries are made by either cloning V genes separately into the display vector, by assembling the V genes using PCR, or by using a single (Sblattero and Bradbury 2000) or double vector (Waterhouse, Griffiths et al. 1993; Smith, Patel et al. 1998) recombination system. In these latter cases the heavy and light chain variable genes are separated by two lox sites and cre recombinase is used to shuffle them. In the double vector system, the heavy chain library is made on a plasmid, the light chain library on a phage and recombination occurs after bacteria hosting the plasmid are infected by the phage. This allows a single round of recombination to occur. In the single vector system, heavy and light chains are cloned into the same vector and bacteria are co-infected at high multiplicity of infection (MOI), with recombination occurring between many different VH and VL chains. With such libraries, reshuffling of variable regions

alternated with selection cycles and/or mutagenesis steps can provide an additional tool for the improvement of binders through affinity maturation.

Why phage antibody libraries?

The display of antibody fragments libraries on phage has some big advantages in obtaining good binders, when compared with the classical production of monoclonal antibodies. Given a good phage antibody library, it should be possible to select and test specific ligands in less time and in larger numbers against any antgen, when compared to the production and testing of monoclonal antibodies. Moreover, some proteins do not give rise to an immune response in animals, either because they are self-antigens or very conserved between different species, while antibodies against some antigens cannot be secreted from hybridomas, because their biological function is involved in the secretory pathway (e.g. endoplasmic reticulum components: in this case the produced antibody could stick to the target in the reticulum before being secreted). These targets present no problems for phage antibody library selections, since phage antibody libraries do not undergo to the same complex functional and structural negative modulation and constraints that is present during the development of a higher eukaryote immune system and the production of antibodies in response to immunization.

Another advantage of the use of antibody libraries is the feasible application of different selection schemes including subtraction schemes (Van Ewijk, de Kruif et al. 1997). A typical selection (biopanning) is made immobilising the target on a plastic surface and allowing the phages displaying variants to bind to the target. Most protein can be adsorbed on a plastic surface, via interactions that are not fully clarified yet, although mostly hydrophobic. Even though it is known that in this way the target can be partially denaturated, this is one of the

most commonly used selection methods. Alternatively, the target can be bound to a resin matrix in a column, biotinylated and then recovered with avidin coated magnetic beads, or bound to another antibody, this last being directly adsorbed on a surface. Therefore, a wide range of possible different ways to make binders interact with the target is available (Gram, Marconi et al. 1992; Clackson and Wells 1994).

Moreover the extraordinary resistance of bacteriophages allows various kinds of more unusual selection schemes. For example, phage antibodies can be selected directly against targets, such as receptors, expressed on the cell surface using either direct binding (Cortese, Monaci et al. 1995) or by internalization (Becerril, Poul et al. 1999). At the farthest end of this type of selections, there are experiments in which a whole library of phage peptides is directly injected into the blood stream of a living mouse, allowing the isolation of tissue specific peptides (Pasqualini and Ruoslahti 1996). Such experiments should also be feasible with phage antibodies.

Furthermore, phage displayed libraries allow one to perform subtractive schemes of selection, otherwise impossible, with the aim of identifying non-shared epitopes in two very similar proteins, protein mixtures or cell types (de Kruif, Terstappen et al. 1995; Van Ewijk, de Kruif et al. 1997). We will provide an example of a simple partially successful subtractive selection between two slightly different isoforms of protein tau, that led to the identification of an anti-tau isoform specific ScFv.

Displaying other proteins on phage

The permissiveness of p3 to the display of foreign proteins is remarkable (see table 13). Over fifty different proteins have been cited in the literature, and these include secreted proteins,

enzymes, the extracellular domains of membrane proteins, intracellular proteins, toxins and a number of protein scaffolds derived from many sources. Virtually, any protein which can be secreted into the periplasmic space of E. coli, is likely to be displayed on phage, and it may be possible to widen the spectrum of proteins which can be displayed by using particular mutants (Peters, Schatz et al. 1994). The main reason to display a protein on phage is to select mutants of that protein which have different properties, usually an increase in affinity or enzymatic activity, although decreases in affinity, which allow the identification of interaction residues, can also be selected for (Jespers, Jenne et al. 1997). This process requires several different steps: the creation of the library of mutants, the selection and screening.

Although several techniques can be used to create a library of mutants, targeted mutations have been used most frequently and with the greatest success (Roberts, Markland et al. 1992; Roberts, Markland et al. 1992; Wang, Yang et al. 1995). A good example, is the selection of specific inhibitors of a number of proteases, including chymotrypsin (Rottgen and Collins 1995), kallikrein (Dennis, Herzka et al. 1995) and tissue factor Factor VIIa (Dennis and Lazarus 1994; Dennis and Lazarus 1994), with a Ki as low as 15pM selected from a library of APP Kunitz domains. Plaminogen activator inhibitor libraries have been also made using error prone PCR (Pannekoek, van Meijer et al. 1993).

Protease inhibitors are not the only example in which the affinities have been increased using phage display. Mutations of human growth hormone at twenty different sites, in five different libraries (four sites per library), selected on the receptor, yielded a derivative able to bind its receptor with an affinity 400 times greater than wild type (Lowman and Wells 1993). The affinity of human IL-6 for its receptor has been increased in similar way five fold (Cabibbo, Sporeno et al. 1995). However, increased receptor binding does not necessarily translate into

increased biological activity. Phage displaying proteins have been shown to be able to bind and activate cell surface receptors at similar molar ratios to soluble protein. This has been reported in several different cases (Buchli, Wu et al. 1997; Merlin, Rowold et al. 1997; Souriau, Fort et al. 1997; Szardenings, Tornroth et al. 1997) and allows the development of selection strategies based on biological activity.

Phage display of cDNA libraries

Common cloning systems used to find specific gene sequences have been based on the screening of cDNA libraries in two main ways:

1) identification of cDNA clones using oligonucleotides probes;

2) immunoscreening of cDNA expression libraries.

Both systems present big limitations. The first relies on the need of a partial amino acidic sequence or homology with known sequences and identifies clones just on the basis of DNA sequence, without a strong relationship to the effective function of the encoded protein.

The second type of screening is similar in concept, but very different in use to standard display vectors. Instead of genotype and phenotype being linked at the level of the individual phage particle, they are linked in the plaque which is used as the basic screening element. Expression libraries can be used in immuno screening experiments in which monoclonal antibodies, polyclonal antisera or ascites are used to test hundreds of thousands of clones, that can be isolated and characterised. However, there are two main differences between the use of expression libraries and display libraries: screening, and not selection, is used; and the numbers which can be screened in an experiment are orders of magnitude lower. As a result, selection

and enrichment schemes based on the nature of the displayed polypeptide are not possible. Moreover, even if in a single bacteriophage plaque genotype and phenotype of the expressed protein are coupled, they are not directly linked in the same useful fashion as in case of phage displayed proteins. Therefore, well established lambda based expression vectors cannot be used for cloning unknown sequences on the basis of direct protein-protein interactions, unless the affinities are very high, and the screening protein is available in large quantities.

A cDNA library displayed on phage would instead allow the identification of a sequence on the basis of the binding or interaction properties of the encoded polypeptide. This would be a great advantage in the effort to understand the function of that polypeptide. However, the display of cDNA libraries presents unique problems. As described earlier, fusion to g3p or g8p is at the N-terminal part of the coat protein. As a result all cDNAs encoding C-terminal protein fragments, even if inserted in the correct frame, will not be expressed due to the presence of translational terminators that will hamper the correct fusion to the downstream g3p and so the incorporation of the predicted fusion protein X-g3P into the phage particle. Moreover, open reading frame fragments randomly fragmented and inserted downstream of the gene 3 leader will have just 1 possibility in 18 to give a functional fusion: there are one in three possibilities that a randomly cut DNA sequence bearing an ORF enters in the frame of the upstream leader, one chance in three that it maintains the frame for the downstream g3 sequence, and one chance in two that it has the correct orientation. These odds can be reduced to one in nine if cloning is directional, and to one in three if the frame at the end of the ORF is unimportant. This can be done by carrying out directional fusions to a C terminus, an approach which has been used with the pJuFo vectors (see below).

So far relatively few phage display cDNA libraries have been described. Allergen derived cDNA libraries have been successfully displayed using pJuFo (Crameri and Blaser 1996), while lambda phage has been used to successfully display a number of cDNA libraries (hepatitis C, brain) (Santini, Brennan et al. 1998). In a direct confrontation between a p3 based filamentous phage (not pJuFo) and lambda systems using the hepatitis C genome, the lambda system proved to be better (Santini, Brennan et al. 1998). While in part this may have been due to the fact that the lambda display system was C terminal, while the filamentous phage was N terminal, the nature of the proteins displayed is also likely to be important. Lambda is an intracellular virus, and filamentous phages are secreted viruses. As a result intracellular proteins (as hepatitis C proteins can be considered) are more likely to be better displayed on lambda and secreted or membrane proteins are more likely to be better displayed on filamentous phage.

The pJUFO system

With the aim of eliminating some of the problems associated with the display proteins at the N terminus of p3, the pJuFo vector was designed (Crameri and Suter 1993; Crameri, Jaussi et al. 1994; Crameri and Suter 1995).

This is a phagemid vector which relies on the strong interaction of two proto-oncogenes products, jun and fos, to allow display at the C terminus. Jun and fos encode for proteins that bind to DNA as dimeric complexes and regulate gene expression. The dimerization is mediated by the interaction of domains rich in leucine residues, called leucine zippers.

The pJUFO vector has been designed in such a way that the cDNAs are not fused downstream of the PelB leader and upstream of g3p, but to the C-terminal part of the fos domain, which in turn interacts with jun fused in frame to the N-terminal part of g3p, as in the usual phage display. The fos-X fusion protein has a leader sequence and is driven to the bacterial periplasm, where it interacts with the jun found at the N terminus of p3. Moreover, two cysteines are present at the extremity of each zipper, giving an even stronger covalent association between the two molecular partners. As a result, cDNA products are displayed using p3 and provide the same link between phenotype (cDNA product) and genotype (phagemid DNA) found in standard g3p vectors

(Figure 14). This system has been used to identify allergens recognised by IgE from allergic patients (Crameri and Blaser, 1996), but there have been few other publications reported.

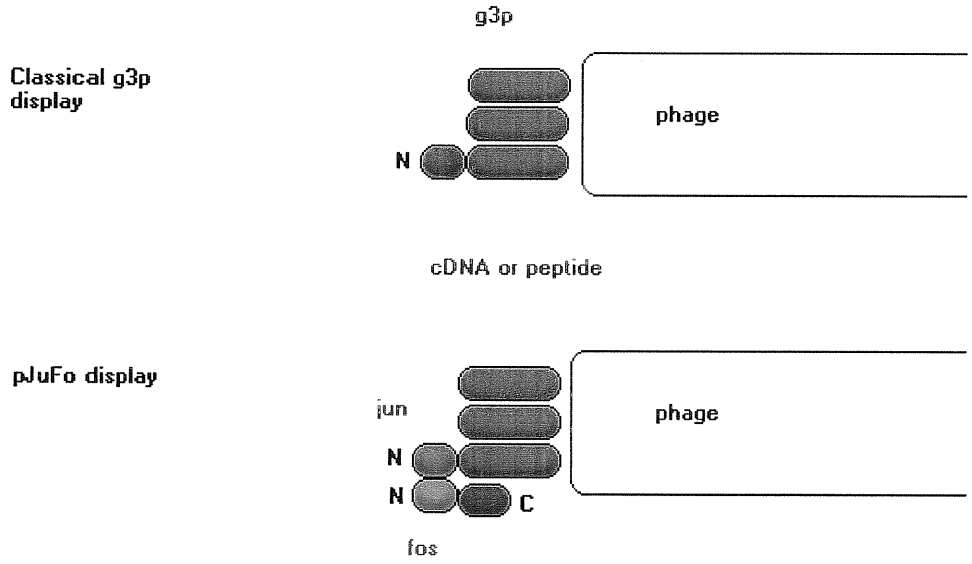


Fig.14. The display of a cDNA product or peptide in the pJuFo vector is obtained exploiting the interaction between the leucin zippers Jun and Fos. The position of the displayed peptide in the fusion protein is C-terminal while in the gene 3 display is N-terminal.

Bacteriophage λ based display systems

More recently, λ and T4 non-filamentous phages have proven to be a suitable choice for protein and peptide display. In particular, λ has been used for surface display of proteins and peptides fused either to the tail protein V or to the head protein D. (Efimov, Nepluev et al. 1995; Mikawa, Maruyama et al. 1996; Jiang, Abu-Shilbayeh et al. 1997; Kuwabara, Maruyama et al. 1997; Santini, Brennan et al. 1998)

Protein D of λ phage is a small major coat protein of 110 aa 11.6 kDa, present in about 420 copies per phage head, is placed outside the capside and is involved in the head stabilisation and maturation. Mutants for D proteins accumulate immature "pre-heads", without being able to package phage genomes.

Protein D has been used both for C-terminal and N-terminal fusions even of large proteins like β-galactosidase. It has been very successful in the identification of interaction partners for intracellular proteins (Sternberg and Hoess 1995; Mikawa, Maruyama et al. 1996; Santini, Brennan et al. 1998; Santi, Capone et al. 2000), perhaps, because, as mentioned above, it is an intracellular phage which develops within the cytoplasm and is released by bacterial lysis.

Molecular diversity techniques in functional genome studies.

With the sequencing of the human (and other) genome(s), a vast amount of data has been produced. One of the most pressing biological problems is to find a suitable way to interpret this data, and to understand the function of thousands of genes, giving rise to even greater numbers of proteins (due to alternative splicing and post-translational modifications).

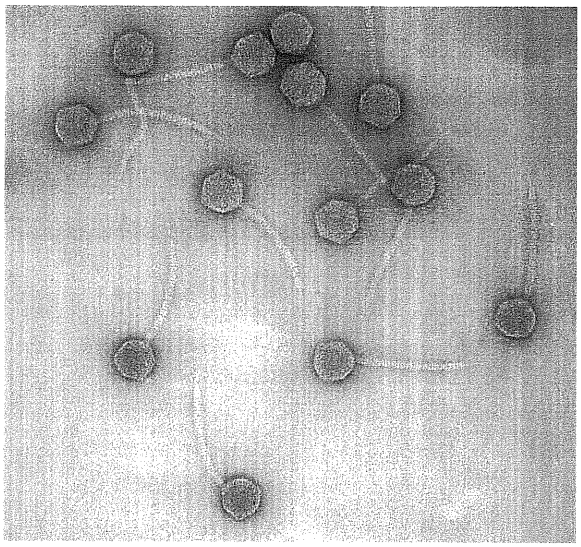
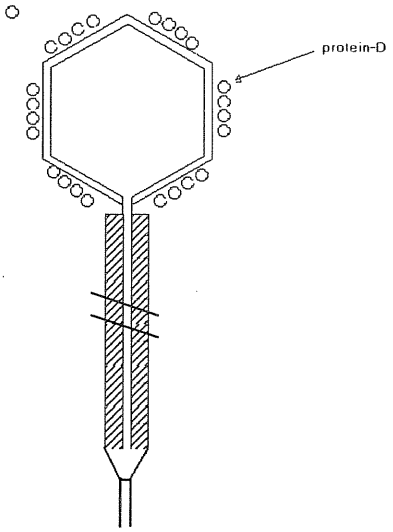


Fig.15. Lamda phages (from Electron Micrograph Library, http://phage.bocklabs.wisc.edu/index.htm) and a schematic view of protein D.



Molecular diversity techniques, which often deal with library sizes in the billions, are particularly suitable for the analysis and interpretation of genomes, almost all of which have fewer than 10^5 genes.

The majority of the studies on the function of gene products will require the use of ligands able to target the gene product. These will play an essential roles in assigning gene function, including the characterization of spatio-temporal patterns of protein expression and the elucidation of protein-protein interactions. The derivation of ligands that recognize protein products of all human genes, such ligands being either antibodies or protein fragments or synthetic peptide would be a crucial target for the whole functional genomic science. Furthermore, the creation of libraries of open reading frames in a format (such as phage display

or yeast two hybrid) which allows interrogation with one or more gene products will help in the untangling of the web of protein-protein interactions involved in cellular processes.

Results

2.1 The monoclonal antibody MN 423

MN423 and the PHFs

Neurofibrillary tangles are considered the second major neuropathological hallmark of Alzheimer's disease. The main constituents of neurofibrillary lesions are Paired Helical Filaments (PHF) named for their characteristic EM structure which shows that PHFs are made of two filaments helically disposed around one another with a periodicity of about 65-80 nm, a width of 10-15 nm at their narrowest point and 65-80 at their widest point, and with a morphology which varies with intracellular location (Crowther and Wischik 1985; Wischik, Crowther et al. 1985). This morphology is not uniform, since a 'fuzzy coat' can be distinguished from a denser core. The former can be stripped off with pronase digestion (Wischik, Novak et al. 1988; Wischik, Novak et al. 1988).

Many efforts have been made to clarify both the ultrastructure and the chemical essence of this component of neurofibrillary pathology. The monoclonal antibody MN423 is one of various results in the attempts to develop specific ligands against the structure or an intimate component of PHFs.

MN423 was derived by immunising mice with an enriched preparation of pronase treated paired helical filaments (PHF, the main constituent of NFTs), and has been shown to decorate PHFs (Bondareff, Wischik et al. 1991; Novak, Jakes et al. 1991) in electron microscopy as well as neurofibrillary tangles in the immunohistochemical analysis of post

mortem AD brain slices (see figure 2 in the introduction). Normal age matched control brains are not stained at all. The ability of this antibody to decorate pronase stripped PHFs indicates that the epitope is intimately associated with the structure of the PHFs. Only by using a formic acid extraction procedure (Novak, Kabat et al. 1993) is it possible to release peptide fragments from the PHF core. The N-terminal protein sequence analysis of these fragments has revealed the presence of six different peptides four of which have the pentapeptide DHGAE at their C-terminus. Of several recombinant peptides produced in the first attempt to map the epitope of this antibody present on tau, only those ending with the amino acidic sequence DHGAE were recognized, regardless of the N-terminal sequence. The removal or addition of a single amino acid to this C-terminal sequence is enough to abolish the reactivity with MN423 (Novak, Kabat et al. 1993). These observations led to the conclusion that the epitope of the antibody is C-terminal, but exactly which feature of the C terminus was responsible for reactivity was not clear.

The fortuitous observation that myoglobin fragments (used as SDS-page markers) derived from CNBr digestion, and ending in GA-Homoserine, were also recognized by MN423, as shown below, led to the conclusion that the sequence –GAE is a minimal epitope

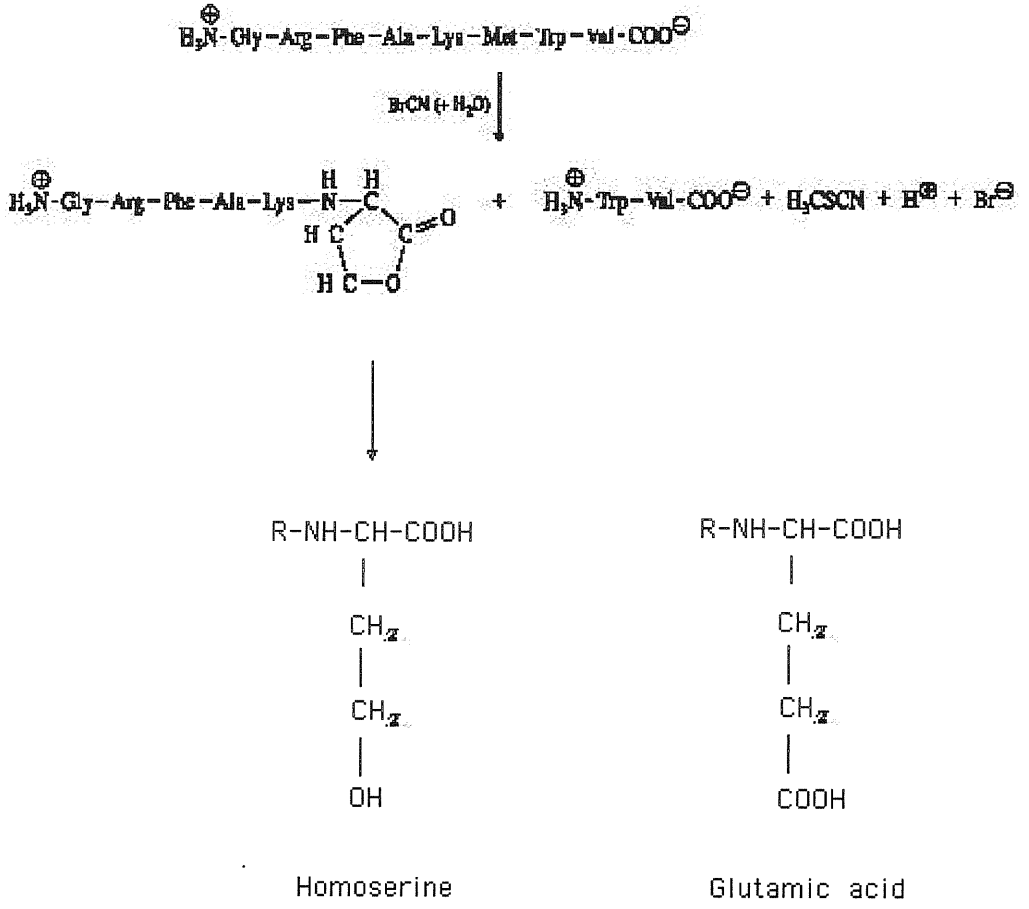


Fig.16. Chemical similarity between homeserine, resulting from a cyanogen bromide digestion, and glutamic acid (modified from D.Sc. thesis, Michal Novak).

recognized by the antibody, since homoserine is chemically similar to glutamic acid. Moreover it is particularly significant that this antibody is able to stain NFTs and all components of neurofibrillary pathology in post mortem AD brain slices without giving any signal in normal control brain slices (Mena, Wischik et al. 1991; Novak, Kabat et al. 1993).

Since this antibody does not recognize either full length recombinant tau or native normal tau it has been described as a specific marker of pathological tau associated with paired helical filament formation (Caputo, Wischik et al. 1992; Novak, Kabat et al. 1993).

While all these observations indicated that this antibody recognized tau truncated at E391, it could not be formally excluded that other proteins were also being recognized. In order to investigate this possibility and further define the epitope specificity of this antibody, a number of experiments were initiated. The questions that we will try to answer in this chapter are:

1)Does MN423 antibody recognise proteins other than truncated tau?

2)What is the precise epitope(s) recognized by MN423?

Western blotting of AD and normal brain with MN423.

In a first attempt to define the antigens recognized by MN423, several western blots of normal and AD brain extracts were carried out using this antibody (for details see Materials and Methods). However, no specific bands could be identified with this antibody, even if a fuzzy staining corresponding to the stacking gel, suggesting the recognition of large aggregated material, was observed (data not shown and Novak personal communication)

Screening of 2 AD brain c-DNA expression libraries with MN423

In order to find out if any other antigens could be recognised by this antibody, two cDNA expression libraries derived from AD patients were screened for the presence of an MN423 positive protein (see Materials and Methods).

The first library used for this purpose was a Clontech commercial library from an AD patient 70 years old. The tissue used was from hippocampus with a post mortem delay of 5 hours, the number of independent clones $8x10^5$, with an average insert size of 1.1 Kb and the vector used was λgt11. About 10^6 clones were screened. Three round of purification screening were performed on the of positive clones.

Of 32 positive clones, 7 were sub cloned, sequenced and characterised. The sequence was the same for all 7 clones. On the basis of this sequence, PCR primers were designed and used to amplify the remaining clones, to see if there was any similarity. The presence of identically sized bands in all remaining clones revealed that all the positive clones had the same sequence.

The sequence found in these clones belongs to the DNA-PK coding sequence. Potentially, this is of great interest, since DNA-PK is one of the kinases identified which is able to phosphorylate tau. However, the recombinant protein encoded by this sequence is β-galactosidase, followed by a piece of DNA-PK in the –1 frame. Just by chance, this additional amino acidic chain ends with the sequence –CYETTEGAA, similar to the previously identified epitope GAE (see figure 17).

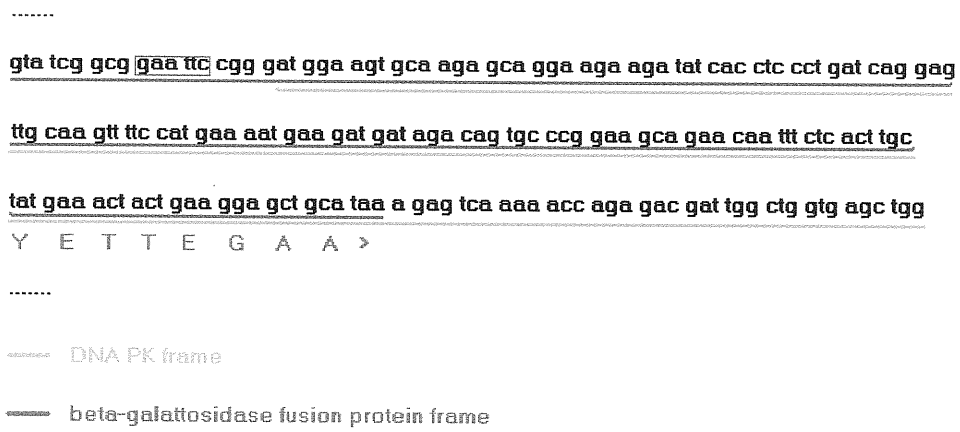


Fig.17. Above is shown the sequence common to all positive clones found during the screening of an AD brain cDNA library. The red box is the EcoR1 cloning site in the β-galactosidase gene. In red is underscored the sequence of the β-galactosidase fusion protein while the correct reading frame of the DNA-PK insert is underscored in green.

This protein is recognised by MN423 in both plaque lift experiments (figure 18) and western blot experiments (figure 19) in which the dGAE peptide (the recombinant fragment of tau, reproducing a peptide isolated from AD PHF preparation) is used as a positive control.

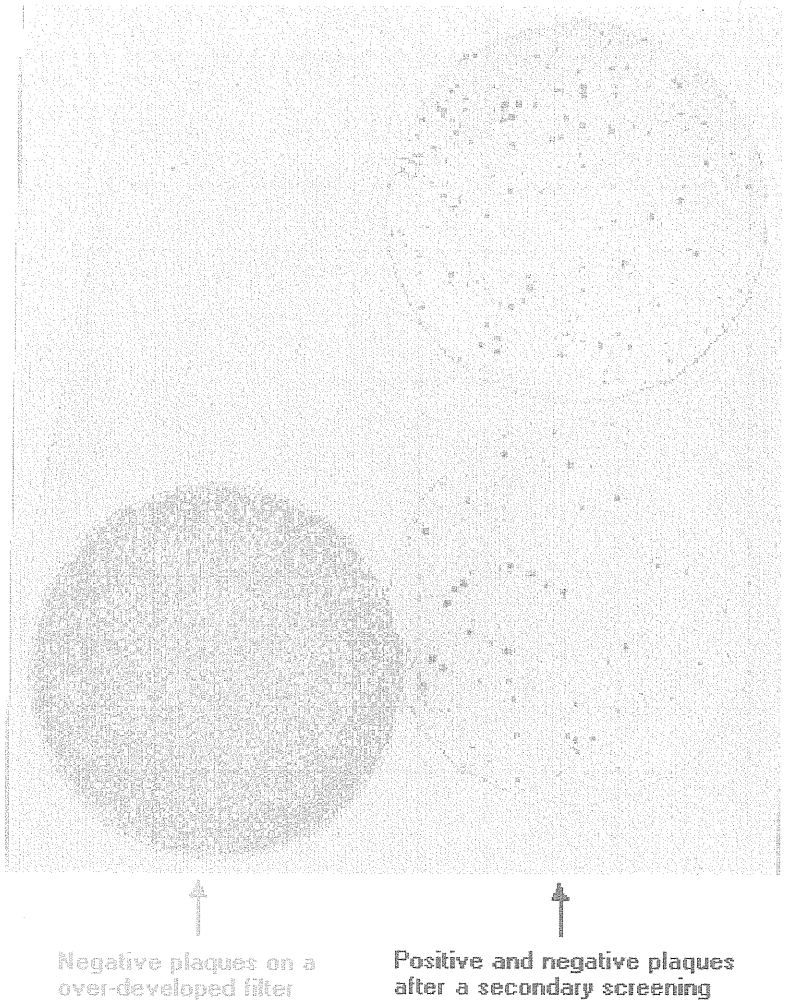


Fig.18. Nitrocellulose filters after plaque lift for screening an AD brain library with MN423 antibody. On the right two filters for the secondary screening are shown. For comparison, on the left, is shown a filter showing only negative plaques. This filter is darker because has been overexposed to the AP substrate and white spots are negative plaques.

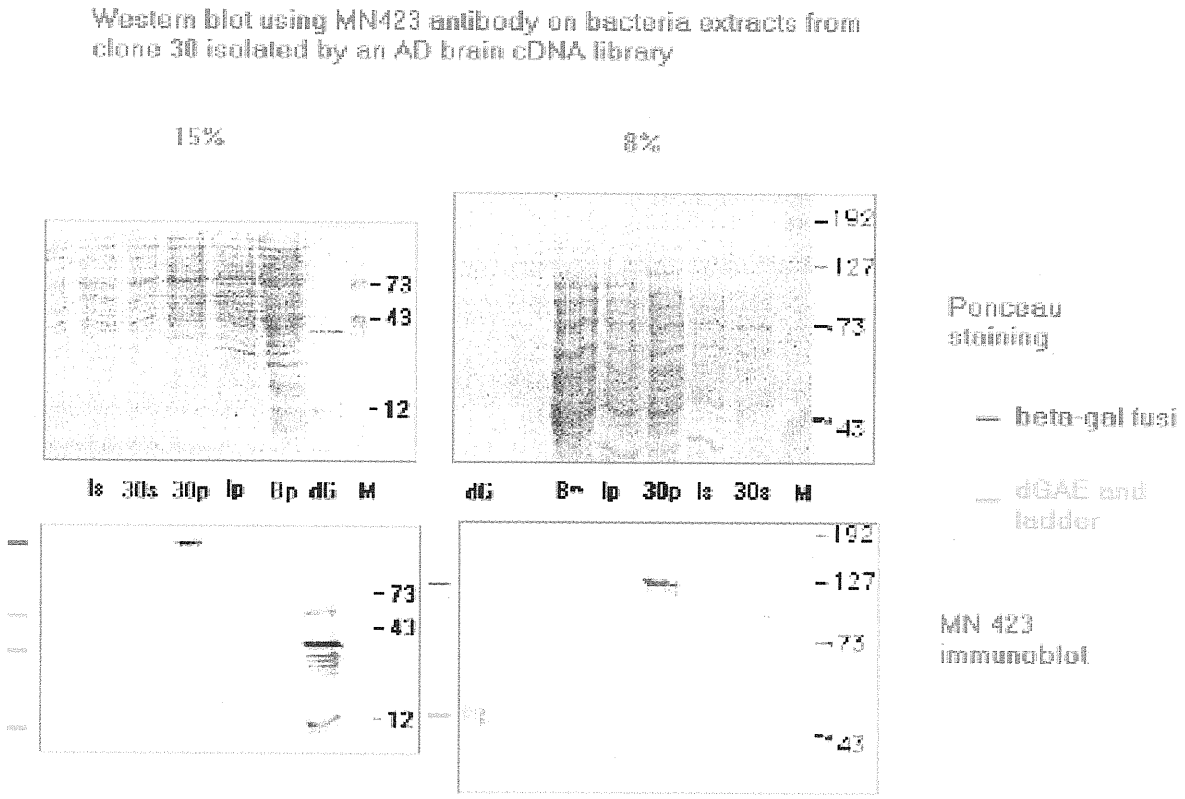


Fig.19. Western blots using MN423 antibody on a protein extracts from a lambda library clone plaque-positive to MN423. Library is from cDNA from AD brain. Legend: 30 = positive clone 30 infected bacteria, l=lambda gt11 vector infected bacteria, B=non infected bacteria, dG= a recombinant purified fragment of tau ending with -GAE, s= supernatant, p= pellet, M=molecular mass marker. The dGAE purified fragment has tendency to forms aggregates even in SDS page. The purified fragment is 12 kD but both gels, 15% and 8%, show additional upper bands.

The second cDNA library was from the temporal cortex of an 83 year old AD patient, containing 10^6 independent clones with average insert size of .8 kb. This was made in a Dutch lab in the lambda Zap express vector. We received an aliquot of this from Anneke Brake. The screening of clones from this library revealed no MN423 positive clones.

A comparison of the sequences of the tau fragment, dGAE, and the ß-galactosidase fusion protein, revealed no similarity beyond the C terminal amino acids.

1) ß-galactosidase-DNA-PK fusion protein > ----CYETTEGAA

2) tau derived recombinant fragment dGAE > ----KAKTDHGAE

Fig.20. C-terminal sequences of the fusion protein β-galactosidase-DNA-PK and the tau fragment dGAE.

The epitope mapping of the Alzheimer specific monoclonal antibody MN423.

When taking this information into account, with that previously described (showing that the addition or removal of a single amino acid was sufficient to abolish reactivity), the surprising conclusion appeared to be that MN423 can recognize at least two different linear C terminal epitopes, namely -DHGAE and -ETTEGAA where the sequences necessary for the recognition appear to be –GAE and –GAA respectively, perhaps with the requirement of some negatively charge amino acids immediately upstream.

Selection of a twelve amino acids random peptide library on MN423

Even if the two known epitopes of this antibody appeared to be such short linear C-terminal sequences, it could not be excluded that there were other features or epitopes which were also being recognized by this antibody.

In order to investigate this possibility, a commercial 12 amino acid random peptide phage library (Ph.D.-12 ™ Phage Display Peptide Library Kit-New England Labs)was selected against the MN423 antibody in a classical experiment of bio panning on a bait protein adsorbed on plastic (immunotubes). After the third round of selection, there was no

increase in output titre, and we were unable to detect positive clones by either ELISA or in a dot blot assay (phage-peptide vacuum adsorbed onto nitrocellulose membrane). Twelve clones were sequenced, of these two were deleted, one was not readable. The remaining nine are shown below.

```
HS SRSKTTIPNPLL GGGS
HS LHKLERLTDTAV GGGS
HS SGIGSKGRGVTL GGGS
HS WARTTRLRHGVF GGGS
HS GDEGWSGHAIDS GGGS
HS VARPTYASGRGI GGGS
HS LALRTSFADHTW GGGS
HS PLATGLKAHERT GGGS
HS IVNLPDNTLGLG GGGS
```

No clear consensus could be detected in these peptide phage, and the conclusion was that true selection was not occurring, suggesting that the epitope recognized by this antibody could not be detected in this library.

Although it cannot be excluded that a linear epitope recognized by this antibody was not present in the library, epitopes with an interesting hydrophobic consensus (tryptophan) were successfully selected against dGAE itself (see the list below), suggesting that the library and the methods used were functional.

```
2)        YHWHWWPQLSAI
3)  10)    WHPWWSAYQLYR
11)        HLNWWNTWLYTP
5)      RIFDPAEWQFKY
8)        HWFSPWTHFVWS
12)          KLWTIETPVYFL
6)        HAHSPVRPHQAH
1)         KCCYYDHLHALS
4)         TTYTWIGPALAG
```

For two clones the sequence was not readable.

These observations lead to the partial conclusion that N terminal or internal epitopes do not appear to be recognized by this antibody, and that the C terminal dipeptide GA at positions –3 and –2, seems to be essential in the definition of the MN423 epitope.

The use of a JUFO based vector for the construction of a C-terminal random pentapeptide library

The mapping of monoclonal antibody epitopes is now predominantly carried out using molecular diversity techniques, phage display in particular. However, present phage display methods are inappropriate for the analysis of epitopes which require a free carboxy terminus. To obtain this kind of display we first tried the pJuFo system. This vector was developed as a system to clone cDNA libraries at the C-terminus of a recombinant display peptide, so avoiding problems related with translation termination sites in cDNAs, as described in the introduction above (Crameri and Blaser 1996; Crameri, Hemmann et al. 1996).

In order to validate the system, two positive control peptide phages were created. The first containing a well characterised monoclonal antibody C-terminal epitope of an anti α-tubulin, YL1/2 (--DHEEF) (Skinner, Bradley et al. 1991; Stammers, Tisdale et al. 1991), while the second was the putative MN423 epitope (--DHGAE).

The first attempts to make such control peptide display phagemid in the standard pJuFo vector failed due to the extreme instability of the vector, which appeared to accumulate deletions between the two repeated sequences found in the plasmid (these contained the lac promoter and leader peptides).

In order to overcome these problems, a modified vector received from Invitrogen (figure 21) was used. This vector was improved in the following ways: the addition of a polylinker and an epitope tag, the deletion of one of the two original lac promoters and the replacement of one of the the two pelB leaders with a gIII leader. These modification eliminated the repetitive sequences lowering the likelihood for homologous recombination.

This new vector proved to be stable when the two positive control peptide phagemid were constructed. In addition to the two C terminal epitopes used, the positive control peptide

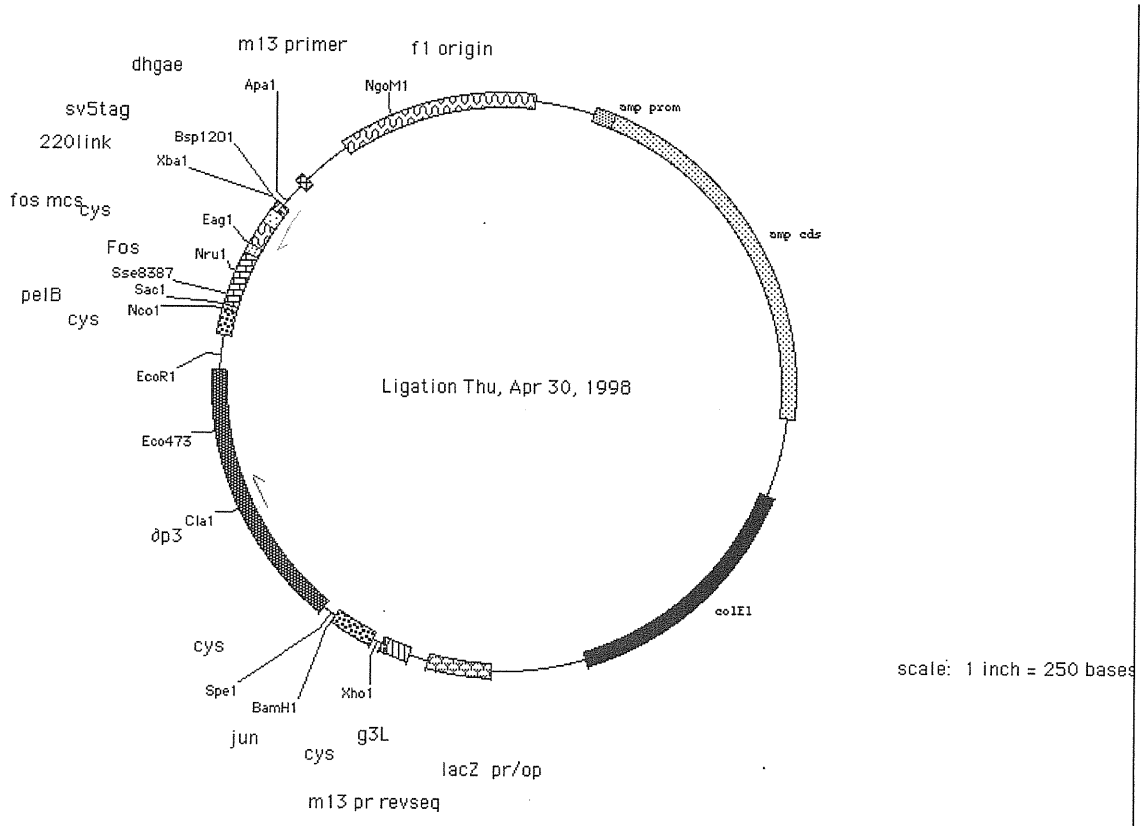


Fig.21. Cad Gene map of the clone 11 made in a new pJuFo redesigned from Invitrogen. The most important new features of the new vector, compared with the original one, are: less repetitive sequences, two different leaders and the presence of only one lac promoter. The two arrows shown in the figure represent the hybridization sites of the oligos used to create the two positive control phage as well as the C-terminal peptide library (see later).

YL1/2 positive control peptide sequence:

....**GKPGSGEGSSGT-** G K P I P N P L L G L – D H E E F
Fos zipper SV5 epitope YL1/2 C terminal epitope

MN423 putative epitope sequence:
....**GKPGSGEGSSGT-** G K P I P N P L L G L – D H G A E
Fos zipper SV5 epitope putative MN423 C terminal epitope

Fig.22. AA sequences of two test clones constructed in pJuFo.

phagemid also contained epitopes for the monoclonal antibody SV5 (Hanke, Szawlowski et al. 1992) which was used as a positive control for expression of the peptide at the C terminus of the fos zipper (see figure 23).

The positive control phages were designed with the following features:

2) each contained a C-terminal epitope, --EEF known to be recognised by YL1/2 antibody or –DHGAE thought to be recognised by MN423;
3) the SV5 epitope as positive control for expression;
4) the fos zipper.

A diagrammatic representation of the epitopes display is shown below.

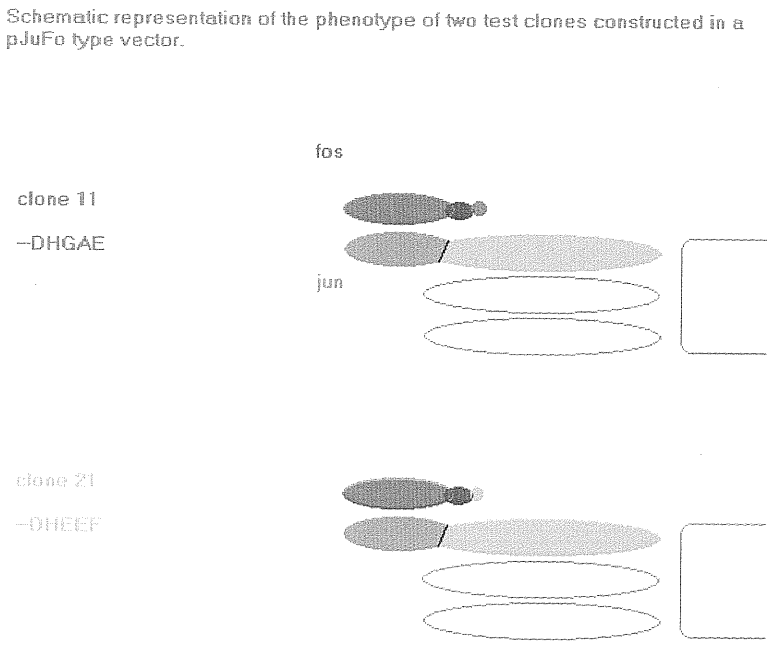


Fig.23. The two clones made to test the selection procedure with a pJuFo vector. Two epitopes were engineered at the C-terminal of Fos. Driven by a leader sequence to the periplasmic space, this should bind the Jun zipper, which is genetically fused to g3p, giving rise to the above structure. The SV5 epitope (here indicated in dark blue) has been fused to Fos just before the C-terminal epitopes.

ELISAs carried out with these two positive control phagemid (with the antibody coated to the ELISA plate) were consistently negative. This result was somewhat surprising, since sequencing showed that the epitopes were correct, and the antibodies had been shown to bind colony lift experiments. Although ELISA is generally regarded as extremely sensitive, nothing is more sensitive than counting bacteria infected by single phagemid. This can only be done by infection, and so by doing a mock selection experiment, in which two phagemid, one bearing the YL1/2 epitope and the other bearing the MN423 epitope, were selected on the corresponding correct and incorrect antibodies, with the numbers of input and output phagemids counted by infection, the ability of these epitope

phagemids to bind their antibodies could be tested at higher sensitivity. The scheme and results of this experiment are shown in figure 24.

As shown in the figure 24, both epitope displaying phages showed an enrichment in biopanning experiment against their correct antibodies, when compared with the incorrect one. In the case of the MN423 epitope, the input / output ratio was $1.5x10^{-2}$ %, while in the case of YL1/2 it was $6.6x10^{-5}$%, this is 3750 (MN423) and 15 (YL1/2) fold better than the incorrect epitope / antibody combination. The enrichment in the case of MN423 is slightly below the very low end of the range of enrichment when antibody phagemid are purified against their own antigens. That found for YL1/2, however, was far lower. We attributed this to the concentration of antibody in the supernatant used in the experiment, which, by experiments in which antibodies are coated in Elisa plates and western blots

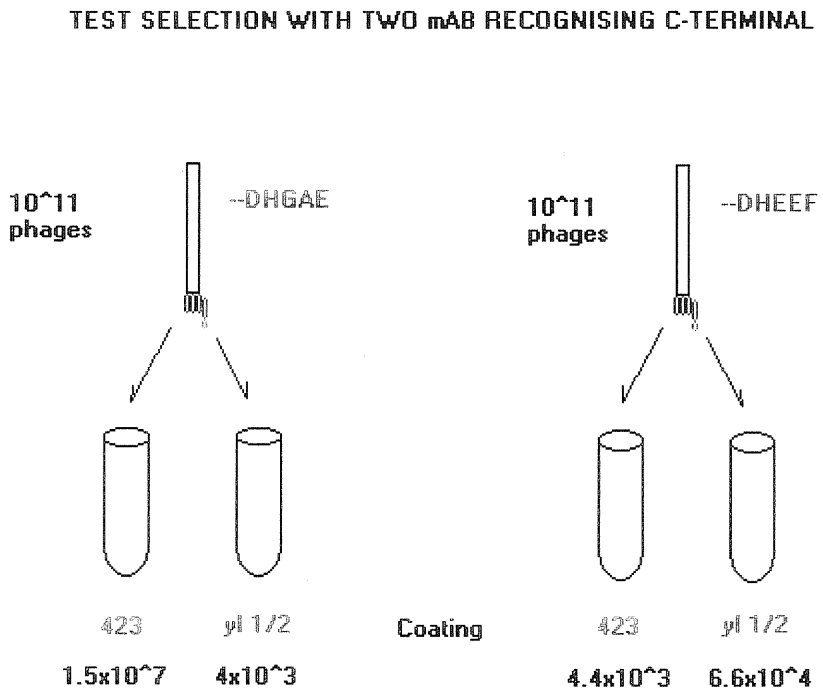


Fig.24. In this scheme a test selection for a possible enrichment of two specific phage-epitopes compared with two non specific ones is shown. Invitrogen pJuFo was engineered to expose two different epitopes (DHEEF and DHGAE) each specific for two monoclonal antibodies, YL1/2 and MN423, respectively. In both cases 10^{11} specific phagemids were used as the input. The output in all four possible combinations is indicated.

(data not shown, see Materials and Methods), was known to be lower than that used for MN423. These positive results show that the phagemids did appear to display their epitopes, and that the antibodies were able to recognise them. However, the lack of an ELISA signal, suggests, either that such display levels are low, or that the affinities of the antibodies for their epitopes, when displayed on phage, were too low to survive ELISA washing procedures. These results also confirmed the fortuitous result obtained with MN423 and the myoglobin fragment, which suggested that the MN423 epitope was purely

a C terminal linear epitope, since binding was demonstrated on two different molecules, in which the only common feature was the GAE tripeptide.

After this preliminary experiment, the numbers seemed to indicate a reasonable selection in favour of the right bait/ligand especially for MN423. So we started the construction of the library. We used degenerated oligos, as shown in figure 25, to introduce a repertoire of random pentapeptide at the C-terminal end of fos zipper peptide, cloning the PCR fragment produced between the two primers using ClaI and XbaI.

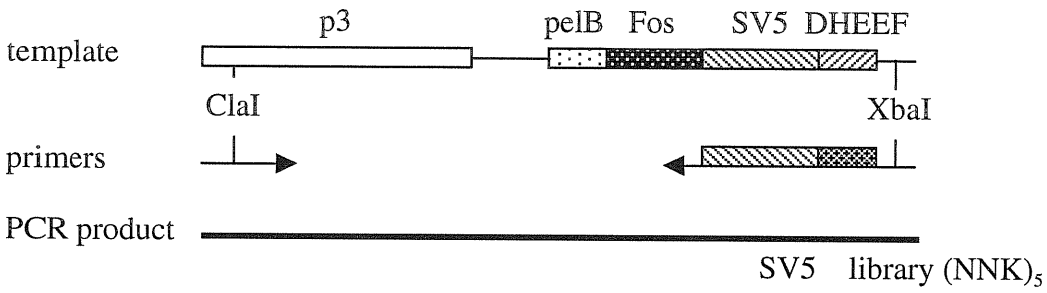


Fig. 25. Use of degenerate oligos for the construction of a random peptides library in pJuFo (see text).

The complexity of a pentapeptide library, as constructed here, is $3.2x10^6$ at the amino acid level (20^5), and $3.4x10^7$ at the genetic level (32 codons - 32^5, considering the use of the sequence NNY). For 99% representation of the amino acid complexity, a library five times greater than the genetic complexity ($1.6x10^8$), while for 90% representation, a library slightly over twice the genetic complexity ($7.7x10^7$) should be constructed (Clackson and Wells 1994). On this basis, it is clear that the library produced here ($3x10^6$) is not large enough, although it is likely that it will approach 99% full C-terminal tetrapeptide representation ($1.6x10^5$ amino acid complexity, requiring $4.9x10^6$ different clones).

After the first set of electroporations, we obtained about $3x10^6$ clones with a vector background of less than 2%. This vector background consisted of the DHEEF sequence corresponding to the YL/1 epitope. To test the proper insertion of the random peptide fragments 5 randomly chosen clones were sequenced. The sequence of these is shown below together with the encoded peptide.

```
1)ctaga tta ctc agc cgc act cca cag g

ctg tgg agt gcg gct gag taa
leu trp ser ala ala glu end

2)ctaga tta cgc cta cac cgc cat cag g

ctg atg gcg gtg tag gcg taa
leu met ala val gln ala end

3)ctaga tta ctg ata cgg cgg cac cag g

ctg gtg ccg ccg tat cag taa
leu val pro pro tyr gln end

4)ctaga tta att cga cgt att cca cag g

ctg tgg aat acg tcg aat taa
leu trp asn thr ser asn end

5)ctaga tta aac cgc acc cgc cag cag g

ctg ctg gcg ggt gcg gtt taa
leu leu ala gly ala val end
```

Codon			
NN		K	
A	11	A	0
G	15	G	18

C	14	C	0
T	10	T	7

Amino acid analysis of C terminal peptide library, created using NNK codon PCR

	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
Expected	2	1	1	1	1	2	1	1	1	3	1	1	2	1	3	3	2	2	1	1
Observed	6	0	0	1	0	1	0	0	0	1	1	2	2	2	0	2	1	3	2	1

Fig.26.Sequences from five randomly chosen clones from the library of random pentapeptides are shown. The sequence reads the reverse strand. Below a nucleotide analysis of C terminal peptide library, created using NNK codons in a PCR reaction.

A crude analysis of the library, on the basis of sequencing 5 clones, showed a relatively even distribution of all four nucleotides in the NN positions, and only G and T in the K position. However, T was relatively underrepresented in both the N and K positions, and G was relatively overrepresented, suggesting that appropriate compensation should be made if a similar library was made again. The amino acid distributions also showed some relative overrepresentations (in ala, asn, gln, val and trp) and underrepresentations, of which arg was the most extreme. These do not reflect the biases in the nucleotide use, and may be due to sampling errors. However, since experiments carried out in parallel, showed that the library was not working, further analysis and sequencing was not undertaken.

In addition, we tried a colony lift assay that would be useful with this vector as an alternative to the ineffective ELISA assay. This was based on the fact that in the vector used, both the peptide JUN and FOS are joined to leader sequences that eventually drive them to the periplasmic space. This was developed using the two positive clones enginereed with the YL1 and MN423 positive epitopes, in which the production of recombinant FOS-peptide was induced with IPTG and then bacteria were either lysed with

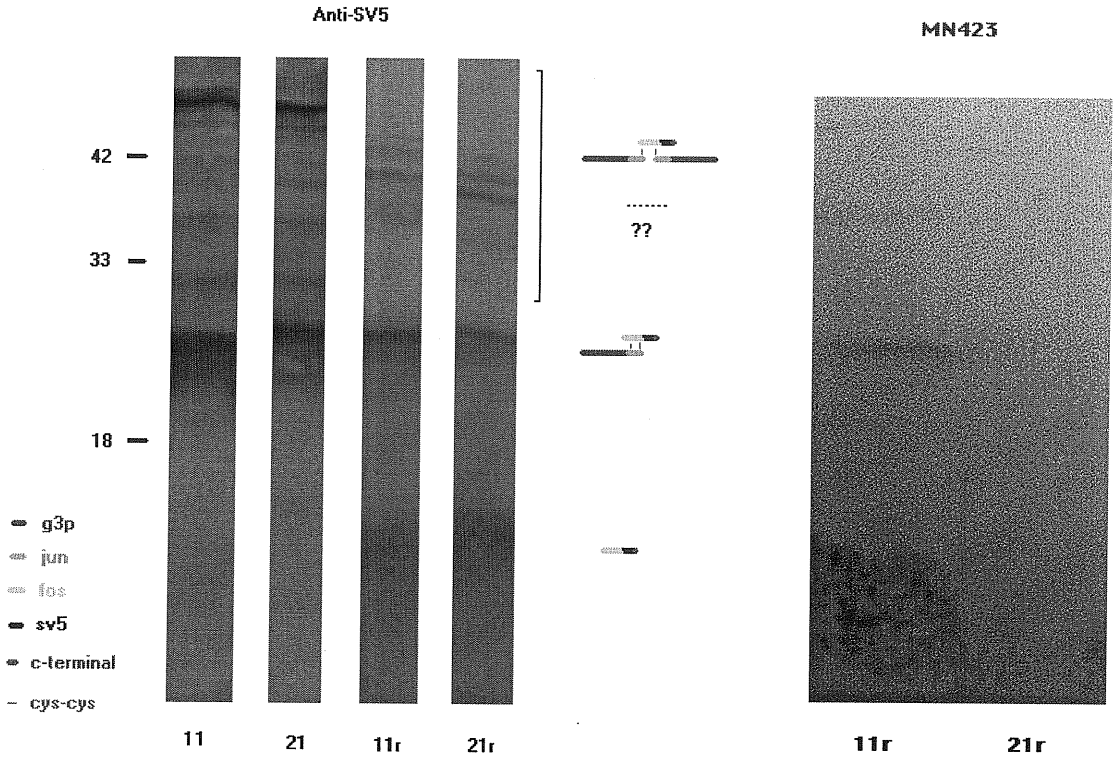


Fig.28. Western blots on the two different test clones engineered in way to be recognised by two different antibodies MN423 and YL1/2, having both C-terminal epitopes. In the clone 11 fos is fused to the sequence --DHGAE at the C-terminal (MN423 epitope) and in the clone 21 fos is fused to the sequence –-DHEEF at the C-terminal (YL1/2) epitope. Both the clones also bear a SV5 tag in the fos-epitope fusion protein which is used for detection (left). The two first lanes (11,21) were run in non-reducing conditions while lanes 11r and 21r were run in the presence of ß-mercaptoethanol. On the right, is shown how 423 recognizes in western in reducing conditions the same length fragments in the clone 11 (-DHGAE) and not in the clone 21 (-DHEEF) used as control.

Using MN423 as the selector adsorbed to immunotubes, the three cycles showed an increase in the output numbers, even when washes were performed with increased stringency for each cycle. This seemed to be a good indication that we were actually enriching for binders. The input and output numbers are given in Chart1-4.

CHCl vapours or infected with helper phage. The rationale for this was that lysis or infection may help in the exposure of the epitope for detection by the antibody. The best exposure and/or reactivity of the epitope in the fusion protein, was found using infection with helper phage (data not shown).

We feel that this may be due to the production of phagemids which are secreted from bacteria and are quickly adsorbed to nitrocellulose filters.

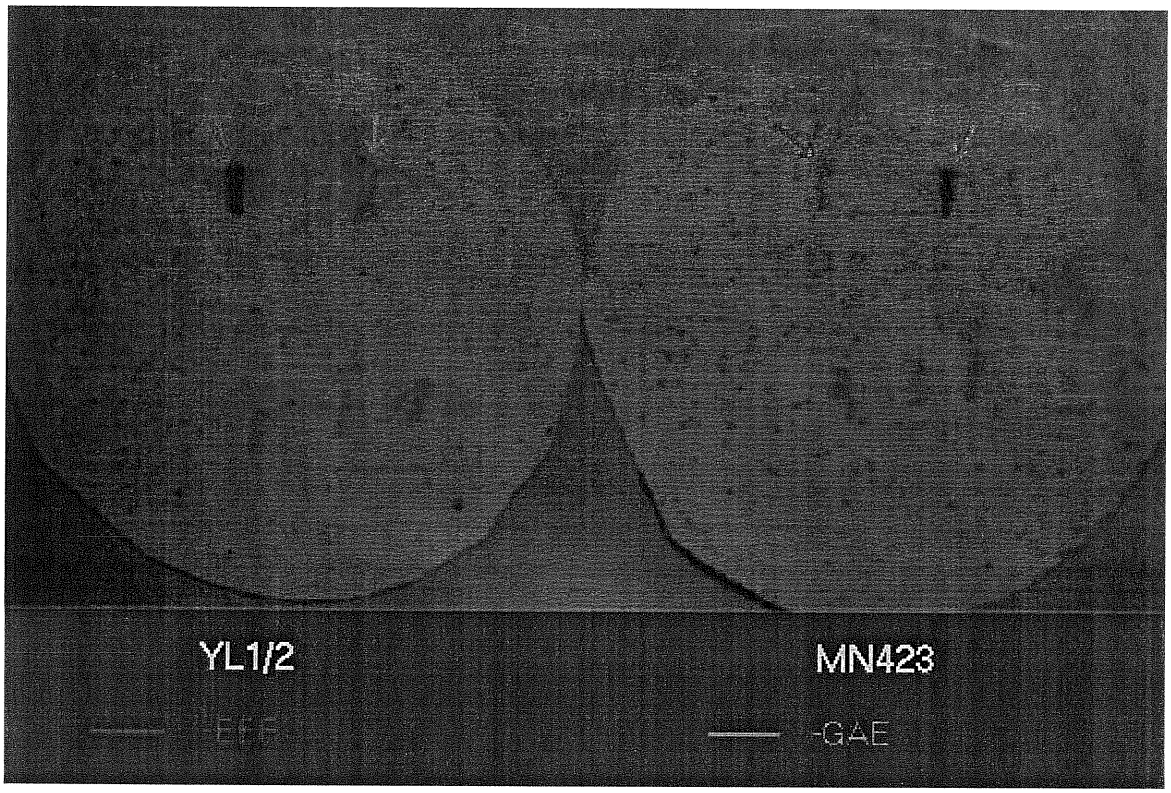


Fig. 27. Colony lift experiments in which the two test pJuFo clones are probed with the respective specific antibody (see text for details). Green color indicates the epitope for MN423 antibody, red color the epitope for YL1/2 antibody. Bacteria bearing the phagemidic vectors have been infected before plating. Colonies in the background come from the unselected library. Then a streak of 1 positive and four negative coltures have been inoculated on the same plate, having in this way a direct comparison between positive and negative clones.

CHCl vapours or infected with helper phage. The rationale for this was that lysis or infection may help in the exposure of the epitope for detection by the antibody. The best exposure and/or reactivity of the epitope in the fusion protein, was found using infection with helper phage (data not shown).

We feel that this may be due to the production of phagemids which are secreted from bacteria and are quickly adsorbed to nitrocellulose filters.

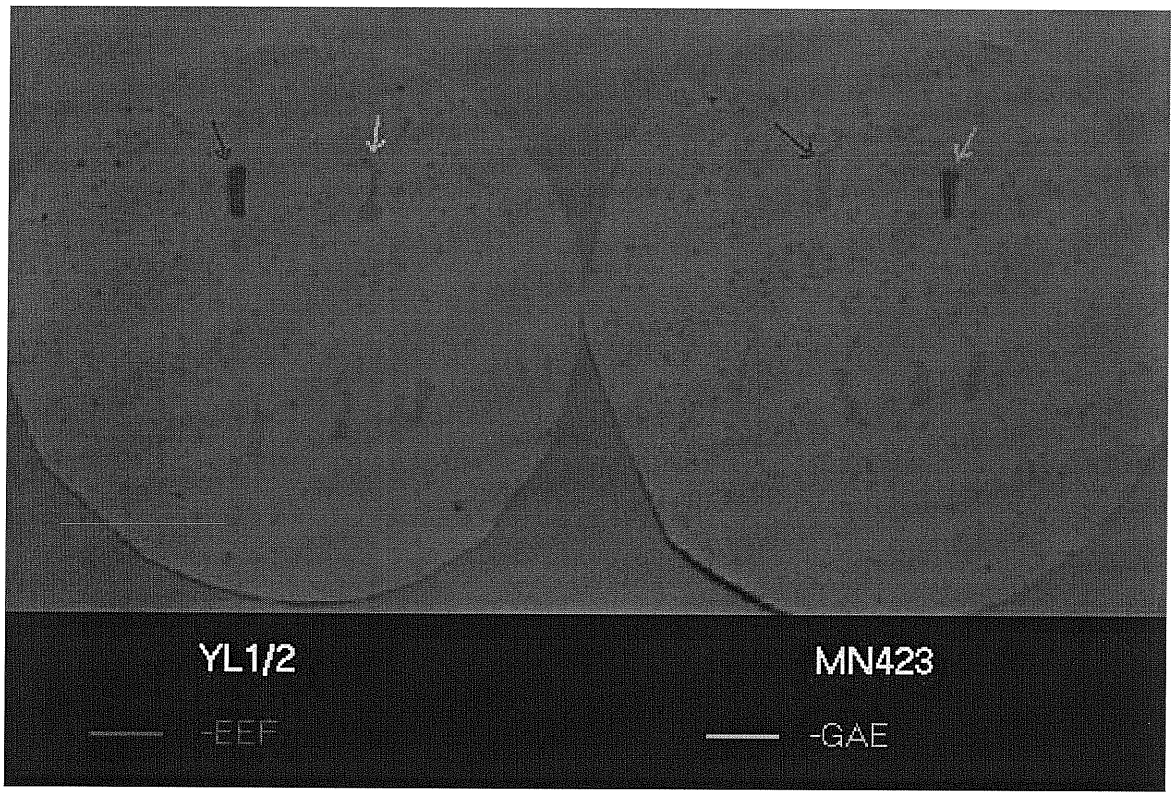


Fig. 27. Colony lift experiments in which the two test pJuFo clones are probed with the respective specific antibody (see text for details). Green color indicates the epitope for MN423 antibody, red color the epitope for YL1/2 antibody. Bacteria bearing the phagemidic vectors have been infected before plating. Colonies in the background come from the unselected library. Then a streak of 1 positive and four negative coltures have been inoculated on the same plate, having in this way a direct comparison between positive and negative clones.

Selections with the pJuFo based random peptide library

The use of this library in biopanning experiments failed to give any enrichment on either of the two monoclonal antibodies used after a number of different experiment, and no colony lift (or ELISA) positive clones could be found after a number of rounds of selection. This was surprising, especially since the one of the two epitopes (DHEEF, the YL1/2 epitope) was over represented in the library, as it was used as the vector for the construction of the library itself. With the known background of 2%, all of which would have been expected to display the YL1/2 epitope, how was it possible that we were unable to fish out a clone represented at such a high level using this monoclonal?

While a number of explanations, to be presented in the discussion, were possible, we wondered whether, notwithstanding the promising results obtained in the control experiments, there were problems related to the display levels of the peptides on the vector. This was examined by western blot experiments, in which we applied both reducing and non-reducing conditions to the same phage samples derived from positive test clones. The results are shown in figure 28.

It is important to remember that SV5 (and YL1/2 or MN423) recognize the very small fos fragment attached to the appropriate epitopes (expected molecular weight 8kD). When the westerns were probed with SV5 under non-reducing conditions, both clones showed multiple high molecular weight bands, which mostly disappeared upon reduction, suggesting (as shown in the figure 28) that aggregates were being formed. One of the most prominent bands under both conditions is a band at 30kD, which most likely corresponds to a single fos with a single jun, which presumably is being held together under reducing conditions by the affinity between the fos and jun peptides. Under reducing condition, a

small fragment of 8 KD appears which probably represents the fos-epitope fusion protein alone. We feel that the most likely explanation for this unexpected behaviour is the formation of single disulfide bonds between more than one fos and more than one jun, rather than as expected (and portrayed in the literature), two disulfide bonds between a single fos and a single jun. Such unwanted disulfide bonds may result in non-functional polymers which perhaps interfere with correct display of the C terminal epitope and so with the ELISA experiment.

The use of a C-terminal random peptide to determine the epitope of MN423.

In view of the disappointing results obtained with the C terminal peptide library made in pJuFo, we felt another system was required. At about the same time as these experiments were being carried out, a C terminal phage display system was described (Cesareni, Montpellier Phage Club Meeting, 1999) which was based on lambda instead of filamentous phage. In addition to the different organism used, this library also had the, perhaps crucial, difference that display was polyvalent, rather than monovalent.

When we carried out a selection with this library (kindly provided by Gianni Cesareni),

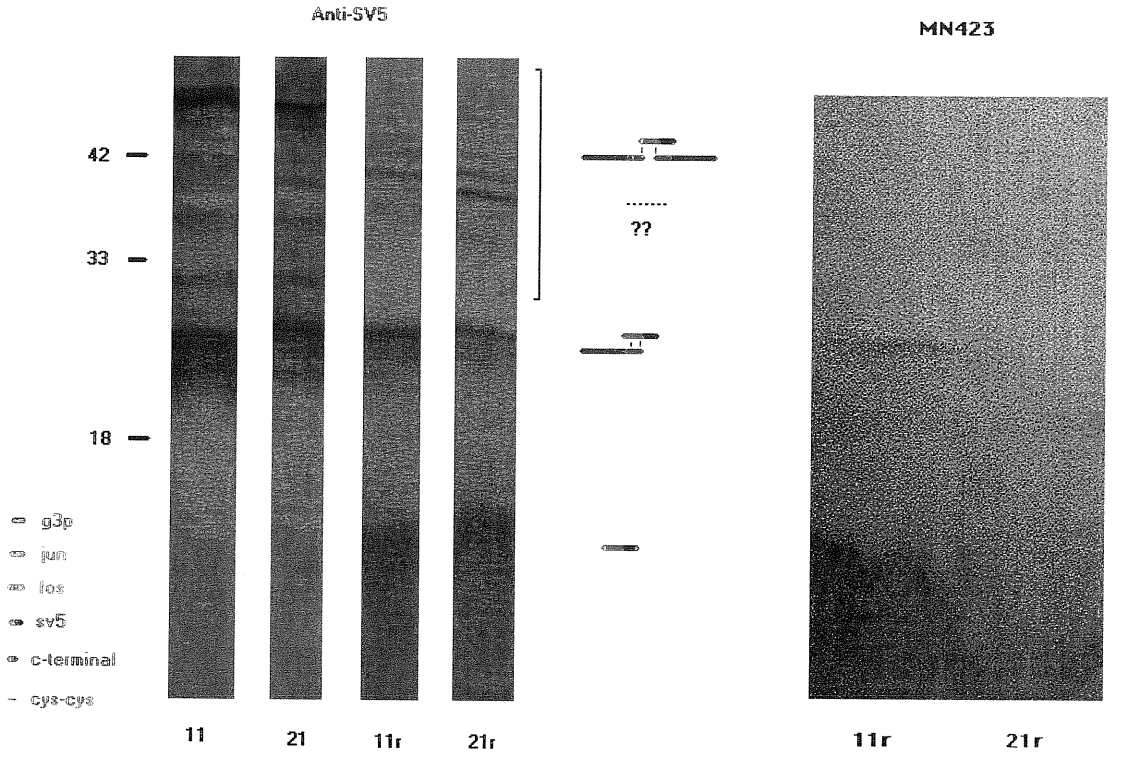


Fig.28. Western blots on the two different test clones engineered in way to be recognised by two different antibodies MN423 and YL1/2, having both C-terminal epitopes. In the clone 11 fos is fused to the sequence --DHGAE at the C-terminal (MN423 epitope) and in the clone 21 fos is fused to the sequence –-DHEEF at the C-terminal (YL1/2) epitope. Both the clones also bear a SV5 tag in the fos-epitope fusion protein which is used for detection (left). The two first lanes (11,21) were run in non-reducing conditions while lanes 11r and 21r were run in the presence of ß-mercaptoethanol. On the right, is shown how 423 recognizes in western in reducing conditions the same length fragments in the clone 11 (-DHGAE) and not in the clone 21 (-DHEEF) used as control.

using MN423 as the selector adsorbed to immunotubes, the three cycles showed an increase in the output numbers, even when washes were performed with increased stringency for each cycle. This seemed to be a good indication that we were actually enriching for binders. The input and output numbers are given in Chart 4.

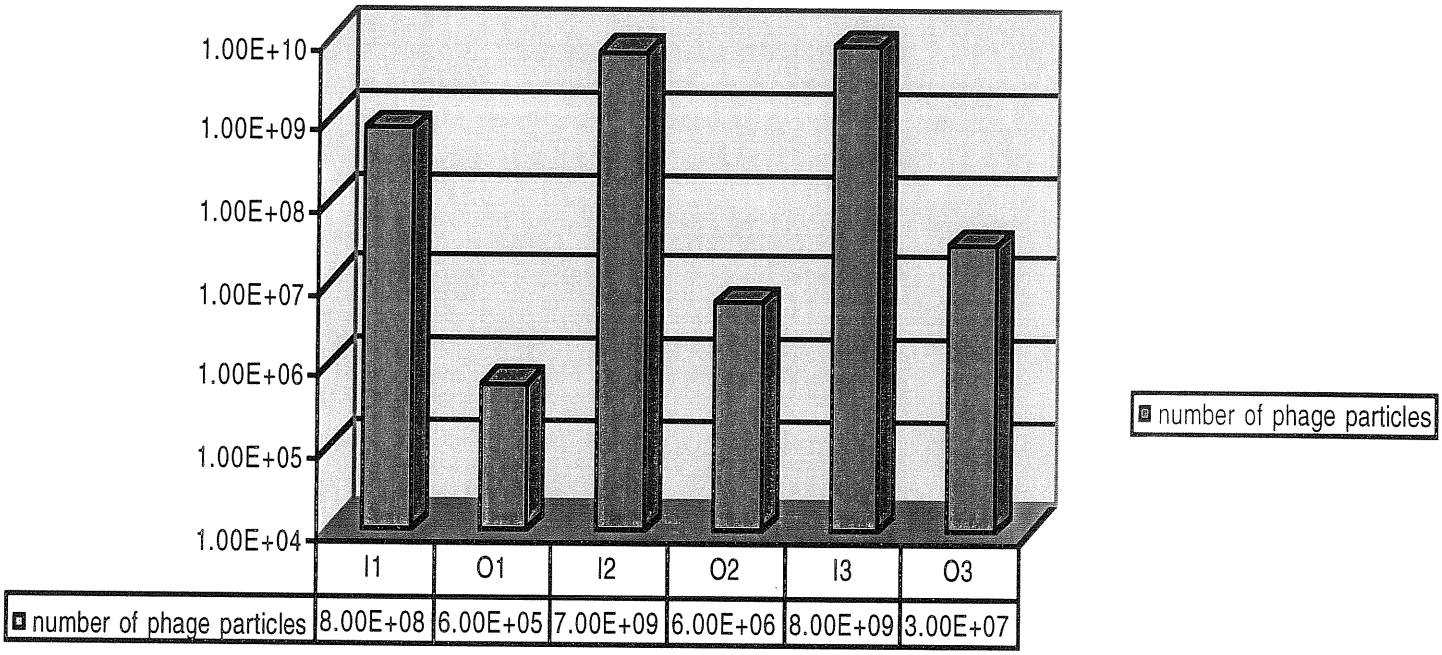


Chart 29. Titer of input (I) and output (O) lambda phage particles before and after each of three round of selections on MN423 antibody adsorbed on solid phase. Washes were made of increasing stringency after each round.

After the third round of selection 20 plaques were isolated and PCR tested for the presence of the insert coding for the peptides, as shown in figure 30.

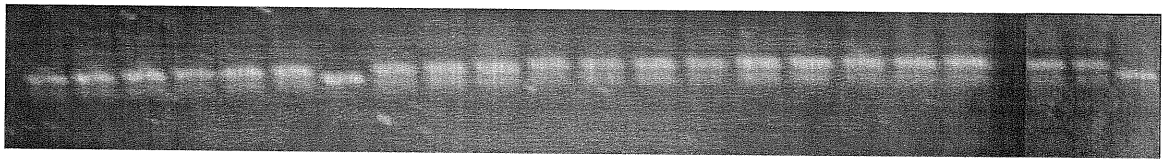


Fig.30. 2% agarose gel showing PCR fragments amplified from 20 lambda clones after three rounds selection on MN423. Primers were choosen to span the region coverng the C-terminal end of λ protein D. Fragments are 383 bp respectively from selected clones bearing the peptides and 345 bp from the empty vector. All analysed clones showed the presence of the insert (383bp) and no duplicate inserts. Lanes 7 and 22 show the PCR from the empty vector (345bp) for comparison.

All clones contained insert, and were sequenced. Of these sequences one was not readable. The amino acid sequence alignment is shown in figure 31.

The red marked peptide corresponds to the sequence of the truncated tau peptide, while the green marked peptide correspond to the epitope discovered by cDNA screening (DNA PK out of frame). Two different alignment with the historically first and second epitopes are shown. It seems clear that the epitope sequence requires some amino acids in certain position, while other positions are more indefinite. The glycine at –3 position is present in all the clones, showing clearly that G at –3 is essential for recognition. Other positions seem to be more flexible. In fact, while the A in the –2 position can exchanged be with a V or I but not others, the –1 position is generally hydrophobic or negatively charged. Moreover, the presence of negative charges upstream of –3 position seems to be of some relevance, revealed by a statistical analysis of the sequences.

The number of times each individual amino acid is found at each position is shown in table 32 and chart 33.

This illustrates very clearly the predominance of glycine at –3, and the alanine at –2, but also brings some other biases into prominence. These become even more clear when examined in a statistical context which compares the frequency of each amino acid at any particular position, and the frequency with which such an amino acid would be expected (tables 34 and 35).

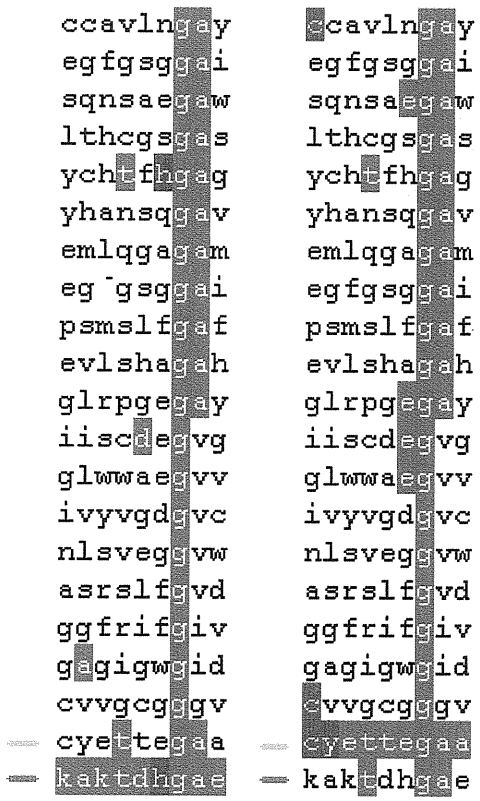


Fig.31. Amino acid alignment of the 19 peptides found after three cycles of selections on MN423 antibody. On the left is shown the alignment with the first found epitope (truncated Tau, ending with -DHGAE), on the right is shown the alignment referred to the second found epitope (DNA-PK out of frame, ending with --CYETTEGAA). In yellow are shown polar or charged groups, in dark blue are marked cysteine and aa with particular structural relevance, and in green simple identities. Below, table 32. The number of times each amino acid is found at each particular position is shown above.

Number of residues found in different positions

AP	-9	-8	-7	-6	-5	-4	-3	-2	-1	T
A	1	1	2	0	2	2	0	11	0	19
V	0	3	1	3	0	0	0	5	4	16
I	2	1	0	1	1	0	0	2	2	9
L	1	3	2	0	3	0	0	0	0	9
M	0	1	1	0	0	0	0	0	1	3
P	1	0	0	1	0	0	0	0	0	2
W	0	0	1	1	0	1	0	0	2	5
F	0	0	3	0	1	3	0	0	1	8
Y	2	0	1	0	0	0	0	0	2	5
T	0	1	0	1	0	0	0	0	0	2
Q	0	1	0	1	0	1	0	0	0	3
N	1	0	1	1	0	1	0	0	0	4
C	2	2	0	2	1	0	0	0	1	8
S	1	2	2	4	3	1	0	0	1	14
G	4	3	1	3	5	4	19	1	2	42
R	0	0	2	1	0	0	0	0	0	3
K	0	0	0	0	0	0	0	0	0	0
H	0	1	2	0	1	1	0	0	1	6
E	4	0	0	0	1	4	0	0	0	9
D	0	0	0	0	1	1	0	0	2	4

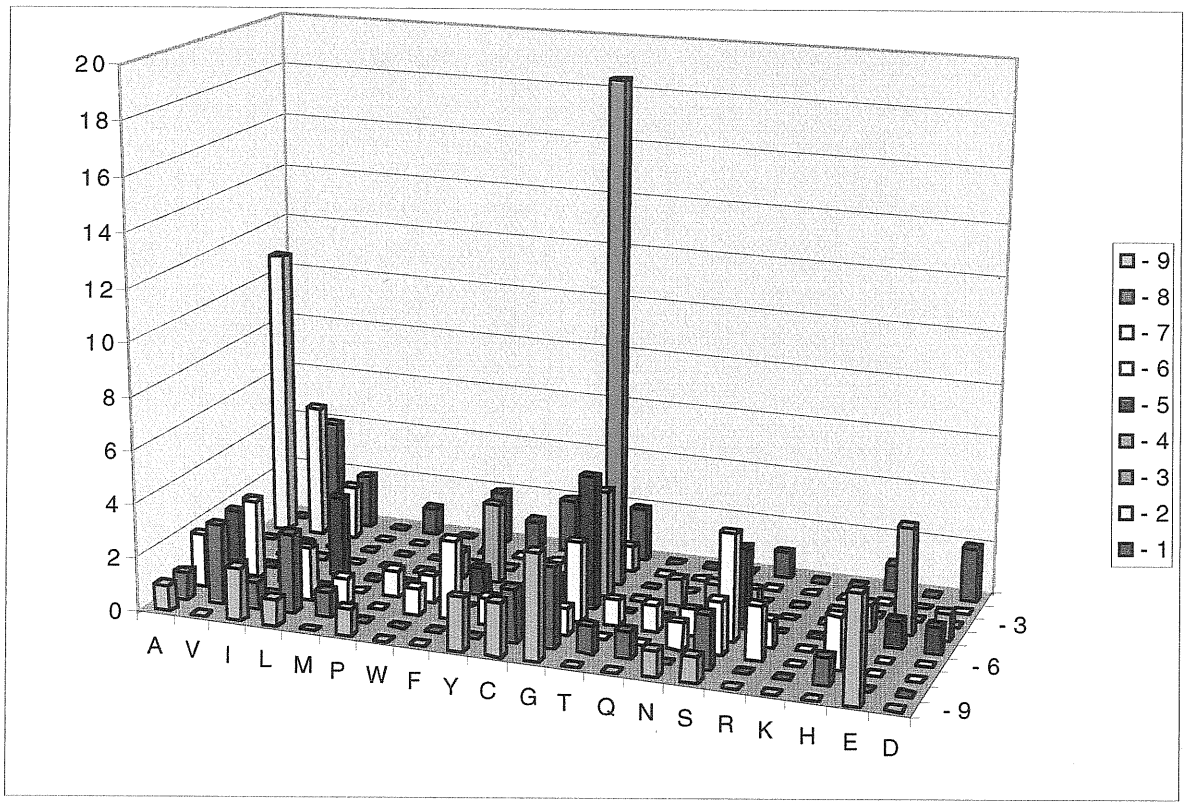


Chart 33. In this chart, aminoacidic residues in 20 selected and sequenced peptides are grouped according to lateral chain: hydrophobic, hydrophilic, basic and acidic and the number of times each amino acid was found at eact position is plotted in relation to the C-terminal. -1 is the last aminoacid in the C-terminal nonapetide, -2 is the previous position and so on. Below, Table 34. Normalised frequencies. 100 is the expected frequency, and amino acids found at significantly higher than expected frequencies are shown in bold.

Normalized frequencies 100=expected frequency

AA	-9	-8	-7	-6	-5	-4	-3	-2	-1	T
A	--81	--81	-163	---0	-163	-163	---0	**-897**	---0	-172
V	---0	-244	--81	-244	---0	---0	---0	-407	-326	-145
I	-326	-163	---0	-163	-163	---0	---0	-326	-326	-163
L	--54	-163	-108	---0	-163	---0	---0	---0	---0	--54
M	---0	-163	-163	---0	---0	---0	---0	---0	-163	--54
P	--81	---0	---0	--81	---0	---0	---0	---0	---0	--18
W	---0	---0	-163	-163	---0	-163	---0	---0	-326	--90
F	---0	---0	**-489**	---0	-163	-489	---0	---0	-163	-145
Y	-326	---0	-163	---0	---0	---0	---0	---0	-326	--90
T	---0	--81	---0	--81	---0	---0	---0	---0	---0	--18
Q	---0	-163	---0	-163	---0	-163	---0	---0	---0	--54
N	-163	---0	-163	-163	---0	-163	---0	---0	---0	--72
C	-326	-326	---0	-326	-163	---0	---0	---0	-163	-145
S	--54	-108	-108	-217	-163	--54	---0	---0	--54	--84
G	-326	-244	--81	-244	**-407**	-326	**1550**	--81	-163	-380
R	---0	---0	-108	--54	---0	---0	---0	---0	---0	--18
K	---0	---0	---0	---0	---0	---0	---0	---0	---0	---0
H	---0	-163	-326	---0	-163	-163	---0	---0	-163	-108
E	**-652**	---0	---0	---0	-163	**-652**	---0	---0	---0	-163
D	---0	---0	---0	---0	-163	-163	---0	---0	-326	--72

AA	-9	-8	-7	-6	-5	-4	-3	-2	-1	T
A	--81	--81	-115	---0	-115	-115	---0	-270	---0	--39
V	---0	-141	--81	-141	---0	---0	---0	-182	-163	--36
I	-230	-163	---0	-163	-163	---0	---0	-230	-230	--54
L	--54	--94	--76	---0	--94	---0	---0	---0	---0	--18
M	---0	-163	-163	---0	---0	---0	---0	---0	-163	--31
P	--81	---0	---0	--81	---0	---0	---0	---0	---0	--12
W	---0	---0	-163	-163	---0	-163	---0	---0	-230	--40
F	---0	---0	-282	---0	-163	-282	---0	---0	-163	--51
Y	-230	---0	-163	---0	---0	---0	---0	---0	-230	--40
T	---0	--81	---0	--81	---0	---0	---0	---0	---0	--12
Q	---0	-163	---0	-163	---0	-163	---0	---0	---0	--31
N	-163	---0	-163	-163	---0	-163	---0	---0	---0	--36
C	-230	-230	---0	-230	-163	---0	---0	---0	-163	--51
S	--54	--76	--76	-108	--94	--54	---0	---0	--54	--22
G	-163	-141	--81	-141	-182	-163	-355	--81	-115	--58
R	---0	---0	--76	--54	---0	---0	---0	---0	---0	--10
K	---0	---0	---0	---0	---0	---0	---0	---0	---0	---0
H	---0	-163	-230	---0	-163	-163	---0	---0	-163	--44
E	-326	---0	---0	---0	-163	-326	---0	---0	---0	--54
D	---0	---0	---0	---0	-163	-163	---0	---0	-230	--36

Table 35. N normalized. This gives a measure of the significance of the variations found.

The results again emphasise the importance of glycine at –3 and alanine at –2. However in addition to these figures (which show the highest levels of significance), there also appear to be a predominance of glycines in the amino acids preceding the invariant glycine (with the exception of position –7), as well as glutamates at positions –9 and –4. Interestingly, the one upstream position (-7) where glycine is lacking appears to have a predominance of phenylalanine.

An alternative approach for mapping C terminal epitopes

The results on the epitope recognition of MN423 were essentially confirmed by experiments carried out by a team led by Michal Novak in Bratislava. These results and those following are presented here for completeness and because they bear directly on the discussion.

Three independent libraries of truncated human tau in the expression vector pET17b were created by PCR. Each library contained bGAE (see table 36), with one of the following C terminal amino acids degenerated: 391 (GAX library), 390 (GXE library), 389 (XAE library). After cloning, and plating, each library was immunoscreened using MN423 to identify positive clones, and so identify the epitopic requirements of this antibody for aminoacids at the last three C terminal amino acid positions known to constitute the minimal MN423 epitope (Novak, Kabat et al. 1993). The total number of colonies on each plate screened as well as number of positive colonies was counted (table 36). In the GAX library more than 50% of the colonies screened were identified as positive, in the GXE library approx 30% colonies were MN423 positive and in the case of XAE library only 5 positives were identified. The number of positive versus negative colonies in each library illustrates that the strictest requirements are found at position 389, this strictness being relaxed towards the C terminal 391 position.

Selected positive clones were used for preparation of dsDNA plasmid DNA for DNA sequencing to identify the C terminal sequence. The number of sequenced clones and their sequences are documented below.

GAX Clones (67 clones)	**GXE Clones (16 clones)**	**XAE Clones (5 clones)**	**GAX, MN423 Negative Clones (12 clones)**
16x GA	3x GTE	5x GAE	12xGAP
11x GAS	5x G E		
9xGA	3x G E		
8x GA	G E		
6x GA	GHE		
5x GAE	GKE		

5x GAH	GSE		
4x GAY	GVE		
3x GAT	GPE		
2x GA			
2x GA			
1x GAD			
1x GAM			

Table 36. Sequences of the 3 C terminal residues obtained by translation of DNA sequence of 423 positive clones. Amino acids are colour coded:
acidic (red), basic (blue), uncharged polar (green), nonpolar (yellow) (from M.Novak).

Affinity analysis of peptides recognized by MN423

MN423 stains neurofibrillary tangles in AD brain, but nothing in normal brain (Mena, Wischik et al. 1991; Mena, Robitaille et al. 1992; Novak, Kabat et al. 1993; Mena, Edwards et al. 1996). In light of the diversity of epitopes which appear to be recognized, this was somewhat surprising. In order to investigate this point further, the affinity of MN423 for a number of different peptides (see table 37) was analyzed by a solid phase competitive ELISA (Harrington, Mukaetova-Ladinska et al. 1991) (figure 38). Affinities for DHGAE-N-terminus, GAEI, HGA,EAG,GAI,GAH, GAW could not be measured.

		Solid phase competitive ELISA	
Name	SEQUENCE	EC50	Hill slope
BGAE	Truncated tau (...-391)	6.7e-9	0.796
Ac5-GAE	CH3-Asp-His-Gly-Ala-Glu	3.07e-6	1.316
DHGAE	Asp-His-Gly-Ala-Glu	5.39e-6	1.271
Ac10-GAE	CH3-Ala-Lys-Ala-Lys-Thr-	1.16e-5	1.295

	Asp-His-Gly-Ala-Glu		
GAD	Gly-Ala-Asp	1.67e-3	0.8535
GAA	Gly-Ala-Ala	2.57e-3	1.581
GAE	Gly-Ala-Glu	1.13e-2	0.8374

Table 37: peptides tested for affinity to MN423 and their sequence and affinity for MN423 (from M. Novak).

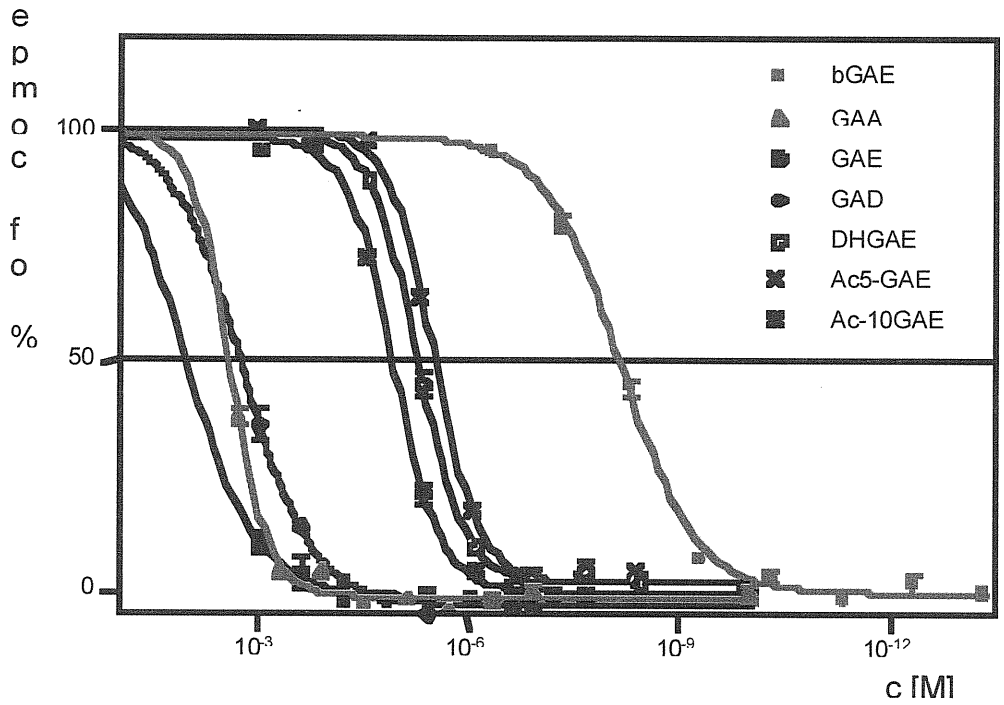


Fig.38. Solid-phase competitive ELISA (Harrington, Mukaetova-Ladinska et al. 1991), (from M.Novak).

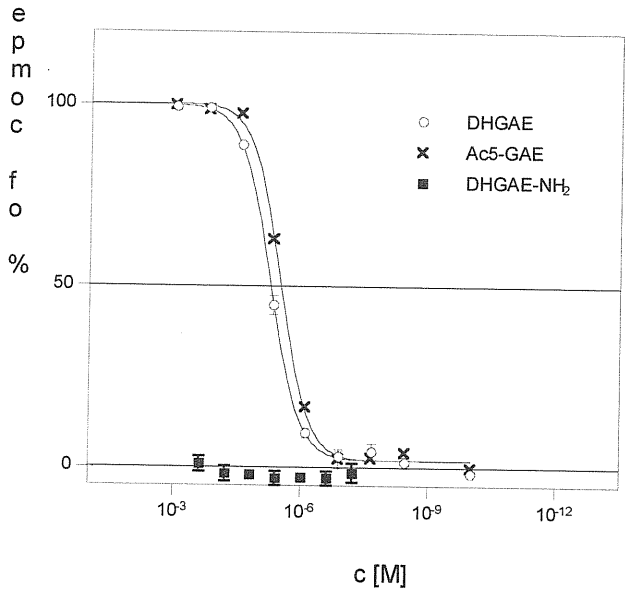


Fig. 39. Comparison of competition curves of peptides DHGAE, Ac5-GAE and DHGAE-NH_2 (from M.Novak).

Of all the peptides tested only six were able to compete with bGAE, but with affinities 10^3x -10^7x lower (figure 38, table 37). Acetylation of the N-terminus led to only moderate increases of affinity, while amidation of the C-terminal carboxyl group led to complete loss of affinity, even for high affinity peptides (figure 39), as did the addition of a single amino acid. Of the tripeptides identified in the selection experiments, only GAA, GAD and GAE were able to bind, albeit with low affinities of questionable significance. Others (such as GAI, GAH and GAW) identified as being potential binders in the molecular diversity experiments had no measurable affinity whatsoever. The results confirm the importance of the C terminus as well as the presence of glycine at position –3. Interestingly, extending the peptide from DHGAE to contain the C terminal ten amino acids of bGAE did not increase the affinity, which remained still 1000 fold lower than bGAE, the 'natural' antigen.

2.2 Tau is processed in vitro by Caspase 3

Tau is processed during apoptosis induced in cerebellar neurons that undergo apoptosis after deprivation of potassium and serum (Canu, Dus et al. 1998). The cleavage can be inhibited by both caspase and calpain inhibitors, and treatment of tau by caspase 3 in vitro reduces the size of tau by about 5 kDa (Canu, Dus et al. 1998).

Three different putative caspase 3 sites, i.e. the amino acidic sequence DXXD (Wang 2000), can be found in tau, and it was not clear which of these was actually targeted by caspase 3. Of these, one is in the middle of the protein, and so cannot correspond to the 5kDa reduction in size, while either of the remaining two, which are found at the N and C terminals of the protein are consistent with a 5kDa reduction of tau protein.

With the aim to better understand which of the two possible of three putative cutting site in the protein was actually processed by caspase 3 we constructed two mutants.

We started from a pSG5 vector bearing DNA sequence coding for tau full length (kindly donated by Michal Novak). From this, we PCR amplified two different fragment in such a way to change, in each mutant, the second aspartic acid of the sequence DXXD in alanine. We designed primers in a way to exploit restriction sites both inside the tau sequence itself and sites flanking tau coding DNA, in the polylinker, to clone back the obtained PCR fragments. The mutants were verified by PCR amplification of the whole tau coding sequence and subsequent fingerprint. The procedure used is shown in figure 40.

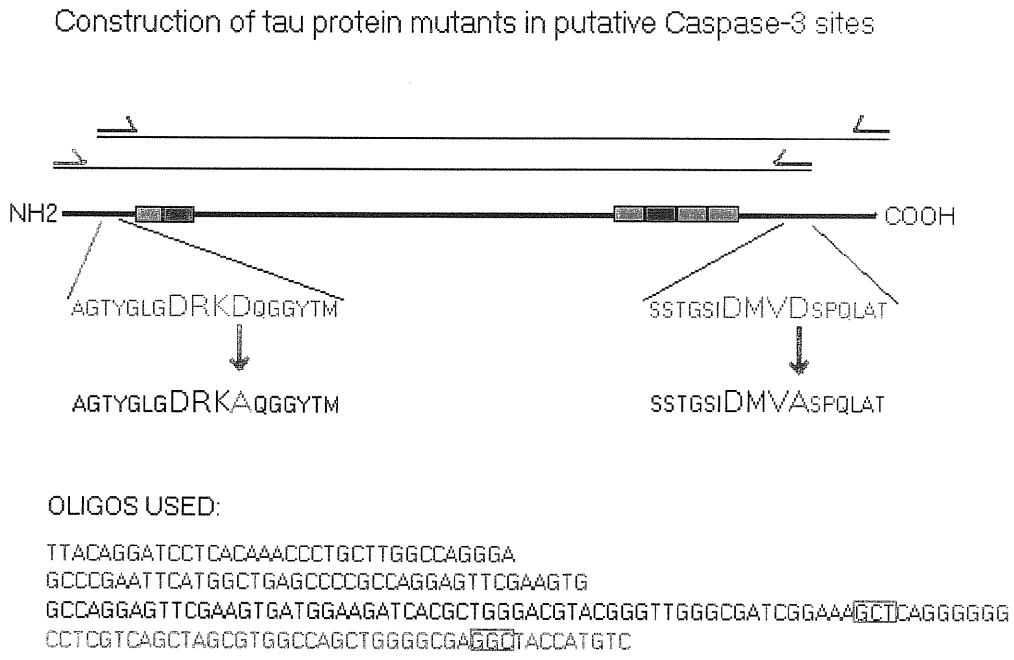


Fig. 40. Here is shown the construction of two mutant tau proteins. Two PCR amplifications were made with oligos designed in way to mutate the second aspartic acid of the sequence DXXD in alanine and containing additional new restriction sites for subsequent test of the cloning. Than the two PCR fragments were sub-cloned in the original pSG5-Tau vector, using restriction enzymes cutting both inside the Tau gene sequence and aside (see Materials and Methods).

Then, the two mutants were in vitro translated and digested with caspase 3. (in collaboration with C. Brancolini, CIB (Trieste)) In a separate reaction mixture also a Caspase 3 inhibitor (N-acetyl-DEVD-aldehyde) was added. The product was finally analysed by PAGE. In figure 41 are shown the results. While the N-terminal mutant shows a digestion product (left side) similar to wild type tau, the C-terminal mutant is not processed at all, indicating that the caspase 3 site recognized when cleaved in vitro is the C terminal site.

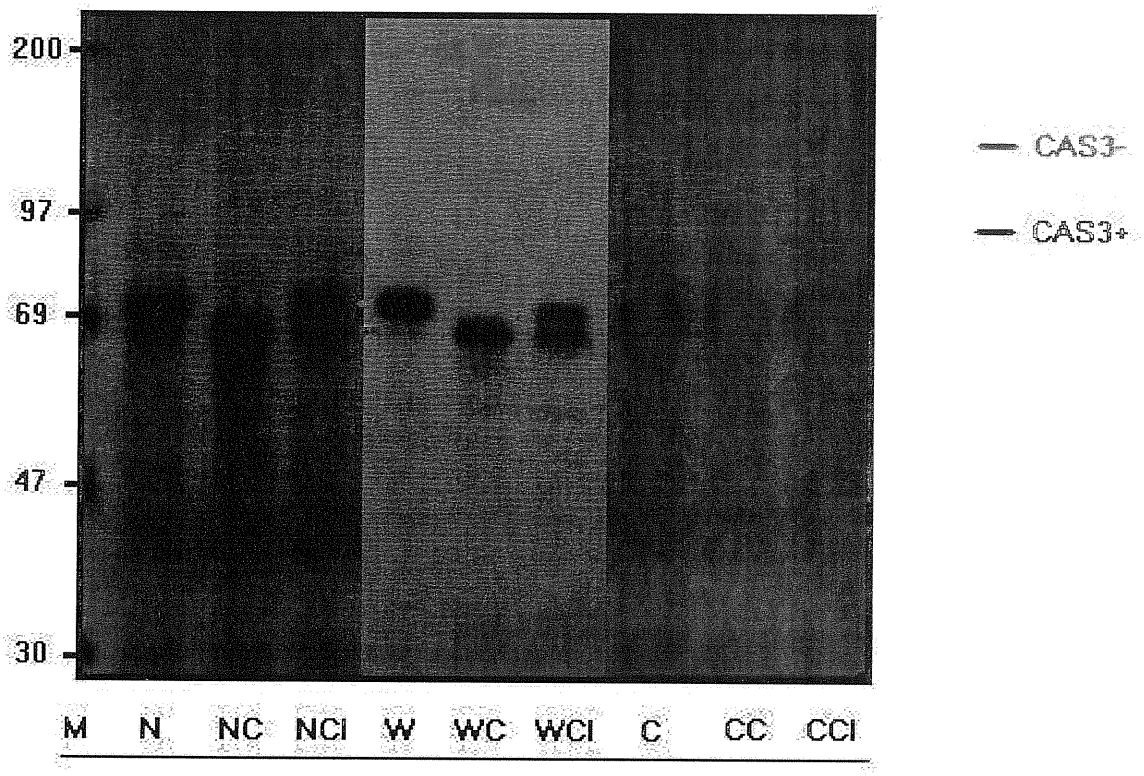
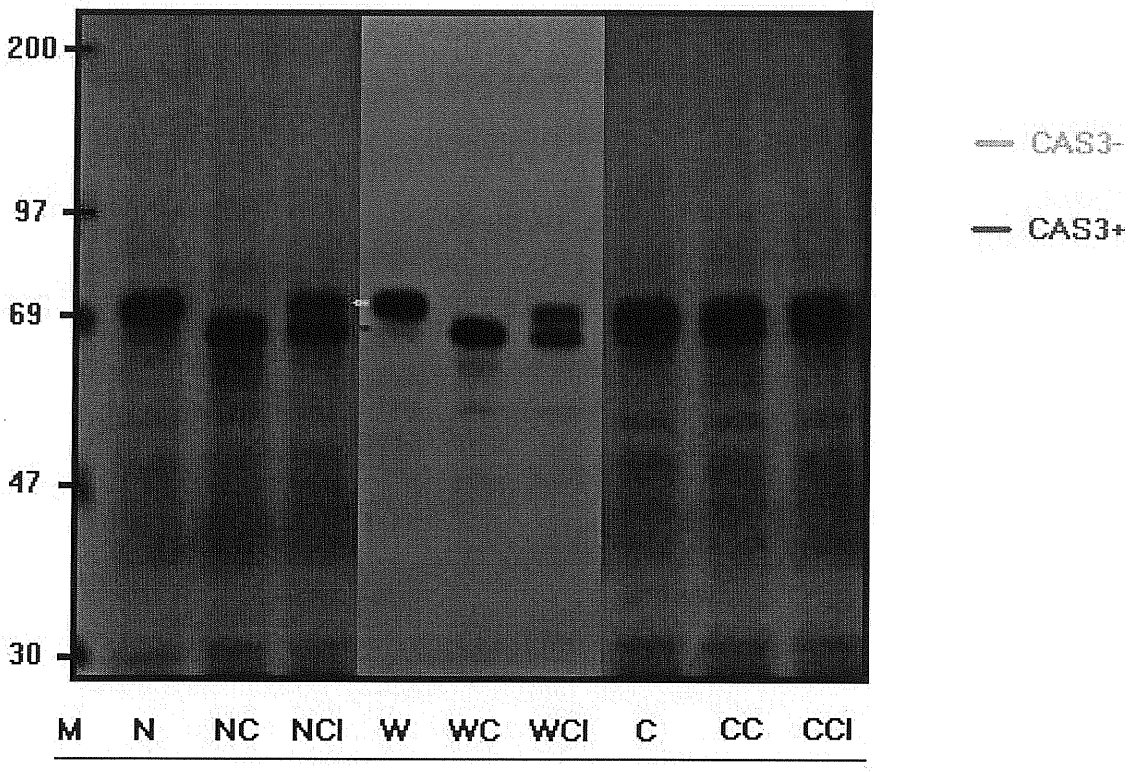


Fig. 41. Gel autoradiography of in vitro translation and Caspase 3 cleavage of the two mutant Tau proteins described above directly compared with the wild type. The Caspase 3 inhibitor used was N-acetyl-DEVD-aldehyde. Legend: M= marker, N= N-terminal Caspase 3 site mutant, NC= N-terminal Caspase 3 site mutant + Caspase 3, NCI= N-terminal Caspase 3 site mutant + Caspase 3 + inhibitor, W= wild type Tau protein, WC= wild type Tau protein + Caspase 3, WCI= wild type Tau protein + Caspase 3 + inhibitor, C= C-terminal Caspase 3 site mutant, CC= C-terminal Caspase 3 site mutant + Caspase 3, CCI= C-terminal Caspase 3 site mutant + Caspase 3 + inhibitor. Green and red dashes indicate the position respectively of uncleaved and cleaved products. The C-terminal mutant is not affected by the protease. The proteolytic cleavage is partially reduced in presence of the inhibitor.



41. Gel autoradiography of in vitro translation and Caspase 3 cleavage of the two ant Tau proteins described above directly compared with the wild type. The Caspase 3 bitor used was N-acetyl-DEVD-aldehyde. Legend: M= marker, N= N-terminal base 3 site mutant, NC= N-terminal Caspase 3 site mutant + Caspase 3, NCI= N-inal Caspase 3 site mutant + Caspase 3 + inhibitor, W= wild type Tau protein, WC= type Tau protein + Caspase 3, WCI= wild type Tau protein + Caspase 3 + inhibitor, C-terminal Caspase 3 site mutant, CC= C-terminal Caspase 3 site mutant + Caspase 3, = C-terminal Caspase 3 site mutant + Caspase 3 + inhibitor. Green and red dashes cate the position respectively of uncleaved and cleaved products. The C-terminal ant is not affected by the protease. The proteolytic cleavage is partially reduced in ence of the inhibitor.

2.3 Towards generating tau isoform specific antibodies by phage display

Tau isoform ratio: an important factor for neurodegenerative diseases

Mutations in the tau gene causing subsequent imbalances in the ratio between tau isoforms have been shown to be causal in FTDP-17 (frontotemporal dementias with Parkinsonism linked to chr 17) (Clark, Poorkaj et al. 1998; Hutton, Lendon et al. 1998; Spillantini, Murrell et al. 1998; Yen, Hutton et al. 1999), a disease in which abundant tau deposits are found in cortex, brain stem and spinal cord.

For AD, notwithstanding a great deal of effort, no mutations have been found in the Tau gene (Roks, Dermaut et al. 1999). Nevertheless we believe that a tool able to discern in a non ambiguous way, between different isoforms and/or between different post-translationally modified tau, would be extremely useful in the study of this and other diseases. For this reason we attempted to develop isoform specific single chains with the goal to eventually use them directly on AD versus normal tissue protein extracts in both western blots or immunohistochemistry. This should allow us to look for possible imbalances in the isoform ratio. Post-translational modification of tau is very complex. Isoform specific ScFvs would ideally also reduce problems related to those tau post translational modifications which alter the behaviour of tau isoforms on SDS-page, rendering the separation between different isoforms difficult and so hampering subsequent reliable identification in brain extracts.

Obtaining tau isoforms specific ScFv

As described in the introduction above, tau is found in six isoforms, due to the alternative splicing of three exons. Conceptually, there are three methods by which one can contemplate attempting to derive phage antibodies which recognize one protein and not one which is very closely related. The first method involves the identification of specific peptides which are found in one isoform but not the other and to use these peptides for selection. Examples of such peptides could be peptides found within alternatively spliced exons (which will be specific for the form in which such exons are present) and peptides which span the two exons flanking an alternatively spliced exon (which will be specific for the form in which the exon has been spliced out). This is diagramatically illustrated below.

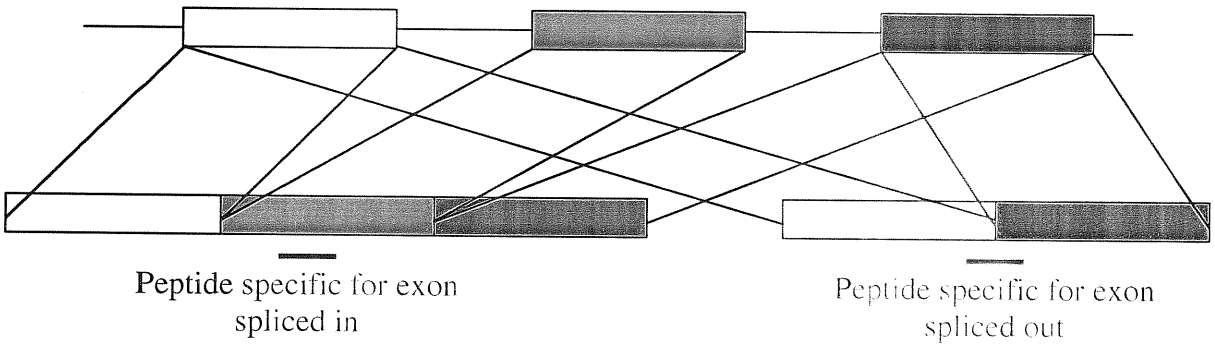


Fig.42. Illustration of specific peptides used to generate probes specific for alternative spliced forms.

The alternative method involves a subtractive approach, in which antibodies are selected against one isoform, in the presence of a vast excess of the other, with the hope that those antibodies which recognize both bind to the isoform in excess. The third method, which may be feasible in conditions in which automation is possible (de Wildt, Mundy et al.

2000; Holt, Bussow et al. 2000), is merely to select against the target of interest and screen against the two forms which are to be discriminated.

The first and second approaches were used with promising results.

Pin-peptide selections

An important issue for this kind of approach has already been already introduced: can peptides serve to find antibodies that recognize the whole protein? And which physical parameters of the protein can influence the choice of peptide for this purpose?

If selection against peptides could be carried out efficiently, it could serve the aim of developing scFvs against virtually every gene product on a genomic scale. In a functional genomics effort, an attempt to find the best way to identify peptide sequences from protein, or genomic sequence, has been made in our lab (Pavlik, submitted). In these experiments, solvent accessibility and flexibility were identified as being the best predictors of peptide antigenicity in a phage antibody context.

For tau, which is thought to be an extended molecule, the structure of which has not been determined, we ran two predictive algorithms. One for secondary structure prediction and the other for solvent accessibility. However, there is a fundamental difference between choosing peptides to select antibodies which should recognize the full length protein, and peptides to select a very specific part of a protein, since there is far less freedom of operation in the latter, as there are very few sequences which fit the criteria of being isoform specific. Even though our peptides were not choosen according to these predictions, but to fit our need to span isoform specific regions in the protein, these data

are nevertheless interesting. In fact, in secondary structure prediction it is possible to see that there is a relation between the presence of more structured parts of the molecules and the assessed or possible roles of those parts. For instance, the four repeat region, known to be essential for microtubule assembly present a higher probability of helical and strand regions. This perturbation of the overall pattern is also seen in correspondence with the N-terminal exons, whose function and possible interaction partners are not known.

Another region of the molecule that appear to be more structured as a helix is the C-terminal part. Solvent accessibility also showed differences between the different known structural features of the protein.

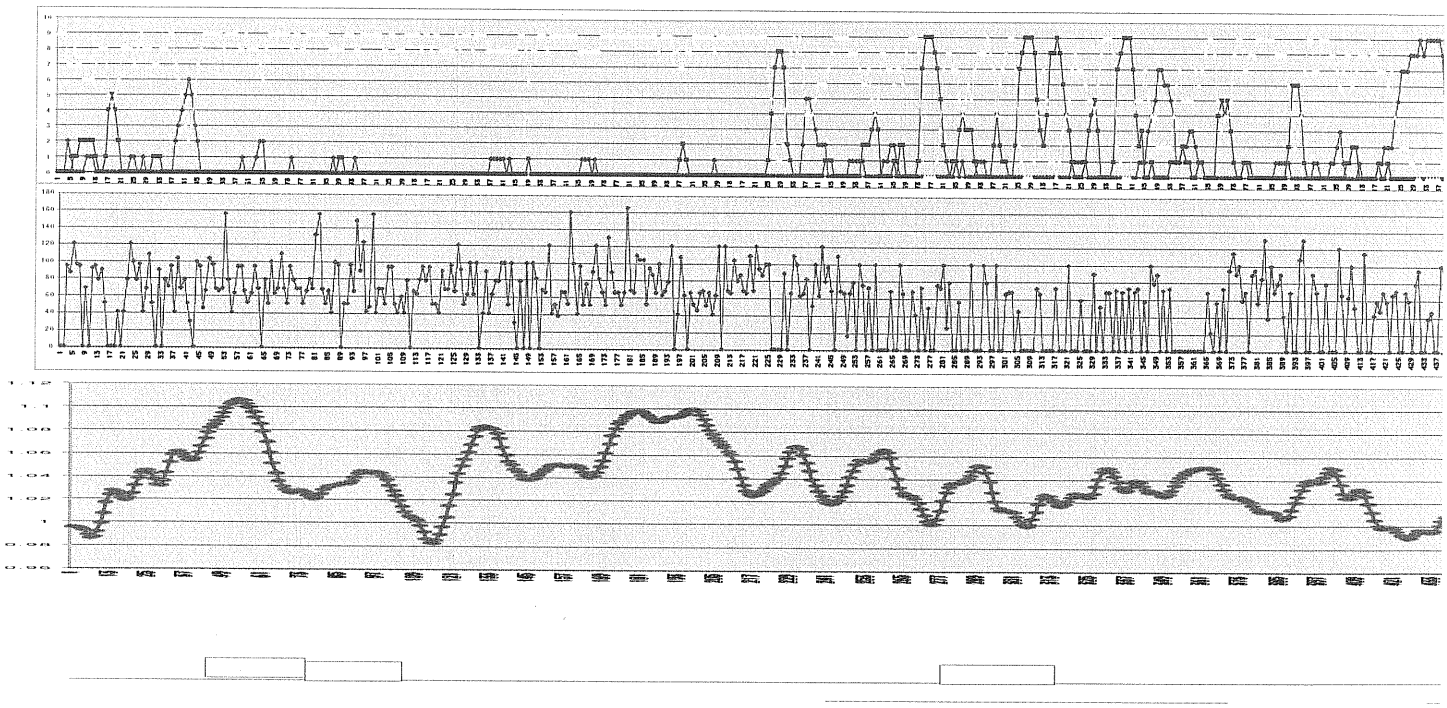


Fig.43. Secondary structure, solvent accesibility and chain flexibility predictions for tau protein. The first two were obtained with a query at http://www.expasy.ch/index.html and further graphically elaborated with Microsoft Excel. In the first panel, dots and lines in blue represent the probability of helix, pink ß strand, yellow others, with the highest probabilities represented by the highest values. In the second panel is shown the predicted

solvent accessibility. In the third panel is shown a graph for predicted chain flexibility values. In this last graph (kindly elaborated by Peter Pavlik), at each corresponding amino acidic positions are plotted average values for the following 15 aa. Underneath the predictions, a schematic view of CNS tau protein is shown with its three alternatively spliced exons and highlighted in red the repeats region and in pink the fragment cleaved by caspase-3 (see Appendix and (Fasulo, Ugolini et al. 2000)).

The length of the peptides used was between 11 and 15 aa, evaluated to be a good enough length to give structure to the peptide and so present an epitope. In the submitted paper cited above, it was found that approximately 30% of 15 amino acid peptides could select antibodies. These were designed to span regions presents in some but not in all isoforms as shown in figure 44. In addition to the exon in specific peptides (E2, E3 and R2), the exon out specific peptides (E2sp, E2/3sp and R2sp), some other peptides were designed which were expected either to be antigenic or which localised to particular tau structures. The peptides in the four different imperfect repeats were kept shorter to avoid using peptides with similarities to other parts of the repeat region, so increasing the possibility of finding repeat specific binders.

There are a number of different options available for antibodies selection on peptides: soluble biotinylated peptides, peptides coupled to a carrier and peptides attached to pins.

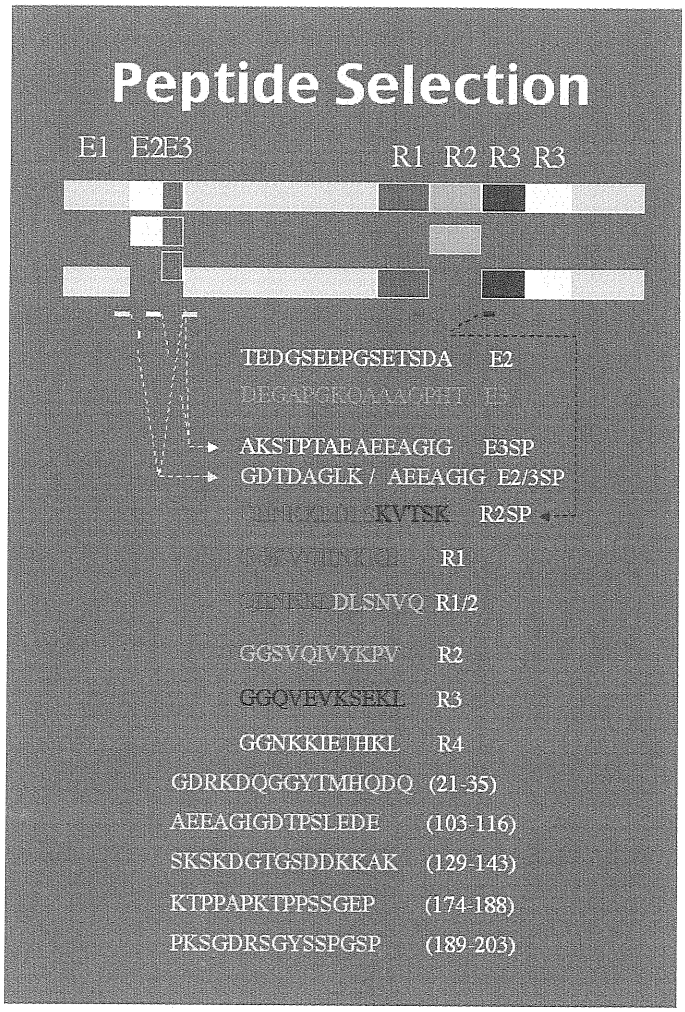


Fig.44. A scheme of peptides used, some designed to be isoform specific.

We decided to use peptides attached to pins, since these had proved to be relatively successful in the functional genomics approach described above and have been used extensively in the past for monoclonal antibody epitopes mapping (Maeji, Bray et al. 1990; Patarapotikul, Pothipunya et al. 1993; Maeji, Bray et al. 1995). These pins are reusable and we used them directly for both biopanning experiments and for subsequent ELISAs to test output clones. Typical peptide-pins are shown below.

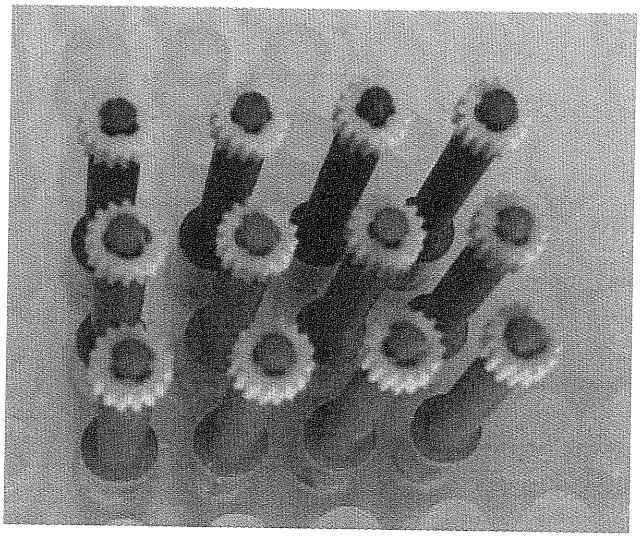


Fig.45. Peptide pins. The peptides are synthesised on the white plastic cogs, with each pin displaying approximately 1μmol of peptide.

Antibodies were selected on such pins (see materials and methods) by incubating a single aliquot of library with all pins simultaneously, washing and then eluting into single wells. Phage were then prepared from single wells and incubated once again with the pin. Pins are easily regenerated, and can be used up to fifty times. Subsequently all interactions occurred in single microtitre wells, so that there was no interaction between different selections, as we have shown that this tends to reduce diversity. Three cycles of selection were carried out, and after the third cycle the polyclonal phage mixtures selected on each pin were tested in ELISA using either full length tau (containing all alternatively spliced exons) or the peptide used for selection. Results are shown in tab 1-5.

These preliminary results show that some of the peptides are able to select polyclonal antibodies which give signals on both the whole protein and peptides. These are promising in the case of R2 which is a peptide specific for the second repeat which is alternatively spliced. However, it is less promising for E2 and E3, the two alternatively

	Selection type														
	Exon-in specific			Exon-out specific			Tau regions common to all isoforms								
Peptide	E2	E3	R2	E3sp	E2/3sp	R2sp	R1	R1/2	R3	R4	21-35	103-116	129-143	174-188	189-203
Predicted flexibility	1.10	1.03	1.00	ND	ND	ND	1.01	1.00	1.03	1.04	1.03	1.02	1.08	1.09	1.09
Solvent accessibility	71.7	81.1	21.7	ND	ND	ND	38.2	37.3	44	57.5	68.3	66.1	71.3	88.4	62.7
Pin Peptide	.425	.452	.555	.428	.706	.610	.534	.608	.544	.564	.752	.412	.747	.577	.626
Tau441 Coating	.111	.086	.356	.291	.914	.099	.448	.105	.307	.077	.087	.213	.213	.314	.210

Table 45. In this table the result of an Elisa experiment on pin-peptides and on tau full length (tau 441) using a polyclonal population of phages obtained after three rounds of selection on pin-peptides is shown. Elisa values and predictions for solvent accessibility and chain flexibility for each peptide are shown in the same column. Prediction on tau chain flexibility, was kindly elaborated by Peter Pavlik at LANL (Pavlik, submitted). ND, not determined.

spliced 5' exons, neither of which give any signal against tau. Interestingly, two of the three peptides which span spliced out exons (E3sp and E2/3sp) give good signals against the full length protein which contains these exons, suggesting that many antibodies are probably recognizing flanking, but not junctional, epitopes. The third exon-out peptide (R2sp) gives a very good signal against the peptide, but no signal against the full length protein, suggesting that in this case antibodies may be recognizing a junctional epitope, although it is also possible that this peptide does not recognize any protein determinants. Of the peptides common to all isoforms, six give signals on the full length tau which are promising, while three (R1/2, R4 and 21-35) give no signal on tau.

Unfortunately, neither these polyclonal phage, nor the peptide pins, survived the trip to Los Alamos, so the second strategy, subtractive selection was attempted.

Subtractive selections using different tau isoforms. An isoform specific single chain.

Although the differences between the different isoforms is relatively small, it was felt that it may be possible to select specific antibodies against different tau isoforms by carrying out subtractive selection schemes in which libraries were depleted against one isoform and selected against another. The simplest scheme used is illustrated in figure 46.

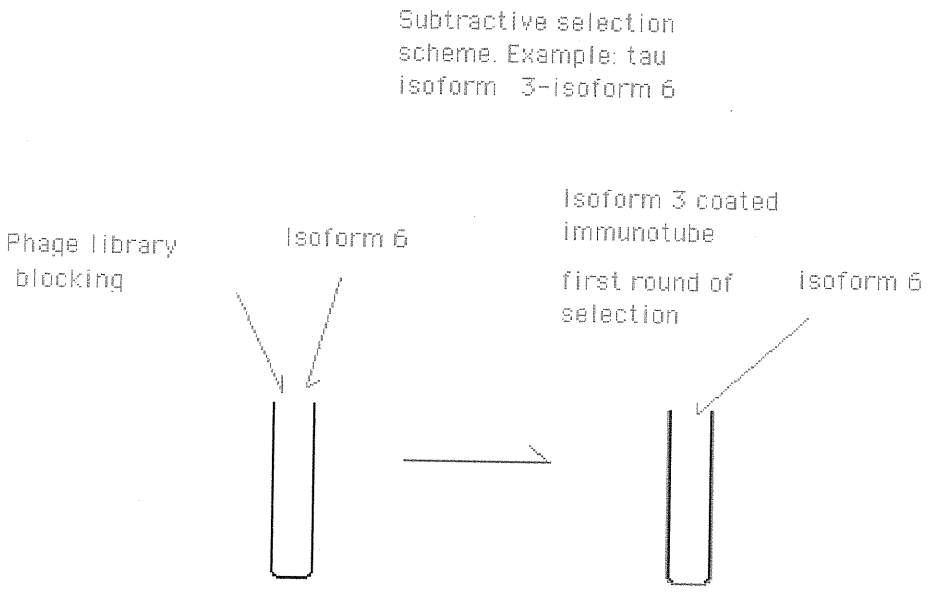


Fig.46. The general scheme that we used for subtractive selection is depicted here. In an tau3-tau6 fashion selection, blocking of the library was made with a 10 to 50 fold of tau6 the concentration of the solution of tau3 used to coat an immunotube. Then the library was biopanned in presence of the same amount of tau6, against tau3 coated onto immunotube. During each step of selection the same amount of tau6 was used in solution with the output from the previous round.

A large number of different subtraction schemes were attempted, illustrated in the table below.

Subtraction scheme Tau isoforms	Library used	Number of positive antibodies	Isoform specific antibodies
3-6	(Marks, Hoogenboom et al. 1991)	6	1
6-3	(Marks, Hoogenboom et al. 1991)	8	0
1-5	(Marks, Hoogenboom et al. 1991)	29	0
5-1	(Marks, Hoogenboom et al. 1991)	18	0
4-5	(Marks, Hoogenboom et al. 1991)	22	0
5-4	(Marks, Hoogenboom et al. 1991)	6	0
1-6	(Marks, Hoogenboom et al. 1991)	ND	0
6-1	(Marks, Hoogenboom et al. 1991)	6	0
5-4	Fd, Marks, unpublished	7	0
4-5	Fd, Marks, unpublished	9	0
5-1	Fd, Marks, unpublished	21	0
1-5	Fd, Marks, unpublished	13	0
1-6	Fd, Marks, unpublished	12	0
6-1	Fd, Marks, unpublished	6	0
Depletion 2,3,4-5	Fd, Marks, unpublished	60	0

Table 47. Subtractive selections were made using two different libraries, one made in a phagemid vector, one in a phage vector (see Materials and Methods).

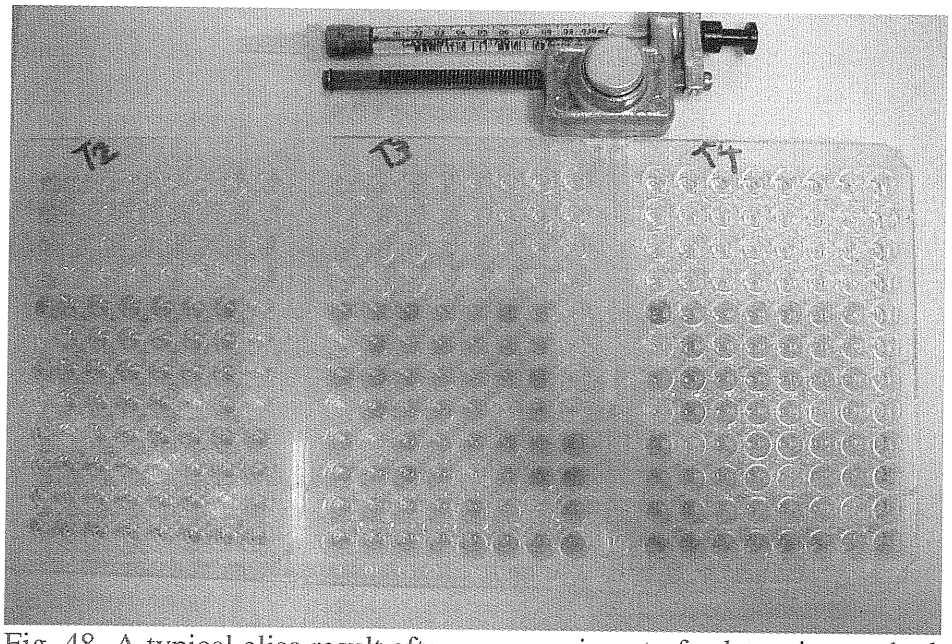


Fig. 48. A typical elisa result after an experiment of subtractive or depletive selection. 60 clones (bottom part of the figure) have been tested against isoforms T2, T3 and T4 probed against two or three isoforms. In this case, the output of a selection carried out using a fd library (J. Marks) is shown. Since in this library all g3p are recombinant and there is no need of a superinfection with helper phage less variability is observed for the same clone in elisa signal. In most experiments, like in this one, no isoform specific ScFvs were found.

Of these, one clone appeared to be isoform specific (see results in table 49). This was able to recognize T2 and T3. These two isoforms, but not others, have the first N-terminal insert in common, indicating that the epitope for this single chain is probably to be found in this part of the molecule, or less likely an epitope whose conformation is induced by the presence of this exon. ELISA results for this single chain on all the different tau isoforms are shown below.

Coating	T1	T1	T2	T2	T3	T3	T4	T4	T5	T5	T6	T6
T3-T6 N3	0.136	0.066	0.241	0.243	0.530	0.310	0.067	0.068	0.054	0.062	0.065	0.064
T3-T6 N9	0.680	1.187	0.844	1.011	0.717	1.019	0.525	0.379	0.782	0.577	0.671	1.010

Tab. 49. Elisa values for the isoform specific ScFv. Elisa wells were coated with the six tau isoforms indicated in the top row. Then a phage supernatant for the isoform specific scFv (T3-T6 N3) plus another anti tau non isoform specific scFv obtained in the same selection (T3-T6 N9) was used in a classical phage ELISA experiment.

As can be seen, scFv T3-T6 N3 only recognises T2 and T3, but not T1, and T4-6, whereas the other scFv (T3-T6 N9) recognises all tau isoforms. The only difference between these isoforms is the presence of exon 3 at the N-terminus. With the exception of T3-T6 N3, all other selected scFvs had the T3-T6 N9 pattern of recognition. This single chain may prove to be useful to understand the function and distribution of exon three in taus expressed in different parts of the brain and the eventual relation of its presence in AD or other neurodegenerative diseases.

Although only one single chain has been selected, that is specific for two of the six tau isoforms, we feel that with more screening, such as automation could provide, it is likely that more clones could be isolated. In particular experiments by Tomlinson et al. (de Wildt, Mundy et al. 2000; Holt, Bussow et al. 2000), which involved the screening of 27000 clones in an array format, are ideal for the high throughput screening which will be required. Our experiment, however, indicates the difficulty of carrying out subtractive selections with phage, and suggests that attempting to carry out such selections on complex mixtures of proteins, such as cell extracts, may be even more difficult, if not impossible.

3. Discussion

Peptide libraries

Peptide libraries are now being widely used for epitope mapping of either monoclonal antibodies (Parmley and Smith 1988; Parmley and Smith 1989; Cwirla, Peters et al. 1990; Balass, Heldman et al. 1993; Smith and Scott 1993; Yayon, Aviezer et al. 1993; Grihalde, Chen et al. 1995; Kola, Baensch et al. 1996) or sera from patients with specific diseases. While the former is relatively straightforward, the latter, although conceptually similar, is practically more difficult, as individual patient sera contain many antibodies able to bind epitopes which have no relevence to the disease under study. For this reason selection on sera from a number of different patients with the same disease is followed by depletion at the nucleic acid level and/or negative screening using sera from normal or diseased controls, this leading to the identification of disease 'phagotopes' as they have termed (Cortese, Felici et al. 1994). This has direct bearing on the latter part of the thesis where the derivation of isoform specific scFvs was attempted, since the different peptide specificities can be compared to the different antibodies binding common parts of tau.

Peptide libraries can also be used to determine binding sites of proteins other than antibodies using the same methodology. For example, the binding specifity of BIP, an endoplasmic reticulum chaperone, was found to consist of a subset of aromatic and hydrophobic amino acids in alternating positions (Blond-Elguindi, Cwiria et al. 1993).

An alternative to using whole proteins is to synthesise individual domains known to be involved in protein-protein interaction. In fact, much of the work which has led to the conclusion that different members of interaction domain families have slightly different specificities has come from work using phage peptide libraries and subsequently confirmed on isolated proteins. SH2 (Songyang 1994; Gram, Schmitz et al. 1997), SH3 (Cheadle, Ivashchenko et al. 1994; Rickles, Botfield et al. 1994; Sparks, Quilliam et al. 1994; Yu 1994; Hoffman, Sparks et al. 1996; Sparks, Rider et al. 1996) and EH (Salcini, Confalonieri et al. 1997) domains have had their binding specificities determined in this way.

Phage peptide libraries, thus, offer the possibility of testing millions of different combinations of peptides in single experiments. However, all such analyses using phage display have been limited to those which are either N terminus or internal, since there has been no simple system which can analyze C terminal epitopes. We attempted to use a widely dispersed filamentous phagemid vector to create a library which would allow this to occur, but found that there appeared to be problems related to display in this vector. It is for this reason that we turned to the use of the lambda peptide library in which the diversity is found at the C terminus of protein D. This allowed us to analyze the epitope specificity of MN423, a monoclonal antibody specific for neurofibrillary tangles in AD.

Analysing the epitope recognized by MN423

The consensus sequence

Two different molecular diversity approaches showed that this antibody recognises a wide range of epitopes with the common feature of a glycine at position -3 and an alanine or valine at position -2. After three cycles of selection with the lambda library, all 19 clones sequences present a common glycine at –3 position, identical to the two first identified epitopes: -DHGAE, -TEGAA. Similarly, when small focussed libraries were created (Novak, personal communication) with diversity within the context of bGAE, the naturally recognized antigen target for this antibody, all clones identified as being positive with MN423 contained a glycine at –3. The conservation at position –2 was less clear, although alanine was present in a high proportion of cases.

It is clear that this glycine, at this fixed position, is a key element for the structure of the epitope. However it is not clear whether this represents a linear epitope with particular amino acid constraints, or a structural epitope defined by the sequence of the amino acids present.

Is there a common structure?

To investigate this possibility, we applied a structural prediction algorithm (Gupta, Anantharamaiah et al. 1993) to the selected peptides to see if they had any common features. Although, such algorithms can be unreliable, especially when applied to such short peptides, they can sometimes provide interesting information, as was the case with these peptides (see table ~~1-6~~ 50).

Peptide sequence	Predicted structure
k a k t d h g a e	H H H H T T H H
c y e t t e g a a	H H H H H H H H
c c a v l n g a y	H H H B H T H T
e g f g s g g a i	T T T T T T T B
s q n s a e g a w	T T T T H H H H
l t h t f h g a s	H B C B H C H T
y c h t f h g a g	B B C B C T T T
y h a n s q g a v	C H H T T T H B
e m l q g a g a m	H H H H T H H H
e g f g s g g a i	T T T T T T T B
p s m s l f g a f	C C H T H H H H
e v l s h a g a h	H B H H H H H H
g l r p g e g a y	T T T T T T T T
i i s c d e g v g	B B B C T T T T
i v y v g d g v c	B B B B T T B B
c v v g c g g g v	B B B T T T T B
n l s v e g g v w	T T T B T T B B
a s r s l f g v d	H H H T H B B C
g a g i g w g i d	T T T T T C B C
g l w w a e g v v	T H H H H H H B B
g g f r i f g i v	T T B B B B B B B

Table 50. The predicted structure, using the algorithm described in (Gupta, Anantharamaiah et al. 1993), of the different selected peptides is given. The following abbreviations are used: H-helix; T-turn; C-coil; B-sheet.

It is possible to observe that in all but two peptides, the predicted structure in the position of the –3 glycine is a T=turn, suggesting that this is perhaps an essential part of the recognition specificity. However, a turn alone is unlikely to provide sufficient specificity, indicating that this, coupled with perhaps the terminal carboxy group is what is being recognized. Nevertheless, what is remarkable about the epitope recognition is the lack of hard defining features beyond the glycine, and its distance from the C terminus. Other peptides containing glycines with a T prediction are also present, although whether these contribute anything to the recognition is uncertain.

It would be interesting to synthesize some of these peptides and determine their structure by NMR, to see whether the presence of this turn is confirmed, and whether binding by the antibody changes its structure in some way. Similarly crystallization of this antibody in complex with a recognized peptide and/or the full length protein antigen, with structural determination, would allow a deeper understanding of the requirements of binding.

Database search for epitope specificity

Given the epitope recognition specificity of MN423, and its extreme specificity for neurofibrillary pathology in AD brain, we searched protein databases at the Expasy web site (http://expasy.hcuge.ch/) for other potential proteins which could be recognized by MN423 on the basis of the peptide sequences identified by peptide libraries. The search pattern used was G[AV]{LRMKPFTQN}>. This requires a glycine at –3, an alanine or valine at –2 and any amino acid except for LRMKPFTQN at position –1.

Some potentially interesting matches found are shown in the table below:

Ref. N.	Name	Sequence
B3AR_HUMAN P13945	BETA-3 ADRENERGIC RECEPTOR. Homo sapiens (Human)	406-408 GVS
FXR1_HUMAN P51114	FRAGILE X MENTAL RETARDATION SYNDROME RELATED PROTEIN 1. Homo sapiens (Human)	619-621 GVS
FXR2_HUMAN P51116	FRAGILE X MENTAL RETARDATION SYNDROME RELATED PROTEIN 2. Homo sapiens (Human)	671-673 GVS

SM32_HUMAN P55855	UBIQUITIN-LIKE PROTEIN SMT3B (SENTRIN 2). Homo sapiens (Human), Bos taurus (Bovine)	93-95 GVY
VE5_HPV5B P26551	PROBABLE E5 PROTEIN. Human papillomavirus type 5b	166-168 GVC
O76035 O6035	POTASSIUM CHANNEL H-EAG. Homo sapiens (Human)	960-962 GAS
O95531 O95531	DJ281H8.4 (UBIQUITIN-LIKE PROTEIN SMT3 LIKE). Homo sapiens (Human)	93-95 GVY
P87914 P87914	ICP0D. Human herpesvirus 1	88-90 GAD

However, the fact that normal human brain shows no staining whatsoever, suggests that none of the proteins above is recognized by MN423, since some of of them, e. g. the Fragile X Mental Retardation Sindrome Related Protein 1, are known to be expressed in brain (Khandjian 1999). This suggests that there is something beyond the epitope specificity identified by the peptide libraries which is contributing to the specificity of this antibody in the staining of AD brain. One interesting observation is that ICP0D, a protein derived from human herpesvirus 1, for which a weak and controversial association with AD has been found (Itzhaki and Lin 1998; Itzhaki, Lin et al. 1998; Dobson and Itzhaki 1999), has a C terminal sequence which could theoretically bind to MN423. Although we feel it is unlikely, it is nevertheless conceivable, that this protein is upregulated in AD brain, becomes associated with neurofibrillary tangles and is recognized by MN423.

The fact that no cDNAs encoding any of these proteins was found during the screening of two cDNA libraries does not completely rule out the possibility of their presence in AD brain. In fact, cDNA libraries are known to bear biases in the complete representation of all cellular mRNAs, especially considering the fact that these have been made from post-

mortem tissues. Furthermore, in the cDNA library which did yield positive clones with MN423 (a commercial AD library purchased from Clontech), the same clone was found 32 times in the million clones screened. This suggests that this library has been heavily amplified, and that its representation is likely to be very far from optimal.

Reconciling the low affinity for peptides with the specificity for AD brain

The lack of staining in normal brain and the specific staining of NFTs in AD brain, suggest that the truncated tau found in paired helical filaments, a post-translational cleavage of tau, known to be specific for AD, is likely to be the primary antigen recognized. This most likely gives rise to the specificity for PHFs and neurofibrillary tangles. This is especially likely, given the 1000 fold higher affinity observed for bGAE (one of the truncated forms of tau recognized by MN423) than the peptides. However, it is difficult to explain this specificity solely at the level of epitope sequence given the clear promiscuity of this antibody for many different peptides, some of which are known to be found in brain proteins (e.g. the Fragile X Mental Retardation Sindrome Related Protein 1). Furthermore, the large difference in affinity between the peptides and bGAE demands explanation. We feel that there are essentially two possible causes:

1) The specificity of MN423 may have two components, the first of which is the DHGAE component identified, which is essential, and the second which is something within tau which is only able to contribute if the first component is present and the effect of which is to increase affinity.

2) DHGAE constitutes the whole epitope, and all differences in affinity are a result of avidity effects caused by clustering of the DHGAE epitope.

Of the two explanations, the second appears to resolve more of the inconsistencies associated with the recognition specificity of this antibody. If the monomeric affinity for DHGAE is $5x10^{-6}$, and that for bGAE is $6.7x10^{-9}$, the thousand fold difference could be due to bGAE aggregation which leads to the presentation of multiple epitopes which can be bound by MN423 as a (dimeric) monoclonal antibody. It is known that bGAE and the other tau truncation products found in core PHF have an extreme tendency to form multimers, even in the presence of SDS, as found in polyacrylamide gel electrophoresis (Novak, Jakes et al. 1991; Novak, Kabat et al. 1993). As a result, even if monomeric bGAE is purified, it will always consist of a small component which is multimeric. This small component will have far higher avidity for MN423 than the monomeric form. In this explanation, the affinity of MN423 for a *single* epitope with the structure G[AV]{LRMKPFTQN}> is likely to be too low to be revealed by any standard procedure such as immunohistochemistry, but the avidity effect caused by the binding of the two binding domains in a full length IgG to two appropriately spaced epitopes on PHFs may be sufficient to be specifically revealed, if indeed the affinity of $6.7x10^{-9}$ is a result of this. This would also explain why, in addition to the problems associated with the pJufo cysteines, it proved impossible to select anything from the pJuFo library. Most phagemid in this library will be displaying single epitopes, and only a very small proportion will be displaying more than one. If the latter are the only selectable forms of the MN423 epitope, then the concentration of appropriate peptides present in more than one copy on a single phage may be too low to allow selection. In the case of lambda, however, display

is multivalent, and so every phage will display multiple copies of epitope and so will be accessible to selection.

The low affinities of the peptides identified by phage display for MN423 suggest that avidity should always be considered when making conclusions about epitope sequences identified using molecular diversity techniques.

Future possible directions on 423

These two possible explanations for the difference in affinity of MN423 for bGAE and the identified peptides could probably be resolved, if a monomeric form of MN423, such as a scFv of Fab, could be prepared. Cloning of the variable regions of MN423 has been unsuccessfully attempted (by Peter Pavlik). It is relatively common to encounter difficulties when attempting to clone hybridoma V genes by V region PCR, due to the presence of multiple antibody genes in the hybridoma cell (probably caused by the fusion of many B cells with the myeloma cell), and heavy mutation in the primer binding sites (Ruberti, Cattaneo et al. 1994). If monomeric forms of the antibody were available, the affinity of the monomeric binding unit for bGAE and the selected peptides could be directly measured. If the explanation proposed above were correct, one would expect to see a large difference between the affinity of the full length antibody and the scFv when attempted on bGAE, but no difference when attempted on peptides.

The role of the structural epitope in the recognition specificity could probably also be further examined by the creation of specific constrained peptides, such as XCDHGAECX, in which a turn induced by the epitope was flanked by two constraining cysteines. This

would allow us to discover whether the free carboxyl group was essential, or whether it was the specific turn structure which was being recognized.

In the end, however, all these experiments do no more than approach the truth. To really identify the bGAE elements recognized by this antibody, it will be essential to carry out structural characterization by X ray crystallography, although this may be difficult, if the measured binding affinity is predominantly mediated by the small amount of bGAE which is multivalent, as a result of aggregation.

Further uses for the lambda C terminal peptide library

The success of this lambda C terminal peptide library in analyzing the epitope specificity of MN423, suggests that this library may also be very useful in other cases in which epitopes are known (or even unknown) to be C terminal. In the past, molecular diversity tools have been either internal or N-terminal in nature. For this reason most screening experiments have been carried out analyzing only this component. Now that a C terminal library is available, it should form the armory of any selection and screening experiment in which new specificities are being identified. It is not uncommon for experiments with standard peptide libraries to yield no conclusive result when attempting to identify monoclonal antibody epitopes. And it has been shown (Craig 1988), that in order to fully characterize a binding protein, it is often necessary to analyze a large number of different peptide libraries, since some libraries do not yield binders, while others do.

In particular, it may also be worth revisiting those experiments in which phagotopes have been identified using patient sera (Cortese, Felici et al. 1994), since the C terminus of many proteins have been shown to be very immunogenic (Babinska, Cierniewski et al.

1984; Milman, Scott et al. 1985; Palker, Scearce et al. 1986; Torrens, Ojalvo et al. 1999), and it is possible that very useful phagotopes are being missed by excluding those which are C terminal.

PDZ domains are small modules found in proteins which mediate protein-protein interactions, and have been shown to recognize short C terminal sequences (Saras and Heldin 1996; Songyang, Fanning et al. 1997; Stricker, Christopherson et al. 1997; Hall, Ostedgaard et al. 1998) . Their binding sites have been identified as being C terminal, and different PDZ domains appear to recognize different C terminal sequences, providing specificity to their interactions which are likely to have biological significance. This library is presently being used to create a database of PDZ binding specificities (Gianni Cesareni personal communication), with the long term goal of creating a catalogue of protein-protein interactions.

However, in addition to known cases such as PDZ domains, it may be useful to screen other small protein modules, since these may also have C terminal recognition components.

The multivalent display used in this vector will also allow the identification of binding of relatively low affinity, which may well account for much of intracellular protein-protein interaction. Furthermore, as lambda is an intracellular phage, the peptides displayed will not suffer from the constraints that have been observed with display on filamentous phage vectors (Peters, Schatz et al. 1994), and may allow the display of peptides which are more cytoplasmic in nature, specifically those which are likely to be recognized by PDZ domains and the like.

Recently a filamentous phage based C-terminal display system has been described (Weiss and Sidhu 2000). This exploits an artificially designed protein which inserts into the phage along with the major coat protein. Although this cannot substitute for wild type p8, it is able to display both proteins and peptides at its C terminus, which could allow it to be used for the same kinds of experiments as described above.

A fragment of tau relevant for apoptosis and neurodegeneration?

Apoptosis has been related to pathological changes in several neurodegenerative disease including Huntington's disease and Alzheimer's disease (Cotman and Anderson 1995; Kusiak, Izzo et al. 1996; Dragunow, MacGibbon et al. 1997; Friedlander and Yuan 1998; Ona, Li et al. 1999; Nicotera 2000). The possibility that tau protein could be abnormally truncated in AD disease is a key argument in many previous studies (Novak, Jakes et al. 1991; Novak, Kabat et al. 1993; Novak 1999). Moreover it has been shown that tau is processed by caspase-3 and calpain in neurons undergoing apoptosis (Canu, Dus et al. 1998) and a tau fragment is able to induce apoptosis when expressed in COS cells (Fasulo 1996; Fasulo, Ugolini et al. 2000). We found that the C-terminal 20 amino acids can be chopped out by caspase-3, in vitro. In the plot of the predicted secondary structure (figure 43) exactly this last stretch of 20 amino acids appears to have a high probability of helix structure, this being this also confirmed by real structural data (Esposito, 2000). This fragment has perhaps a precise biological role in apoptotic cascade of events, even if it has not previously investigated by the comprehensive analysis previously made (Fasulo, Ugolini et al. 2000). We believe that the function of this 20 aa peptide should be further

investigated for a possible role in apoptotic processes, especially since it is specifically removed during apoptosis.

cDNA display – background

While cloning of cDNA into lambda vectors (Collins and Hohn 1978; Hohn and Collins 1980) for detection by either hybridization or expression (Lerch, Frank et al. 1989) has been relatively succesful, the same cannot be said of the phage display of cDNA fragments. Although in principle, this should be relatively straightforward, the implementation of such display libraries has been far more difficult than their proposal. There are two main obstacles. The first is that when cloning random fragments into a protein such as p3, where only the N terminus can be used, only a very small proportion of clones will be in frame, and hence fused to p3. This is due to the problems of correct orientation (1/2), correct frame entering (1/3) and leaving (1/3) the open reading frame, as well as problems related to those clones which will be non-coding because either the 5' or 3' untranslated region is cloned. The second obstacle is that as filamentous phage is a secreted phage, polypeptides must cross the bacterial inner membrane to be efficiently displayed. This tends to be easier for secreted proteins and the extracellular domains of membrane proteins, although a number of cytoplasmic protein domains have also been displayed (see table in introduction). The rules which govern protein export, and hence display, are not completely understood, although some interesting observations regarding the role of charge just after the leader sequence have been made (Peters, Schatz et al. 1994). A number of different vectors have been developed to overcome these problems,

but no universal solution has emerged yet. In general these solutions are of two kinds: either change the display protein, or change the phage. The vector described by Jespers et al. (Jespers, Messens et al. 1995), uses p6 to clone cDNA fragments at the C terminus. This has the advantage that the frame leaving the cDNA fragment is irrelevent, since it is not required to connect to the display protein. This reduces the proportion of clones which are non-functional because they are out of frame to 1/3 for directional cloning and 1/6 for random cloning. However, p6 is a relatively hidden protein on the phage, and direct comparisons between the use of p3 and p6 appear to show that display levels are lower in the p6 vectors (Hufton, Moerkerk et al. 1999). The system recently described by Weiss et al. (Weiss and Sidhu 2000), works on a similar principle, but uses an artificial protein to provide C terminal display within the context of the major coat protein, p8. The pJufo system attempts to solve the problem in a similar fashion using p3 display, but instead of display at the N terminus of p3, cDNA fragments are displayed at the C terminus of a fos polypeptide which interacts with a jun fragment at the N terminus of p3 (Crameri and Suter 1993; Crameri, Jaussi et al. 1994)). This has been used to identify allergens recognised by IgE from allergic patients (Crameri and Blaser 1996) and one genomic library from E. Coli (Palzkill, Huang et al. 1998), but no other libraries have been published with this system.

Display at the C terminus of proteins from intracellular phages, such as lambda (Jamieson, Kim et al. 1994; Sternberg and Hoess 1995; Dunn 1996; Mikawa, Maruyama et al. 1996; Kuwabara, Maruyama et al. 1997; Santini, Brennan et al. 1998; Santi, Capone et al. 2000), T4 (Efimov, Nepluev et al. 1995; Jiang, Abu-Shilbayeh et al. 1997), P22 (Carbonell and Villaverde 1996; Carbonell and Villaverde 1998) and P4 (Lindqvist and Naderi 1995)

have been proposed as alternatives. Such systems would be expected to be more efficient for the display of intracellular proteins, which sometimes have problems traversing the inner bacterial membrane. This has been shown to be the case for display of the hepatitis C genome (a small genome of 8kB), where lambda was far better than filamentous phage (Santini, Brennan et al. 1998). This lambda vector is the same kind as that used for the peptide library described here and has also been successfully used to display a cDNA library from human brain and mouse embryo (Santi, Capone et al. 2000).

Vectors designed for cDNA display can also be used to display genomic fragment libraries. These differ from cDNA libraries in that if the library is sufficiently large, representative fragments of all genes, with the exceptions of those which span exons, should be displayed, whereas cDNA libraries are limited to those genes expressed in the tissue used to prepare the library. Furthermore, expression levels do not need to be normalised. The display of genomic fragments to date has only been carried out with bacterial genomes, which lack introns. Fibronectin, fibrinogen and IgG binding open reading frames have been selected from Staphylococcus aureus (Jacobsson and Frykberg 1995; Jacobsson and Frykberg 1996; Jacobsson and Frykberg 1998; Zhang, Jacobsson et al. 1998) while alpha-2 macroglobulin, IgG and serum albumin binding domains have been selected from the Streptococcus genome (Jacobsson, Jonsson et al. 1997), all of which have been carried out with p3 or p8 based filamentous phage based vectors.

A single display sytem which is suitable for all proteins may not be feasible. In fact, it may be necessary to use two (or even more) display systems to obtain complete protein representation, each system balancing the biological biases of the other(s).

Potential ways to improve cDNA display using the pJuFo concept

The concept employed in the pJuFo vector is very attractive, and yet papers using this system have only described the display of allergen cDNA products. No other libraries have been described. This could be because allergens are the most suitable kinds of proteins which can work with this system, since they are usually small, highly stable, secreted proteins and the antibodies that recognize them are high affinity IgE.

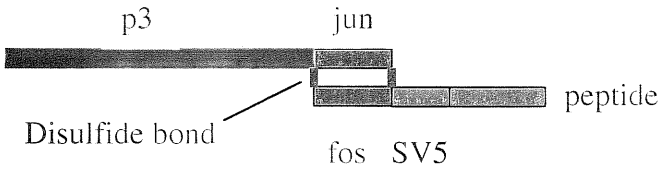
In the test selection performed on the model peptides, we showed that we were able to obtain a limited specific enrichment. This is likely to be for two reasons:

1) If 423 (and YL1/2) need more than one epitope to bind with sufficient affinity to survive a selection (the affinity for MN423 for DHGAE is 5μM), at least two clustered epitopes will be required. In a 3+3 vector very few phage will have two epitopes. In fact, considering that in a normal 3+3 phage display system 1-10% of phages display one copy, only 0.01-1% of phages will have two copies of the antigen, and this number may drop even more due to the other problems associated with the pJuFo system (see below), further complicated by the need for another interaction step (fos and jun). This low display of clustered epitopes would hamper ELISA detection and selection, for the very small numbers of phages able to bind coated antibody.

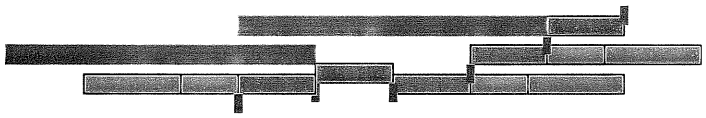
2) The presence of cysteines at either end of the jun and fos zippers clearly results in problems due to the creation of aggregates. These cysteines were included in the vector to create a covalent attachment between the jun and the fos, after they had bound by non-covalent forces. In theory, this is a very attractive idea. However, our westerns blots in non-reducing conditions show that instead of the expected simple jun-fos product, an entire set of polymers is present, the vast majority of which resolve to a few bands upon

reduction. Given that our detection scheme involved the use of SV5, an epitope which is only present on the fos-peptide fusion, this indicates clearly that the small fos-peptide product is associating with many larger products to create aggregations which are likely to cause problems and may mask the peptide epitope.

Theoretical pJuFo product



An example of a complex which could be formed because of the presence of the two cysteines.



In addition to those bands which were visible on the western blot (from the SV5 epitope in fos-peptide), there may be others which are even more complex which contain multiple copies of the p3-jun (invisible in westerns because they do not have the SV5 epitope), and this analysis takes no account of the three cysteines found in the truncated p3 used, one of which will always be unpaired. In the presence of the two unpaired jun cysteines this is likely to complicate matters still further.

Depending upon how great a problem all these cysteines are when it comes to displaying peptides, one obvious and relatively easy improvement would be to pair them or eliminate

them. This would probably be most easily done by using full length p3 instead of the truncated form and using only one, or no cysteines to join jun and fos zippers.

Exploiting the same display concept, but using a different approach, one could think about the use of different interacting, relatively small and stable, pairs of proteins to provide the interaction between p3 and the C terminally displayed peptide or protein. For instance, the immunoglobulin CH3 region usually forms homodimers. This has been modified to give stable heterodimers using an elegant mutagenesis and selection scheme in which a designed mutant "knob" has been probed with a phage display structure guided library of "hole" mutants (Atwell, Ridgway et al. 1997). The identified pair of CH3 mutants heterodimerize with high affinity and specificity without the need for disulfide bonds and could therefore be used in a pJufo context. Another alternative would be the use of VL and VH from a scFv known to associate with high affinity. One could also keep jun and fos, but improve their affinities for one another by introducing combinations of mutations in the interaction surfaces, and using these mutants in a mutual exchange bait-prey, in phage display selections. Similar experiments to improve the association capabilities for the formation of a fos homodimer have also been carried out (Porte, Oertel-Buchheit et al. 1995).

Subtractive selection of antibodies

So far techniques based on differences between tissues and/or cells have been largely exploited to find genes that are differentially expressed in many different conditions and situation. The majority of them are based on the detection of differences in the cellular pool of mRNAs. A recent review of the most advanced of these can be found in (Jurecic

and Belmont 2000). We believe that there is a major problem in this approach related with the often weak correlation between mRNA and protein abundances (Anderson and Seilhamer 1997). Nevertheless, techniques like DD-PCR, SSH applied to cDNA libraries, SAGE and microarrays have provided the instruments for interesting and challenging observations and discoveries. However, a reliable system able to detect differences in the actual proteome state of a cell, tissue or organism is lacking. Such a system would be of enormous importance for a number of vast fields such as pathology, development and differentiation, structural genomics etc. In particular, subtractive selections of ScFv specific for one tissue/cell/organism and not another, promises to be a key technique for the future study of biology, which is likely to go beyond the proteome and allow the study of post-translational modifications as well. Today this kind of selection is still to be fully developed, even if some nice results have already been obtained (de Kruif, Terstappen et al. 1995; Van Ewijk, de Kruif et al. 1997), in which antibodies specific for certain cell types have been derived.

As opposed to the above mentioned nucleic acids based differential techniques, antibody subtractive selections would be capable of detecting differences in a sort of proteome based "epitope differential display". In an ideal "epitope differential display" even differences absolutely not detectable with changes in transcription and translation machinery (i.e. a protease activity, triggered by an apoptotic stimuli, or a phosphorylation event) would be detected and, even more important, would directly produce a specific biochemical tool, the ScFv, specifically suitable for further studies.

Of course, stepping from considering DNA molecules to considering protein molecules for such an approach, is somehow as to step from a mono dimensional problem to a three (or even greater) dimensional problem, in terms of complexity.

If a DNA molecule requires a set of oligos to be amplified in a PCR reaction, the same is not true with a protein or a protein domain or a post translational modification. The problem is somehow shifted to the recognition of such structure, the sensitivity of such recognition and eventually the possibility to perform differential display of the structural characteristics identified. In this light, it is clear that a reliable system to select protein isoform/cell/tissue/organism specific ScFv would be the perfect shortcut to solve this high complexity problems.

In our experiments we have exploited the availability of high quality ScFvs libraries to understand the feasibility and reliability of subtractive selections of ScFvs. To reduce biases due to antigen concentration and similar problems we kept our model as simple as possible: two isoforms of six of the same protein were used in each subtraction. Both schemes of subtraction or depletion were used. In the first case, the selection was carried out in presence of an excess of the isoform we wanted to subtract. Whereas in depletion schemes we tried to pre clean the library from all the unwanted isoform unspecific binders (for details see Materials and Methods). We were able to fish out just one isoform specific scFv. We believe that this could have been due to several reasons. First there is the possibility that the library we used simply does not cover a large enough universe to give the specificity that we are searching for. However, given the large numbers of different antibodies which have been selected from this library, this seems unlikely. Another possibility is that tau, which has not been crystallized and that, from predictive algorithms

seems to have somehow a "poor structure", simply cannot generate enough differences to give more isoform specific ligands. However, the fact that these differences are present at the amino acid level, does not exclude the possibility of selecting linear epitopes, although in the case of exon 10, which is one of four imperfect repeats, it may be difficult to select specific scFvs which do not recognize the other repeats. This was the basis for the peptide based approach, which should be feasible, even if it may be necessary to alternate selection between the peptide and tau, as has been carried out for the phosphorylated form of the transcription factor, E47. ScFvs could only be selected against this epitope when the phosphorylated peptide and the phosphorylated protein were used alternately, but not when either was used alone [Lu, 1999 #2458].

While the peptide approach is very attractive for suspected differences which can be identified at the genome level (alternatively spliced exons, alternative start or stop sites), it misses the whole world which is potentially accessible to this technique if selection can be applied directly to tissue extracts or cell surfaces. For it is only when true subtractive selections can be applied at this level, that subtleties which go beyond the genome, and which play important roles in differentiation, development and pathology are likely to be identified. To carry this out, however, significantly improved subtractive schemes must be developed, to avoid our experience of screening 223 different clones to find a single one which recognized the subtracted species in a system which was very simple. Differences between tissues in different states of differentiation are likely to be far more complex and challenging.

Once such a scheme has been implemented, the task of identifying the epitopes recognized by specific subtractive antibodies will not be trivial, and will probably require

the development of high throughput mass spectroscopy approaches, similar to those already described (Siegel, Allen et al.).

Materials and Methods

Bacterial strains used

BL21 DE3 F⁻ *hsd*s *gal*(λcI*ts*857 *ind*1 Sam7*nin*5 *lac* UV5-T7gene 1)

DH5αF' (Gibco BRL): F'/*end*A1 *hsd*R17 (r_K^- m_K^+) *sup*E44 *thi*-1 *rec*A1 *gyr*A (Nal^r) *rel*A1 Δ (*lac*ZYA-*arg*F)U169 *deo*R (F80*dlac*Δ(*lac*Z)M15)

BB4 *gal*K2, *gal*T22, *hsd*R514, *lac*Y1, *mcr*A⁻, *met*B1, *sup*E44, *sup*F58, *trp*R55, Δ(*arg*F-*lac*) U169, [F' *lac*I^q *lac*ZΔM15, *pro*AB, Tn10]

Note: tn10 confers tet^r

BB44 These cells were derived from BB4 cells in Gianni Cesareni's laboratory and selected to be resistent to an unknown lytic phage contamination (Paola Vaccaro, personal communication).

TG1 *sup*E *hsd* Δ 5 *thi* Δ (*lac-pro*AB)F'(*tra*D36*pro*AB$^+$*lac*I^q*lac*ZΔM15)

Y1090r⁻ *ara*D139, *hsd*R (r_K^- m_K^+), *mcr*A⁻, *rps*L, *sup*F, *trp*C22::tn10, Δ*lac*U169, Δ*lon*, (pMC9).

XL1-Blue MRF' (*mcr*A)183, (*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, thi-1, *rec*A1, *gyr*A96, *rel*A1, *lac* [F' *pro*AB *lac*IqZM15, Tn10]

Buffer compositions

AP buffer= 100 mM NaCl, 5mM $MgCl_2$, 100mM Tris-Cl pH 9.5.

DTT 1M=3.09 g DTT in 20 ml of 0.01M Sodium acetate (pH5.2), store at –20.

TE=10mM Tris-Cl, 1mM EDTA, various pH.

PBS 10X=2g KCl, 2g KH_2PO_4, 80g NaCl, 11.5 Na_2HPO_4 for one liter solution.

Autoclaved.

MPBS= 2% or 4% dryed skim milk in 1X PBS

PEG-NaCl=20% w/v PEG, 2.5M NaCl, filter sterile.

SM buffer=5.8 g NaCl, $MgSO_4$ 2g, 50ml of 1M Tris-Cl pH7.5, 5ml of 2% gelatin (Fluka 48722) solution. Autoclaved .

Carbonate buffer=100 mM $NaHCO_3$ pH9.6

Western transfer buffer=2.9 g glycine, 5.8 g tris base, 0.37 g SDS, 200ml methanol in one liter of solution.

SDS loading buffer 6X or SLB=7 ml of 4X Tris-Cl/SDS, 3.0 ml glycerol, 1g SDS, 1mg bromophenol blue. β-mercaptoethanol is added to the sample at 2-5% v/v.

Tris-glycine running buffer 5X=15.1g Tris base, 94g glycine, 50 ml 10% SDS in one liter, pH 8.3.

TBS =50mM Tris-HCl (pH7.5), 150 mM NaCl. Autoclaved and stored at RT.

TBST=TBS+tween20 at 0.05-0.1% v/v

MTBST = 5% dryed skim milk in TBST

4X Tris-Cl/SDS buffer = 6.05g Tris base, pH at 6.8 with 1N HCl, 0.4 g SDS, filter.

Media and plates

LB
10 g bactotryptone (Difco)
5 g yeast extract
5 g NaCl
15 g agar (for plates)
To 1 L with deionized water.
Autoclave.
Top Agar
1L LB +15g agar (10 mM $MgSO_4$ was added before plating for lambda). Aliquots of 50 ml
Agarose Top
1L LB+ 1g $MgCl_2$ hydrated + 7g agarose. Autoclave. Aliquots of 50 ml.
SOB
20 g bactotryptone (Difco)
5 g yeast extract
0.5 g NaCl
10 ml of 250 mM KCl solution
5ml of 2mM $MgCl_2$ solution
15 g agar (for plates)
To 1 L with deionized water.
Autoclave
2xTY
16 g bactotryptone
10 g yeast extract
5 g NaCl
15 g agar (for plates)
To 1 L with deionized water.
SOC
2% w/v glucose in SOB

For each of the plates and media, ampicillin, kanamycin or glucose may be added to the following final concentrations:
Kanamycin: 25μg/ml
Ampicillin: 100μg/ml
Tetracycline 15μg/ml
Glucose: 1-2%
Such plates are indicated as 2XTY amp/glu, etc.

Antibodies used

7.51 (a generic anti tau antibody), YL1/2 (antitubulin) and MN423 (anti tau bGAE and dGAE), monoclonals, were kindly donated by Novak (Novak, Wischik et al. 1989). SV5 was kindly donated by Dr. Randall (Hanke, Szawlowski et al. 1992).

As secondary antibodies:

For MN423, SV5 and 7.51 detection: Dako, goat anti mouse IgG monoclonal AP conjugated, used at 1:2000 dilution (western, colony and plaque lift screening) or HRP conjugated for ELISA or ECL.

For Yl1/2 detection: Dako, rabbit anti rat IgG monoclonal AP conjugate, used at 1:2000 dilution (western and colony lift screening) or HRP conjugated for ELISA or ECL.

For Elisa experiments: Pharmacia, mouse anti M13 monoclonal HRP conjugate, used at 1:5000 dilution.

Phage display libraries selections

ScFvs phage libraries used

Two ScFv phage libraries were used to select ScFvs against tau and in subtractive selections on tau isoforms. These were both kindly provided by Jim Marks. One was published in (Sheets, Amersdorfer et al. 1998) and was made in pHEN (size=$6x10^9$), the second is unpublished and was made by subcloning the "Sheets" library into fdDOG (size=$5x10^8$).

Peptides libraries used

For peptide selections on MN423 and on the dGAE fragment of tau protein a commercial library of 12-mers was used (Ph.D.-12 Phage Display Peptide Library Kit, New Englands Biolabs). A library of C-terminal random peptide was made in an improved pJuFo vector (kindly provided by Invitrogen) – see results for details of construction. With this library selections against MN423 and YL1/2 were unsuccessfully attempted.

λ display peptide library

The library of random C-terminal 9-mer peptides was made in bacteriophage lambda (Gianni Cesareni, Universita' di Tor Vergata, Rome) and an aliquot was kindly donated for our selections. This library has random nonapeptides fused to the C teriminus of the gene D protein and was made by PCR using an oligonucleotide with diversity encoded by $(NNK)_9$. The gene D protein is a major component of the phage head and serves to stabilize the head during DNA packaging in virions. There are approximately 420 copies of protein D per mature λ phage.

Selection of filamentous phage-antibody libraries (M13 immunotube biopanning, one cycle)

To a 75 x12 mm Nunc-immunotube (Maxisorp; Cat. No. 4-44202) 1 - 2 ml of antigen (at 10-100μg/ml) in PBS were added and incubated overnight at 4 °C to allow protein adsorption to the plastic surface. The different tau isoforms were used at 5-10μg/ml. The next day tubes were washed 2x with PBS-Tween-20 (0.1%), 2x with PBS (by simply pouring the solution in and pouring it immediately out again). Following this, tubes were preblocked by filling to the brim with PBS containing 2% skimmed milk (MPBS). Tubes were sealed with Parafilm and incubated at room temperature for at least 45 min (ideally 2 hours) to block. Phages were prepared in the following way: 500-800 μl of PEG

concentrated phage in PBS (~10^{11}-10^{13} phage particles, depending upon the cycle number) were added to 500µl PBS and 1 ml of 4% MPBS, and incubated for 30'-1 h at room temperature. After blocking tubes were washed 2x with PBS-Tween-20 (0.1%), 2x with PBS. The phage mixes were transferred to the immunotubes, the tubes were sealed with a cap or with parafilm, incubated 30 min at room temp on an under and over turntable and then let to stand for at least a further 1.5 hrs at room temp. During this phase, phage were selected by the antigen. Tubes were washed with 20 washes PBS-Tween-20 (0.1%), followed by 20 washes PBS. Each washing step is performed by pouring buffer in and out immediately. This is best achieved using a squirt bottle. Phage were eluted from the tube by adding 1 ml 100 mM triethylamine (140 µl per 10 ml water; this has a pH of 12) and mixing for no more than 10 minutes on an under and over turntable. Phage viability decreases with longer elution times. After the elution time, eluted phage were immediately neutralized with 0.5 ml 1.0 M Tris-HCl, pH 7.4, and transferred to another tube. It is convenient to store selected phage/phagemid particles at 0-4°C. Displayed antibody/p3 fusion protein is gradually proteolysed during storage, so it is best to perform phage rescues and selections on two consecutive days.

Rescue of selected phage output

300 µl of eluted and buffered phages were used to infect 5ml of DH5αF'cells at 0.5 OD_{600}, by incubating for 30 minutes at 37°. After infection, infected bacteria were plated out on 2xTY amp/glu plates and incubated at 30° overnight. The following day, colonies were resuspended by scraping from the plates with a glass rod in 1-2 ml of LB amp and harvested in a 15 ml Falcon tube. This bacterial suspension was mixed well and 10µl were

grown in10ml 2xTY amp/glu at 37° to an OD_{600} of 0.5. M13K07 helper phage was added (twenty fold excess – 10^{10}), and the culture was left for 30 minutes at 37°. After infection the culture was centrifuged, resuspended in 2xTY amp/kan (the omission of glucose allows antibody expression and the presence of kanamycin allows selection for those bacteria infected with helper phage) and grown ON at 30° to produce phage particles used for the next cycle of selection. The day after, cultures were centrifuged at 3000 rpm 4° for 20', and the phage particles in the supernatant werePEG precipitated and finally resuspended in 1ml PBS. 200-500 µl of this phage preparation was used for the next selection cycle.

PEG precipitation of phages

To the phage solution to be precipitated, 1/5 volume of PEG NaCl solution (20% w/v PEG, 2.5M NaCl) is added, left 40' on ice, centrifuged 3700 rpm 4°, resuspended in 1ml cold PBS, centrifuged again for 5' in an Eppenderdorf rotor at 4°, to eliminate residual bacteria, and transferred to another tube. This procedure can be repeated ("double PEG precipitation")

ScFv phage selections on pin-peptide and pin regeneration

Peptides synthesised on plastic pins were obtained from Chiron Technologies. The amount of peptide on the plastic pins is 1 - 5 µmole (approx. 1.5 – 7.5 mg of a 15mer). Most peptides used were 15mers. Pins are reusable and after each selection cycle or ELISA experiment, were regenerated using 0.1% beta-mercaptoethanol in 1% SDS, 0.1 M sodium phosphate pH 7.2. at 60° in a sonication bath for 10', washed once with methanol and air dried. For selections, pins were first washed in PBS, blocked in 4%

MPBS for 1 hour and then incubated with the phage single-chain antibody library for 2 hours on an oscillating platform at RT. The washing steps were done twice in PBST for 30 seconds follwed by once in PBST for ten minutes, and twice in PBS for 30 seconds followed by once in PBS for ten minutes for the first round of selection and with increasing stringency (up to 5-6 short washes followed by the 10 minute wash for the second and third rounds). These washes were all carried out in a large volume of buffer, with all pins washed in the same buffer solution. Binders were eluted by adding 200µl DH5α F' bacteria grown to OD 0.5 in 2xTY amp/glucose 1% to each well of a microtitre plate corresponding to each pin used. The plate containing the pins (see figure 45) was then placed into the microtitre plate in such a way that each peptide pin, with attached antibodies, was immersed in the bacterial solution. After 30 minutes incubation at 37°C (to allow infection), the pins were removed, regenerated as described above, and 10^9 helper phage were added to each well and incubated for a further 30' minutes at 37°. Bacteria were spun down and resuspended in 2xTY amp/kan (this allows expression of the scFv on the phage), and the 96 well plate was incubated overnight at 30°C shaking at 250 RPM. Phage containing supernatants were harvested and used directly for the next round of selection or alternatively PEG precipitated, resuspended in sterile PBS and stored at –80° in 20% glycerol.

In the first round of selection asingle aliquot of phage antibody library was incubated with all pins simultaneously. In subsequent rounds, the pins were only incubated with the corresponding eluted and amplified phagemid in the microtitre well.

Subtractive selections

To the general above described procedure for phage antibody selections, we applied the following changes. Tau CNS isoforms will be referred to using the following numbers: T1=381aa isoform, T2=410 aa isoform, T3=441 aa isoforms, T4=383 aa isoform, T5=352 aa isoform, T6=412 aa isoform. For subtractive selections on tau isoforms we utilized two different main schemes 1) subtraction and 2) depletion.

1) Subtraction. In a T3-T6 subtraction, for example the following scheme was used, 5μg/ml of isoform T3 (selector) was coated overnight at 4° on plastic (Nunc) immunotube in PBS, the library was blocked in 2% MPBS in the presence of 50-250μg/ml of isoform T6 (subtractor). Subsequent selection on the solid phase target T3 was carried out as described above.

2) Depletion. T5 is the shortest isoform, and so contains all the parts of tau common to all other isoforms, without the extra exons. T2 contains the two amino terminal exons, T3 contains the two amino terminal exons and the C terminal exon, and T4 contains only the C terminal exon. This method was used for (T2– T5, T3– T5 and T4 – T5) subtraction schemes. In this case the library is first blocked with 2% MPBS, then panned 3 to 5 times, each incubation being of 45' (without amplification steps) against the T5 (subtractor) coated immunotubes (coating and blocking as for a standard selection). After these passages, the library is panned against the T2, T3 and T4 isoforms (selectors) coated immunotubes in a classical selection protocol. Both the population of phages, e.g. phages bound to tubes coated with the subtractor, during the depletion step, and phages bound to selectors coated tubes, were harvested. Depletion

was carried out only prior to the first round of selection, and two subsequent rounds were carried out on each protein.

Growth and rescue of phagemidic particles in 96-well micotitre plates.

The following method was used for growth of large numbers of clones for preliminary screening for binding activity by methods such as ELISA. Colonies were inoculated into 150 µl 2xTY, 100 µg/ml ampicillin, 2% glucose in 96-well round-bottomed plates (Nunc) and grown with shaking (270 r.p.m.) overnight at 30°C. The plates were covered with lids and fixed in a microtitre plate holder. The incubator contains a flask of water to avoid dessication. This plate is termed the master plate. Next day, a 96-well transfer device was used to inoculate (twice) from this master plate to a fresh 96-well plate containing 150 µl 2xTY, 100 µg/ml ampicillin, 1% glucose per well in round-bottomed 96-well plates (Nunc). Bacteria were grown 2.5 hrs, 37°C, shaking. The master plate was stored at -70°C afer adding 50 µl 60 % glycerol per well.

To each well of the inoculated plate 50 µl 2xTY, 100 µg/ml ampicillin, $2x10^9$ pfu M13K07 helper phage (0.1 µl of a $2x10^{13}$ pfu/ml stock per well) were added. The ratio of phages to bacteria should be around 20:1. Infection occurred for 30' at 37°.

Plate were spun at 2700 rpm for 10 min in a swinging bucket rotor for microtitre plates (Beckman) and the supernatant removed with a vacuum device. The pellet was resuspended in 150 µl 2 x TY, 100 µg/ml ampicillin, 25 µg/ml kanamycin and grown overnight at 30°C shaking at 270 rpm. Next day, plates were spin at 2700 rpm for 10 min and 50µl supernatant used per well for phage ELISA.

Coating of antibodies onto immunotubes.

In all cases coating of SV5, MN423, 7.51 and YL1/2 was made using hybridoma supernatants or (for MN423) ascites. We considered a valid indication of the average amount of antibody present in this fluids the following: 50 μg/ml for hybridoma supernatant, 5 mg/ml for acites as described in (Harlow and Lane 1988). Each batch of supernatant and ascites was tested usually in western blots (see above for details) purified recombinant tau and/or tau fragments for 7.51 and MN423, cell extracts for YL1/2 (see above for details). Supernatants from 7.51 and MN423 hybridoma were used in 1:20 –1:50 dilutions, while Y1/2 was used in 1:5 dilution.When absorption to plastic surface was required (immunotube coating) the coating procedure was verified in an ELISA assay, coating the antibody overnight, usually in 1 to 1 volume ratio supernatant /PBS in Nunc Maxisorp plates at 4° and probing with a sheep or rabbit HRP conjugated antimouse. For MN423 western tested ascites, dilutions between 1/1000 and 1/100 in PBS were used. Coating was tested in ELISA, using an anti mouse (for 7.51, MN423 and YL1/2) HRP conjugated directly to reveal coated mouse IgGs. See below for details of how ELISA was carried out. This test was considered indicative also for the coating in Immunotubes. YL1/2 is a rat hybridoma, but can be detected with anti-mouse sera.

Phage-ScFv Elisa

96 wells plates (Nunc-Immuno TM Plate, MaxiSorp TM Surface) were coated with 100 μl per well of protein antigen used for selections (an amount of 5-10 μg/ml, for all 6 tau protein isoforms, works well). This amount can be used for many proteins, but for some proteins, higher amounts are required, e.g. lysozyme in the order of mg/ml). Coating was

made usually in PBS leaving overnight at 4°C. This works for tau. For some proteins, if PBS does not work, 100 mM sodium hydrogen carbonate pH 9.6, may be effective. Wells were rinsed 2x with PBS-Tween-20 (0.1%), 2x with PBS, and blocked with 120 µl per well of 2% MPBS, for at least 30 min at room temperature. Plates were washed by submersing the plate into PBST and removing the air bubbles in the wells by agitation, or alternatively using a squirt bottle quickly filling each well. Wells were rinsed 3x with PBS-Tween-20 (0.1%), 3x with PBS, then 50 µl 4% MPBS was added to all wells followed by 50 µl culture supernatant containing phage antibody (usually grown in a 96 wells plate – see preceding protocols). Phage supernatants can also be previously concentrated if required by PEG precipitation. After 1.5 hrs incubation at room temp, the supernatants were discarded and wells were washed 3x with PBST (Tween-20 0.1% v/v) and 3x with PBS. 100µl anti fd-phage HRP conjugated monoclonal (see above for details, diluted 1/5000 in 2% MPBS) were added to each well and incubated for 1hr at room temperature. After incubation, the secondary antibody was discarded, and the wells washed 3X with PBST 0.1% and 3X with PBS. Finally 80µl of TMB (Sigma, T2885) were added to wells, in the dark for 2-20 min. The reaction was quenched with80 µl of 2N H_2SO_4 and plates were read at 450 nm.

Pin-peptide phage elisa

Pins were first washed in PBS and blocked in for 45' on a shake table. Pins were incubated with phages in 2% MPBST 0.5% for 1 1/2 hour on a shake table at RT, washed four times with PBST, incubated 1h with a suitable dilution (see above for details) of a secondary anti -M13 antibody HRP conjugated (Pharmacia) and washed four times with PBST. Pins were developed in 200 µl (this amount can vary depending on the plate used,

it is worth to check it before the experiment, the volume should cover the tip of the pins) of TMB (Sigma) per well in a 96 well plate for 5', and removed from the plate. The reaction was stopped with 100µl 2N H_2SO_4 and plate absorbance measured in an Elisa reader at 450 nm.

Fingerprinting positive clones

In some case, phage identified as being positive by ELISA were fingerprinted by carrying out PCR on their scFv and digesting them with BstNI. In general, different fingerprint patterns represent different antibodies. If fingerprinting n clones, a restriction enzyme mix containing the following was made up:(n+1) x 17.8µl water; (n+1) x 2µl restriction enzyme buffer (NEB buffer 2); (n+1) x 0.1 or 0.2µl restriction enzyme (0.1µl for HaeIII HC and 0.2µl for BstNI); (n+1) x 0.2µl BSA (10mg/ml). 20µl of the mix were added to each well of a 96 well plate with10µl of PCR mix from different clones to each well. Close wells with plates caps strips. 2-3 h for BstNI at 60°C. Samples were then run on gel (2% Metaphor agarose in 0.5 X TBE) for fingerprint analysis.

Selection of the 12-mer peptide library (Ph.D.-12 NEB)

This library was selected against two different target: MN423 and the tau fragment dGAE. All steps with this library were made using DH5αF' bacteria, instead of ER2537 as suggested by the manifacturer. Coating was made as described above. About 10^{10} phage from the library (10µl) were diluted in 1ml TBST, incubated for 60' RT in the coated tube, sealed with parafilm on a rotating wheel. Tubes were washed as above inTBST.

Bound phages were eluted with 100 mM triethylamine, buffered as above. 500µl of eluate was amplified infecting 10ml DH5αF' at OD 0.5, as above, growing them in a culture volume of 30ml incubating infected cells 5-6 hours, shaking 270 RPM. The day after, phages were PEG concentrated, as above. Pellet was resuspended in 800µl TBS. 200-400 µl were used to enter the next cycle of selection. Each fraction was titred plating infected cells, at various dilutions, in an agarose top layer, were slow growth plaques were counted.After three rounds of selection, single plaques were picked with a sterile toothpick, transferred in diluted cultures (1ml or 10ml) of DH5αF' (1:100 of an ON culture). Then bacteria were incubated 5-6 hours. Bacteria were spun down as above. Supernatants were trasnferred and spun again. Supernatants were titred and used for ELISA or phage blot experiments. 500 µl of phage stock, was PEG precipitated and used for DNA extraction and sequencing.

Selection of a λ random nonapeptides library (λ immunotube biopanning)

Nunc immunotubes were coated overnight at 4° with 1 ml of a 1-10:1000 dilution in PBS of previously western blot tested 423 ascites. Tubes were washed once in SM Tween 20 0.5% plus once in SM before and after blocking, saturated with 3% BSA in SM for 1h on rocking platform at RT. The library was blocked by making it 1% BSA and incubating for 1 hour at room temperature. 10 µl aliquots of the library (~$2x10^9$ phage particles) were added to each tube and incubated for 1.5 hour on an oscillating platform, at 4°. Tubes were washed seven times (short washes) with SM Tween 20 0.1 %, five times (short washes) with SM alone and once with SM for 5' on an oscillating platform. Phage particles bound to the antibody were eluted by adding 1 ml overnight BB44 bacteria (from

Gianni Cesareni) and incubating for 15'-30' at 37° standing. Infected cells were mixed with 8 ml molten top agar 40, 0.2% Maltose, 10 mM $MgSO_4$ and poured onto 2XTY tet plates (BB44 are tetracycline resistant). The following day plaques were harvested by washing the plates with 10 ml of SM, on an oscillating platform for 2h at RT or 4h at 4°. 0.5g NaCl was added to the phage suspension and after 1h on ice, the phage were centrifuged for 10' at 13000 rpm at 4°. The supernatant was harvested and the phage were purified by PEG precipitation and resuspended in 400 µl SM containing 20µl chloroform. 200 µl were used for the next cycle of selection, which was carried out as described above. Aliquots of both output and input were stored and used for titration.
20 of the clones selected after three cycles were sequenced at the Los Alamos National Labs genome sequencing facilities, using the BigDye Terminator sequencing system.

PEG precipitation of lambda phage

0.5 g PEG 8000 were added to10ml of phage supernatant from a plate in SM medium. After incubating for 1 hour on ice, the phage were centrifuged for 10' at 8000 rpm at 4°, and resuspended in 400 µl SM. 20 µl chloforom were added, and the solution centrifuged for 8000 rpm at 4° to remove bacterial debris.

Preparation of protein samples

Tau isoforms production and purification

The six tau isoforms were produced according to a slightly modified version of the protocol described in (Kontsekova, Cattaneo et al. 1995). pET vectors each bearing a tau

isoform were transfected or electroporated into BL21 DE3 cells. Overnight cultures were diluted 1/100 into 500 ml LB. After reaching 0.6-1 OD_{600}, IPTG to a final concentration of 0.5 mM was added to each culture, and grown for a further three hours. Bacteria were pelleted, resuspended in 1/20 - 1/10 of initial volume ice cold PBS, boiled 5', centrifuged at 15000g for 20'. The supernatants were filtered through a 0.45 μm filter disc and tested for the presence of tau, by running aliquots on 12% SDS-PAGE, then staining gels with comassie blue and testing by western blot using 7.51 (a generic anti-tau monoclonal antibody, which recognizes an epitope in all tau isoforms, M. Novak personal communication). A comparison between comassie stained gel and western blot was made to evaluate purity of the samples. This purification procedure relies on the finding that essentially all E. coli proteins denature upon boiling, whereas tau remains soluble.

Human brain extracts.

Crude brain extract were prepared by homogenizing tissue directly in 1X SDS loading buffer (Laemmli 1970) at a ratio of 5 to 10 v/w of sample to SLB in a glass to glass Dounce homogenizer or electric homogenizer on ice. Samples were centrifuged at 3000 rpm in a Beckman centrifuge, and the supernatants were syringed through a 0.6 X 25 mm needle (to disrupt DNA). β-mercaptoethanol 2-5% v/v was added and samples were stored at -20°. Aliquots of samples were then boiled for 5' before running on gels.

λgt11 clone extracts

50μl of Y1090r$^-$ cells from an overnight culture were infected with the phage clone of interest for 20' at 37° at an multiplicity of infection of 5 to 10. Infected bacteria were inoculated in a 5ml volume of LB media, grown for 2h, and induced with a final

concentration 0.2- 0.5 mM IPTG and grown for an additional 3 h. Bacteria were centrifuged, resuspended in 100µl 1X SLB and run on SDS-PAGE. Supernatants were also pooled and analyzed by SDS-PAGE.

Proteins from clones made in pJuFo.

Phagemid clones in DH5α F' cells from an overnight liquid culture or plate were allow to grow to an OD of 0.8, then IPTG to a final concentration of 0.5 mM was added and bacteria grown for additional 3 hours. Aliquots were centrifuged and pellets resuspended in 1X SLB.

For phage production clones were growth to OD_{600} in 10 ml 2xTY amp/glu at 37°, infected with helper phage at 37° and incubated for 30 minutes at 37°C, IPTG 0.5mM final concentration was added and phage were grown overnight or 5 hours at 30°. Several experiments were made also without IPTG induction with no discernible difference.

Reducing/non-reducing conditions

To obtain reducing conditions, 1 to 5% ß-mercaptoethanol was added to protein samples with loading buffer, while buffer without ß-mercaptoethanol was used to run proteins in denaturing non-reducing conditions.

Western blots

Protein extracts were run on SDS page according to (Laemmli 1970) and transferred to nitrocellulose according to (Towbin, Staehelin et al. 1979). The electrophoresis apparatus used for most gels was from Hoefer (Mightly Small II). Most gels were minigels between 7.5 and 15% acrylamide, prepared with recipes found in (Sambrook, Fritsch et al. 1989) and assembled and run using the Mightly Small II apparatus, with a current flux of 15-20 mA per gel. The transfer of proteins from the acrylamide gel to the nitrocellulose membrane was made using a grafite electrode semi dry apparatus (Pharmacia), using a current of about 0.7mA/square centimeter, soaking a sandwich made with 3mm paper, nitrocellulose and gels in the transfer buffer described above for 5' before the transfer. The sandwich was prepared by placing 3 pieces of 3MM (Whatman) paper fitting the gel size, on the anode, then a nitrocellulose filter in contact followed by the gel and an additional 3 pieces of 3MM paper on the top, in contact with the cathode.

PJuFo colony lift experiments.

Clones bearing pJuFo phagemid test clones or the library were plated at 100 to 1000 CFU for overnight growth, depending on the kind of experiment to be performed, on 2xTY-amp 90 mm plates. The following day a dried sterile 10 mM IPTG saturated nitrocellulose filter was applied to the plates containing colonies. The colonies were allowed to adsorb to the filters for 10' at 37°C, filter positions on the plate were marked and the filters peeled off and placed on new 2xTY-amp plates and allowed to grow for an additional three hours. We tested different ways to determine the greatest peptide exposure (as determined by signals on the colony lift) using either bacterial lysis by chloroform vapour

(Sambrook, Fritsch et al. 1989) or infection with helper phage, with the latter appearing to give the best signals. Helper phage infection for this purpose was carried out by growing bacteria containing pJuFo clones to OD 0.5 in 2XTY amp and infecting with helper phage for 30' at 37° at a multiplicity of infection of 20:1, before plating. After the last growth step, filters are removed from plates, washed in TBST 0.05% and processed as above for immunodetection.

Immunodetection

(The following procedure was used with slight variations also for plaques and dots). After the transfer of proteins to nitrocellulose filters, the filters were blocked in TBST+ skim milk 5% w/v for 40', incubated with MN423 or 7.51 for 1h, ascite or hybridoma supernatant dilutions in MTBS, washed 4 times in TBST over 15', incubated with a secondary enzyme conjugated antibody (see above for details), and washed again 4 times in TBST over 15'.

For HRP detection: I+II ECL (Amersham) solutions were mixed, applied to the blot, and incubated for 60-120 seconds. Excess solution was blotted off with a piece of paper and the filter was exposed to X-ray film for various times ranging from 15 seconds to 1 hour. Films were developed.

For AP detection: 66µl of solution II (see below) in 10ml 100 mM TRIS pH9.5 were mixed with 33µl of solution I (see below). The filter was exposed to this solution and the signal followed in real time (4-10'). The reaction was stopped with water.

I) BCIP (0.5g in 10 ml 100% dimethylformammide, from Boheringer)

II) NBT (0.5g in 10 ml 70% dimethylformammide, from Boheringer)

Lambda cDNA libraries immunoscreening

λgt11 library and/or clones plating/titering

This procedure used to obtain plaques is essentially the same for both initial library screening or subsequent purification (secondary and tertiary screening) of positive clones. In a cDNA library primary screening, plaques can be in large number on a plate while it is better to have a smaller number of plaques in further steps, while isolating single clones.

From a master plate, one isolated colony of Y1090r$^-$ cells was inoculated into 10ml LB broth+10mM $MgSO_4$+maltose 0.2% and incubated at 37°C shaking 200 rpm overnight or until OD_{600} reaches 2.0.

To 200 µl of the overnight culture 10 µl of SM buffer containing λ phages either from a library dilution or isolated plaques were added. As many as 8000 small plaques could be screened on a single 90mm plate during a primary screening. Infection was obtained by incubating λ infected cultures at 37° for 15'. After infection, 3 or 8ml of LB top agar (3ml for 90mm and 8ml for 130mm) at 45°C containing 10mM $MgSO_4$ and 0.2% maltose were added, mixed and poured onto pre-warmed LB plates, swirling the plates to allow even spreading of the agar. Plates were cooled at RT to make the top agar solidify prior to incubating in the inverted position at 42°C. Plaques were usually visible after 4-5 hours.

Library/clones immunoscreening.

Nitrocellulose (Protran Nitrocellulose 90 mm round) filters, previously saturated in a 10 mM IPTG solution and dried, were gently placed on the top agar containing plaques. The plates were incubated with the filters for 3.5 hours. Parallel filters could be obtained by

adding a second IPTG saturated filter for an additional 3.5 hours on the same plates after the first filter had been removed, if plaques are not confluent. Such parallel filters were required when probing with 7.51 in addition to MN423.

Filters were marked by stabbing them through the agar with a needle soaked in black ink in three asymmetric points. Plates were cooled at 4° for 10'. Filters were removed and rinsed twice in TBST 0.5%.

Filters were blocked in 5% milk TBST 45', incubated for 1h in 1:20-1:50 dilution of MN423 hybridoma supernatant in MTBST, washed 3-4 time over 15' with TBST, incubated for 1h with a secondary goat anti-mouse AP conjugated antibody (see above), washed 3-4 time over 15' and finally developed using NBT-BCIP substrate in AP buffer (see above), usually for 5-10' were enough. All above steps were carried out at RT on a rocking platform.

Cloning

A standard restriction digest was made as following: 5 µl of buffer 10X (usually NEB buffers),10-50 units of restriction endonuclease (usually from NEB), 0.5-3 µg of DNA, dd water to 50 µl, incubate 3h-ON at 37° . For cloning digestion product was gel purified, for testing directly run on gel.

A standard PCR reaction is described below.

Ligations were made using the following mix: 0.5-2µg vector purified and sometime CIP (calf intestinal alkaline phosphatase) treated, a molar ratio 5:1 amount of the insert DNA, 50-400 units T4 ligase, T4 ligase buffer (NEB, 1X =50 mM Tris-HCl (pH 7.5), 10 mM

MgCl2, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin), dd water to 50 μl or less, at 16° ON.

Construction of a random C-terminal pentapepeptide library in pJuFo.

As a starting point, we received a modified pJuFo vector from Invitrogen. This vector was improved in the following ways: the addition of a polylinker and an epitope tag, one of the two original lac promoter was deleted and one of the the two pelB was replaced with a gIII leader. These modifications reduced the repetitive sequences present in a previous version of the vector, lowering the probability for intra-vector homologous recombination and deletions. From this improved vector we constructed two test clones both bearing the SV5 tag and each bearing a different tripeptide C-terminal epitope. Primers used for this step were:

5'primer > TAT GTA GAT CTG CGG CCG CTT CCG GAG GGT CGA CCA G

3'primer (MN423) 1> CAT ACC GGT ACC TCT AGA TTA TTC GGC ACC GTG ATC CAG GCC CAG CAG TGG GTT TGG GAT TGG TTT GCC AGT ACC GCT CGA GCC TT

3'primer (YL1/2) 2> GAT ACC GGT ACC TCT AGA TTA GAA CTC TTC GTG ATC CAG GCC CAG CAG

With the 5' primer and the MN423 primer, using pJuFo as a template, a PCR fragment was produced which contained the 220 linker, the SV5 tag and the –GAE epitope (recognized by MN423). This was cloned into the modified pJuFo using XbaI and ClaI to create pJuFo-GAE. When the correct clone had been obtained, the 5' primer and the YL1/2 primer were used to create a PCR fragment containing the –EEF epitope

(recognized by YL1/2 antibody) which was cloned into pJuFo-GAE using XbaI and ClaI to create pJuFo-EEF. With these two clones test selections were carried out.

The library was constructed in pJuFo-EEF by cloning a PCR fragment created using the two oligonucleotides below and pJuFo as a template. XbaI and ClaI were used for cloning.

VL5 > GGTGCTGCTATCGATGGTTTC

VL3> GAATGGGCCCTCTAGATTAMNNMNNMNNMNNMNNCAGGCCCAGCAGG TG

M=A/C

The PCR reaction for the construction of the library (Mullis, Faloona et al. 1986; Mullis and Faloona 1987), was made in 6 volumes of 50 µl using Taq polymerase and the supplied Taq buffer, 10X from Perkin Elmer. Oligos and dNTPs in same concentrations as the reaction described below. The program for the thermal cycler using this primers was the following:

1) 5' at 94°

2) 1' at 94°

3) 1' at 60°

4) 1' at 72°

6) go to 2) 29 times

7) 10' at 72°

8) 4° 24h

PCR fragments, homogenous in length but heterogeneous in sequence, were run on a 1.5% agarose gel and purified with the Qiagen Gel extraction Kit. The vector (clone

bearing the C-terminal epitope –EEF) and the PCR product were cut with the same endonucleases, XbaI and ClaI (New England Labs) in NEB buffer 4 in an overnight digestion at 37°C. Both the digested DNAs (fragment and vector) were again gel purified. Approximately 3µg (40µl) of insert were ligated with 3µg (10µl) of vector (molar ratio 5:1) in a 60 µl reaction with 400 units of T4 ligase (NEB), in the following buffer: 50mMTris-Cl pH7.5, 10mM $MgCl_2$, 10 mM DTT, 1mM ATP, 25 µg/ml BSA. Ligation proceeded overnight in a water bath at 16°. Ligase was inactivated by incubation for 10' at 70°. 1-2µl of ligation mix (100-200ng) were electroporated into 100 µl electrocompetent cells (see below). Then electroporated cells were grown 1h at 37° before plating on 2xTY amp/glu plates.

We obtained about $3x10^6$ clones. The vector alone ligation control plates gave a number consistent with the presence of $6x10^4$ vector colonies in the whole library, representing a vector background of 2%. 5 randomly chosen clones were sequenced (see results). All presented the expected randomized 5 C-terminal amino acids at the end of Fos. Nevertheless a more detailed analysis of this library was not performed, because parallel experiments, described in the test, showed that the library could not be used.

Tau mutants

pSG5 expression vector bearing tau coding sequence was kindly provided by M. Novak. Two Vent-PCR reactions were made using this as a template. As shown in figure 40, two PCR product were obtained using this construct as template. Primers used are shown in figure 40. Oligos concentration was of 10 pmol/µl used as 20X, dNTPs at 10 mM used as

20X, buffer used was (final concentrations):10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100.

The PCR program was the following:

1) 5' at 94°

2) 1' at 94°

3) 1' at 58°

4) 1':30'' at 72°

6) go to 2) 29 times

7) 10' at 72°

8) 4° 24h

The PCR bands were gel purified (as described above) and the following digestion reactions were made:

pSG5-tau and PCR product 1 with SacI and BstBI (NEB buffer 4). ON 37°

pSG5-tau and PCR product 2 with HindIII and NheI (NEB buffer 2). ON 37°

This digestions produced two tau fragments of about 200 bp in both the PCR products and the vector. The gel purified fragments from the PCR (mutated in codons highlighted in red in figure 40, the N-terminal mutant D25>A25, the C-terminal mutant D421>A421) were ligated back into the gel purified vector. Each oligo internal to the tau coding sequence was designed with one additional restriction sites each (Pvu1 and Pvu2). This allowed the subsequent verification of the correct cloning, digesting a full length tau mutant PCR product.

Electrocompetent cells

DH5αF' cells were prepared for electroporation in the following way. From an overnight liquid culture or plate, cells were inoculated in 200ml 2xTY, grown at OD 0.5, chilled on ice, spinned down at 3000 rpm 10' at 0°, resuspended in 200ml cold sterile water, chilled on ice 10', spun down as above, resuspended in 1/2 original volume in cold sterile water, chilled on ice 10', spun down as above, resuspended in 25ml sterile 0° 10% glycerol, chilled on ice 10', resuspended in 800µl sterile 0° 10% glycerol, aliquot in 40-60 µl (is possible to store aliquots at -80°). Electroporations were performed with the following settings 1800V, capacitor 25 µFD, pulse controller 200 ohms, controlling the time constant (4.6). After the pulse 1ml of SOC media was added quickly to the cuvette, cells harvested and grown at 37° for 1h before plating.

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