In fulfillment of the requirements for a doctoral degree in the subject of
Biochemistry and Molecular Biology

Generation of scFv recombinant antibodies with the help of the phage display system against the microtubule associated protein Tau and the kinase MARK

Thesis submitted to the
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Table of Contents

Abstract

Alzheimer's disease (AD) has two hallmarks: the extracellular amyloid plaques (AP) consisting mainly of the ß-amyloid peptide and the intracellular neurofibrillary tangles (NFT) containing mainly of Tau protein. The Alzheimer Tau is hyper-phosphorylated, and this abnormal phosphorylation is an early signal for neurodegeneration in AD. The function of Tau binding to microtubule (MT) and stabilizing them is regulated by phosphorylation. One of the kinases that plays a role in this process is the microtubule-affinity-regulating kinase (MARK).

In this study, I used the phage display recombinant antibody technique to generated new antibodies recognizing phosphorylated Tau and MARK in order to develop diagnostic tools for recognizing MARK in diseased tissue, transgenic animal models and Alzheimer Tau in the cerebrospinal fluid (CSF).

I established a phage display antibody library based on the hybridoma cells which produce Tau-1 monoclonal antibody. From this library, I obtained the scFv–Tau193-204 antibody which has the same properties as Tau-1 monoclonal antibody.

From the Griffin.1 library (a gift from Prof. Winter), I obtained four scFv antibodies against Tau. The scFv-TauS235p and scFv-TauS320p/S324p antibodies are phosphorylation-dependent. The scFv-TauS320p/S324p can distinguish the Alzheimer Tau from the control Tau; the scFv-TauS235p antibody showed by immunofluorescence that Tau in mitotic CHO cells is phosphorylated at Ser235. The scFv-Tau13-24 and scFv–Tau349-360 are phosphorylation-independent, the scFv-Tau13-24 antibody is human Tau–specific and can therefore be used to distinguish endogenous Tau from human Tau in transgenic animal models.

From the Griffin.1 library, I obtained two scFv antibodies against MARK. The scFv–PanMARK antibody recognizes all MARK isoforms, whereas the scFv–MARK1–535-549 is MARK1-specific. The scFv-PanMARK antibody can detect the overexpressed MARK in CHO cells by immunofluorescence.

By gene manipulation, I made tetramerized scFv antibodies. Comparing the monovalent scFv antibody to the tetravalent scFv antibody, I could show that the tetravalent scFv antibody has a 10-fold higher functional affinity.

Zusammenfassung

Die Alzheimersche Krankheit (Alzheimer's disease, AD) zeigt zwei Merkmale: die extrazellulären Amyloid-Plaques (AP), die hauptsächlich aus β-Amyloid-Peptiden bestehen und die intrazellulären neurofibrillären Bündeln (intracellular neurofibrillary tangles, NFT), die hauptsächlich aus dem Tau-Protein bestehen. Dises Tau ist hyperphosphoryliert und die abnormale Phosphorylierung stellt ein frühes Signal für Neurodegeneration in AD. Die Funktion von Tau, Modulation der Stabilität von Mikrotubuli (MT) bindet und stabilisiert, wird durch Phosphorylierung reguliert. Eine der Kinasen, die eine wichtige Rolle in diesem Prozeß spielt, ist die Mikrotubuli-Affinität-regulierende Kinase (microtubule affinity regulating kinase, MARK).

In meiner Arbeit wurde die rekombinante Antikörper Phagen-Display Technologie für die Erzeugung neuer Antikörper verwendet, die phosphoryliertes Tau und MARK erkennen. Damit sollten diagnostische Werkzeuge zur Detektion von MARK in erkrankten Geweben, transgenen Tiermodelen und von Alzheimer-Tau in der cerebrospinallen Flüssigkeit (cerebrospinal fluid, CSF) entwickelt werde.

Ich etablierte eine Phagen-Display-Antikörperbibliothek aus Hybridoma-Zellen, die monoklonalen Antikörper gegen Tau-1 produzieren. Aus dieser Bibliothek erhielt ich den Antikörper scFv–Tau193-204, der die gleichen Eigenschaften wie der monoklonale Antikörper gegen Tau-1.

Aus der Griffin.1-Bibliothek (ein Geschenk von Prof. Winter) erhielt ich vier scFv-Antikörper gegen Tau, wobei scFv-TauS235p und scFv-TauS320p/S324p eine phosphorylierungabhängige bindung zeigen. Der scFv-TauS320p/S324p kann das Alzheimer-Tau von Kontroll-Tau unterscheiden. In der Immunofluoreszenz zeigte der scFv-TauS235p-Antikörper, daß in mitotischen CHO-Zellen Tau an Ser235 phosphoryliert ist. Die scFv-Tau13-24 und scFv-Tau349-360 sind phosphorylierungsunabhängig. Der scFv-Tau13-24-Antikörper ist spezifisch für menschliches Tau und kann somit zur Unterscheidung des endogenen Tau von menschlichem Tau in transgenen Tiermodelen verwendet werden.

Aus der Griffin.1-Bibliothek erhielt ich zwei scFv-Antikörper gegen MARK. Der scFv-PanMARK-Antikörper erkennt alle Isoformen von MARK, während der scFv-MARK1-535-549 MARK1-spezifisch ist. Der scFv-PanMARK-Antikörper kann in der Immunofluoreszenz die überexprimierte MARK in CHO-Zellen detektieren.

Mit Methoden der Genmanipulation erzeugte ich tetramerisierte scFv-Antikörper. Verglichen mit einem monovalenten scFv-Antikörper zeigen tetravalente scFv-Antikörper eine zehnfach höhere funktionelle Affinität.

1. Introduction

The aim of this thesis is to contribute to the diagnosis of the neurofibrillary pathology in Alzheimer's disease (AD) by generating novel antibodies against Tau protein. Alzheimer's disease is the leading form of neurodegenerative diseases, its incidence has increased rapidly over the past century, because of the aging population. No cure is currently available and even diagnosis of the early stages is difficult, because the disease manifests itself only in the human brain. Therefore many laboratories throughout the would are developing methods for identifying the molecular and cellular origins of the disease with the aim of finding methods of prevention or treatment.

1.1. Alzheimer's Disease (AD)

Alois Alzheimer described the disease, which now carries his name, for the first time in 1907 (Alzheimer, 1907). In general, it appears as a neurodegenerative disorder in aging, accompanied by the loss of cognitive functions and short-term memory, speech problems and at the end motoric disabilities (McKhann et al., 1984).

If Alzheimer brains are analyzed postmortem, two types of pathological protein deposits are clearly seen (Braak and Braak, 1997; Rosenwald et al., 1993; Kobayashi et al., 1992), the extracellular amyloid plaques (AP) consisting mainly of ß-amyloid peptides (Glenner and Wong, 1984; Glenner et al., 1984; Masters et al., 1985), and the intracellular neurofibrillary tangles (NFT) containing mainly Tau protein (Goedert et al., 1996; Mandelkow and Mandelkow, 1998; Mandelkow, 1999).

It has become increasingly clear that AD is a heterogeneous group of sporadic and hereditary dementia (Trojanowski et al., 1997). The hereditary AD is also called familial AD (FAD). Many factors have been found playing a role in the onset of AD. As summarized in Figure 1,. FAD relates to three genes: APP, PS1 and PS2. Mutations in the APP (Amyloid Precursor Protein) gene on chromosome 21 account for a small number of FAD kindred (Selkoe, 1994; Wisniewski et al., 1994). Mutations in the genes encoding two novel membrane spanning proteins known as PS1 (Presenilin 1) on chromosome 14 and PS2 (Presenilin 2) on chromosome 1 account for the majority of FAD cases (Levy-Lahad et al., 1995; Sherrington

et al., 1995). A risk factor for sporadic AD is linked to the Apolipoprotein E (ApoE) gene on chromosome 19. The homozygosity for the ApoE4 allele confers an increased likelihood of developing AD by age 80 (Saunders et al., 1993).

A current hypothesis holds that mutant genes (APP, PS1 and PS2) in a patient lead to the elevation of Aß peptide, a fragment of APP, and to the accumulation of characteristic AD brain lesions AP and NFT, and they merge into final common pathways leading to neuron death and dementia in AD (Trojanowski et al., 1997).

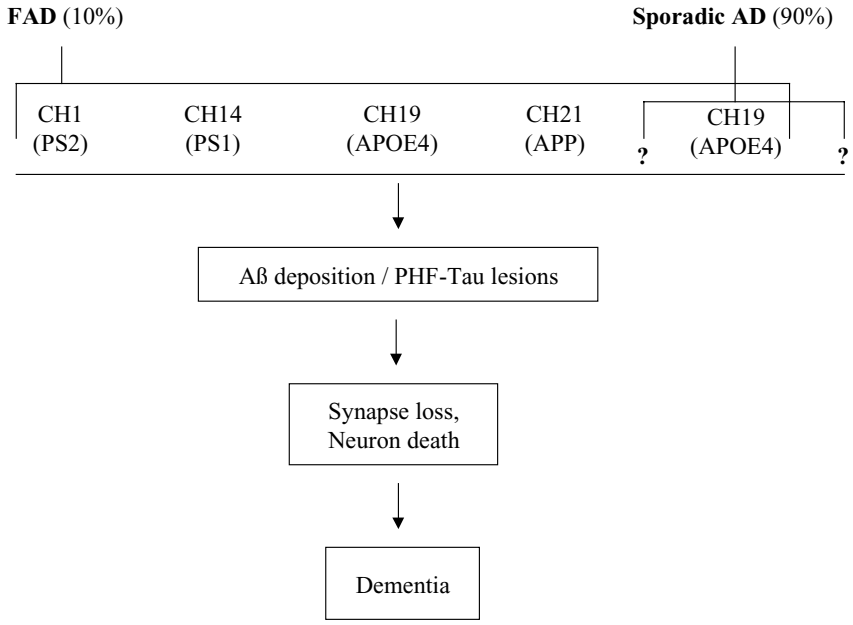

Figure 1. Genetic risk factors of the AD phenotype. The sequence of events shown here is hypothetical. FAD accounts for about 10% of all AD cases, and these hereditary forms of AD are linked to mutations in the PS2 gene on chromosome 1 (CH1), the PS1 gene on chromosome 14 (CH14), and the APP gene on chromosome 21 (CH21) while the ApoE4 allele (ApoE4) on chromosome 19 is a risk factor for the development of FAD as well as for sporadic AD. (Adapted from Trojanowski et al., 1997).

1.2. Tau protein

Tau protein is a microtubule associated protein (MAP), stabilizing the microtubule network (Mandelkow and Mandelkow, 1995). As shown in Figure 2, the C-terminal part of Tau is called “assembly domain”. It contains a region which has three or four homologous sequences which are called “repeats”. The repeat region is important for the interaction between Tau and microtubules (Goode and Feinstein, 1994; Littauer et al., 1986; Mandelkow et al., 1995). The flanking regions (P2 and R’) are also important for the binding of Tau to microtubules

(Gustke et al., 1994; Butner and Kirschner, 1991). The N-terminal part of Tau is called "projection domain", it is probably used by microtubules as a spacer (Chen et al., 1992; Zingsheim et al., 1979), or as an anchoring domain for enzymes (Sontag et al., 1999).

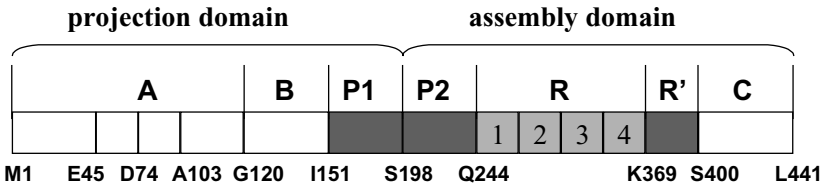

Figure 2. Tau domain structure. Full length Tau contains 441 amino acids. The N-terminal part (from Met1 to Ser198) is called "projection domain", the C-terminal part (from Ser198 to Leu441) is called "assembly domain". R is the repeat subdomain, A, B, P1, P2, R' and C are other subdomains. (Adapted from Gustke et al.,1994).

1.3. Microtubule affinity regulating kinase (MARK)

Phosphorylation is a post-translational modification of Tau. Tau is hyper-phosphorylated in Alzheimer brain (Sternberger and Sterberger, 1983; Blanchard and Ingram, 1989; Zhang et al., 1989; Roder and Ingram, 1991), suggesting that phosphorylation plays an important role in the onset of AD. The main phosphorylation sites are serine-proline (SP) and theronine-proline (TP) motifs (Biernat et al., 1992; Gustke et al., 1992; Lichtenberg-Kraag et al., 1992; Mandelkow et al., 1996), they can be phosphorylated by the glycogen synthase kinase 3 α and ß (GSK3α and ß, Mandelkow et al., 1992; Berling et al., 1994), the cyclin dependent kinases (CDK2 and CDK5, Baumann et al., 1993; Paudel et al., 1993) and the mitogen activated protein kinases (MAPK, Drewes et al., 1992; Mandelkow et al., 1993).

Besides SP and TP sites, there are other Tau sites which can also be phosphorylated by different kinases such as PKA (Litersky et al., 1996), PKC (Correas et al., 1992), CK1 (Pierre and Nunez, 1983) and CAM Kinase (Steiner et al., 1990). In particular, Ser262, Ser293, Ser324 and Ser356 in KXGS motifs of the repeats are phosphorylated by the microtubule affinity regulating kinase (MARK), which strongly reduces the binding of Tau to microtubules (Biernat et al., 1993; Drewes et al., 1995, 1997 and 1998; Trinczek et al., 1995; Illenberger et al., 1996;). MARK consists of different isoforms depending on the species. In rats, MARK1 and MARK2 have been found, whereas in mouse EMK (97% identical to MARK1) and in human p78 (75% homologous to MARK1 and 67% to MARK2) have been detected (Drewes et al., 1997). They share common characteristics, for example, as Figure 3 shows, MARK1 has the N-terminal header domain, followed by the catalytic domain; the T region is a

membrane-targeting motif; the ubiquitin-associated (UBA) domain which may relate to the degradation or regulation; the spacer domain and the C-terminal tail may relate to the binding to different membrane compartments (Drewes et al.,1998). The activation of MARK can be achieved by the phosphorylation of the regulatory loop in the catalytic region.

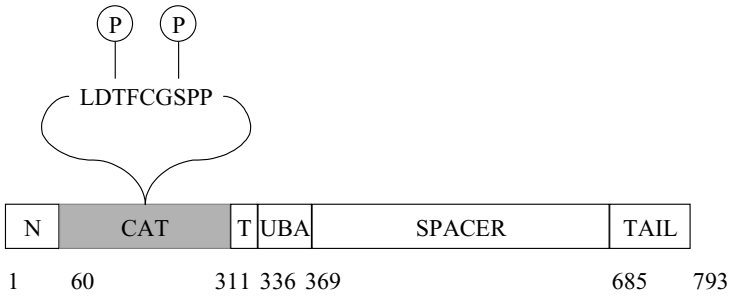

Figure 3. MARK structure. CAT is the catalytic domain, it contains a regulatory loop which has two phosphorylation sites. The T domain is a membrane targeting motif. UBA is the ubiquitin-associated domain. The spacer domain is large and presents the least homology among the different MARK isoforms. At the two ends, there are the N-terminal header and the C-terminal tail. (Adapted from Drewes et al., 1998).

1.4. The phage display recombinant antibody system

The phage display method was introduced by G. Smith (Smith, 1985). For the first time, he fused a portion of the gene encoding the EcoRI endonuclease to the minor capsid protein pIII , which is present on the surface of bacteriophage M13. Afterwards, within a few years, many applications of this technology were established. Till now, the phage display of peptides and proteins on the surface of bacteriophages represents a powerful new method for carrying out molecular evolution in the laboratory, it is applicable in a wide range. Some of them are listed as follows (Barbas, 1993; Francisco and Georgiou, 1994; Kay and Hoess, 1996):

Phage display of natural Peptides

- Mapping epitopes of monoclonal and polyclonal antibodies
- Generating immunogens

Phage display of random peptides

- Mapping epitopes of monoclonal and polyclonal antibodies
- Identifying peptide ligands
- Mapping substrate sites for proteases and kinases

Phage display of proteins and protein Domains

- Directed evolution of proteins
- Isolation of high-affinity antibodies

cDNA expression screening

As illustrated in Figure 4 (McCarferty and Johnson, 1996), antibodies have a tetrameric structure consisting of two identical heavy chains and two identical light chains. The region which defines the binding specificity of an antibody is referred to as the variable domain and is at the N–terminal end of each chain. The association of a heavy- and a light- chain variable domain (V_H and V_L, respectively) forms a heterodimeric molecule, termed an Fv fragment, which determines the binding specificity of the antibody. Antibodies can be expressed in *E. coli* in the form of an Fv fragment by expressing separate V_H and V_L domains, or in the form of an Fab fragment by expressing separate V_H–CH1 and V_L–C_L domains; a signal peptide will direct these chains into the periplasmic space where they associate to form an Fv or Fab fragment. Fab fragment is stabilized by the disulfide bridge between C_H1 and C_L domains. One problem with this approach is that the Fv fragment dissociates in some cases. One solution to this problem is to join the V_H and V_L genes with DNA encoding a flexible linker peptide. This gives rise to a single protein with covalently linked V_H and V_L termed a single chain fragment variable (scFv) molecule.

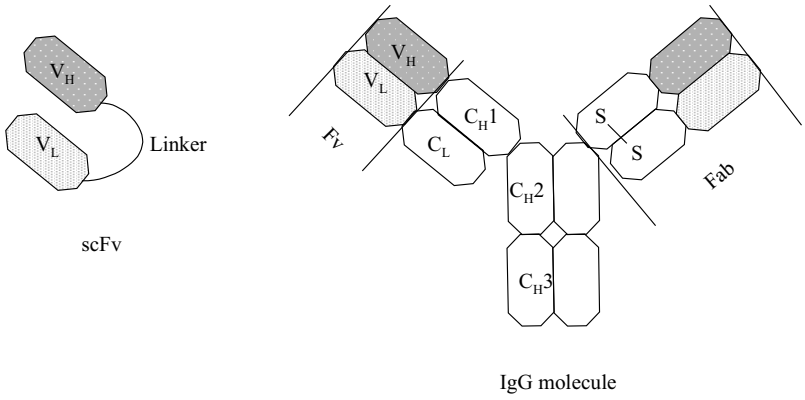

Figure 4. Structures of an IgG molecule (the right side) and scFv molecule (the left side). The globules represent the various domains in the heavy chain and the light chain. The binding specificity of the antibodies is determined by the V_H and V_L segments. The linker of the scFv is a flexible peptide. (Adapted from McCafferty and Johnson, 1996).

In this study, we focused our work on the recombinant antibody in the form of scFv. There are two key points in the phage display recombinant antibody system, one is the construction of the phage display antibody library, the other is the selection of a target antibody out of the phage display antibody library. These two points, together with other points in the system, are described in detail as follows.

1.4.1. Cloning of scFv

The cloning of a single chain fragment variable (scFv) gene is the first step for the phage display recombinant antibody system. The principle of the scFv cloning is summarized in Figure 5. It contains four steps: messenger RNA (mRNA) isolation, cDNA synthesis, V_H and V_L preparation and scFv assembly (Hoogenboom et al., 1992). Briefly, the mRNA is isolated from antigen-immunized animal spleens or from hybridoma cells. The mRNA is transcribed to cDNA by reverse transcriptions (RT). V_H and V_L are prepared by polymerase chain reactions (PCR) with primers which consist of corresponding sequences intrinsic to most antibodies to ensure that nearly all species of antibodies can be obtained. The scFv gene assembly is performed by linking the V_H and V_L with a linker containing the respective conserved sequence. Till now, the complete scFv gene is obtained, and it will be used to construct the phage display antibody library in the next step.

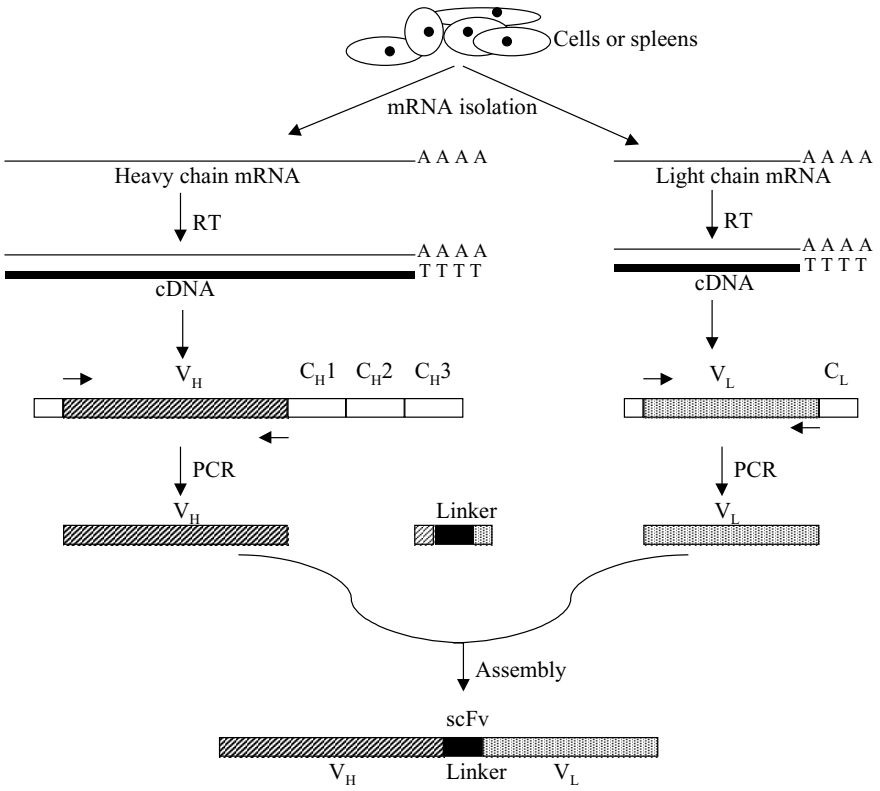

Figure 5. The cloning of a single chain fragment variable (scFv) gene. The mRNA is isolated from hybridoma cells or spleens; then it is transcribed to cDNA by reverse transcription (RT). The cDNA is amplified by polymerase chain reactions (PCR) with V_H-specific primers or V_L-specific primers (symbols of ← and → represent primers). Finally, the linker, which has a part of V_H sequence at the 5'-end and a part of V_L sequence at the 3'-ends, is used to link V_H and V_L to build the complete scFv gene.

1.4.2. Construction of the phage display antibody library

As shown in Figure 6, the phage display antibody library construction includes four main steps: restriction digestion, ligation, transformation and phage rescue. The pCANTAB5E vector and the scFv gene are digested with the same restriction endonucleases, then they are ligated and the ligation product is used to transform *E. coli* TG1 which is the host of phages. The phage rescue (i.e. the phage reproduction) is performed together with the helper phage M13KO7. Due to the fact that replacement of the part of gene3 with the exogenous scFv gene in the pCANTAB5E vector leads to noninfectious phages (Smith, 1993; Armstrong, 1996), this wild-type gene is now supplemented by the helper phage genome. After the phage rescue, the phage display antibody library is obtained, it consists of a number of antibody species. If one kind of antibody is preferred, this library has to be selected by the antigen.

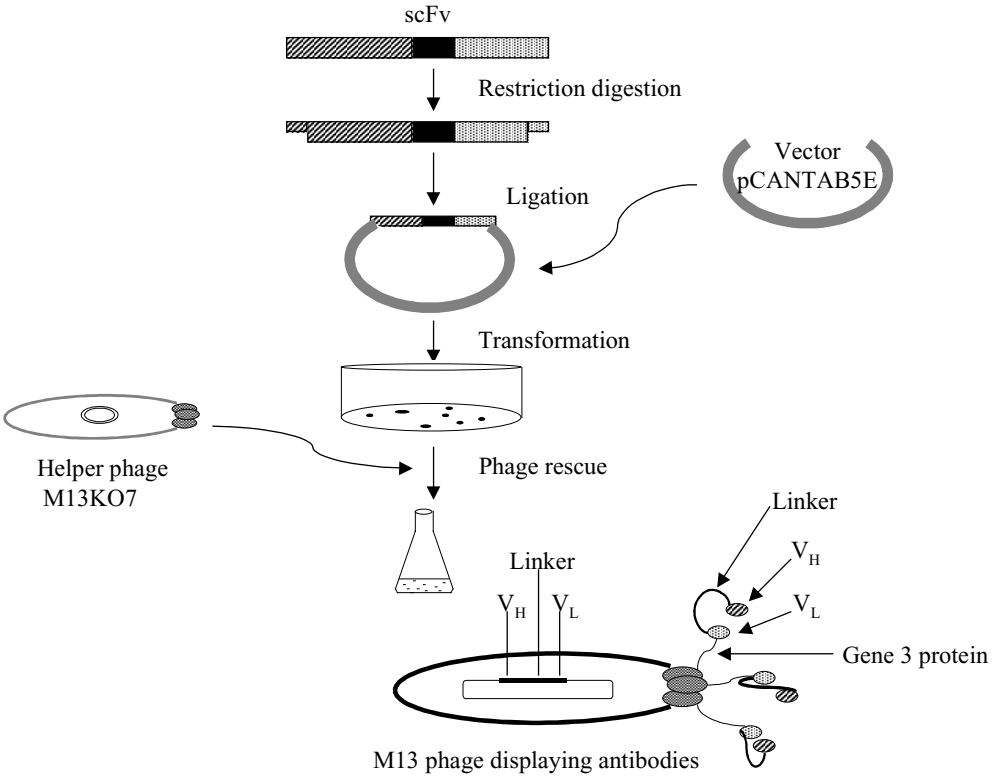

Figure 6. The construction of a phage display antibody library. After the restriction digestion, the pCANTAB5E vector and the scFv gene are ligated. The ligation product is then used to transform *E. coli* TG1 cells, and only the transformed TG1 cells grow on the Agar plate with Ampicillin. Finally, the transformed TG1 cells are infected by the helper phage M13KO7 to obtain all genetic information in order to reproduce M13 phages. The antibodies, which are fused to gene3 phage coat protein, are displayed on the phage surface. The scFv antibody sequentially has the V_L, the linker and the V_H.

1.4.3. Selection of phages

Usually, an specific antibody against one defined antigen is required, thus one step is necessary to get rid of the antigen-unspecific phages from the phage display antibody library, this is the so-called “selection”. The selection of phages is in principle an affinity purification of the target phages. A typical selection strategy is illustrated in Figure 7. Briefly, the antigen is coated on the solid surface, for instance, a plastic well. Then antigens are incubated with the phage display antibody library. The target phages recognizing the antigen will bind, whereas the unspecific phages will be washed out. The *E. coli* TG1 cells are added to let them be re-infected by the target phages. Finally, these re-infected TG1 cells are used to rescue the new phages displaying the selected antibody candidates. If necessary, the second selection cycle will start from this new phage batch; likewise, the third cycle will base on the new phage batch from the second cycle. Round by round, the target phages finally will be enriched to a detectable level.

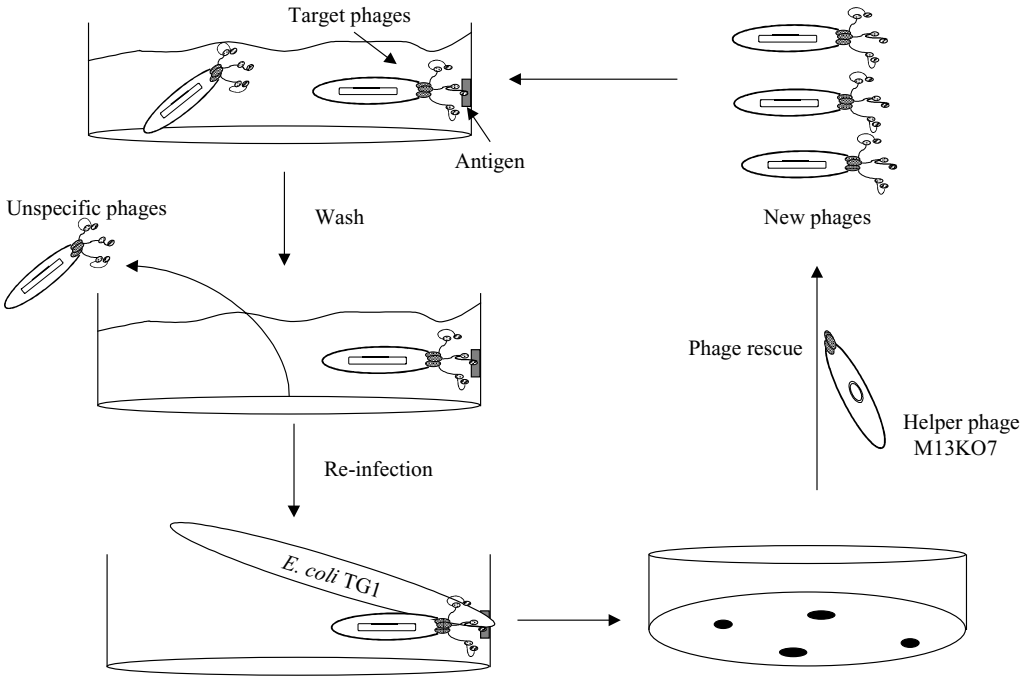

Figure 7. The selection of phages. A phage display antibody library is incubated with the coated antigen in a plastic well. The antigen-specific phage binds to the antigen, the unspecific phage does not bind and is washed out. The *E. coli* TG1 cells are directly added into the well to achieve the re-infection (alternatively the phage can be eluted, and then re-infects the TG1 cell in another tube). On the Agar plate with Ampicillin, only re-infected TG1 cells can grow, and these TG1 cells are then infected by the helper phage M13KO7 to continue the reproduction for the new phages. If necessary, the new phage display antibody library can go on to another one or more rounds of selection.

1.4.4. Screening of phages

Since the enriched phages can never be completely composed of the target phages, one additional step, the so-called “screening”, has to be performed in order to obtain finally a single specific clone as illustrated in Figure 8. Briefly, at the last round of selection, instead of going ahead to reproduce the new phages, the re-infected *E. coli* TG1 single clones on the Agar plate (see Figure 7) are transferred to wells so that each well has only one single TG1 clone. A part of TG1 cells in each well is transferred again to another corresponding well and the phage rescue is performed. A part of the reproduced phages in each well is detected by performing ELISA with the antigen. According to the positions of the positive ELISA signals, the remaining TG1 cells and phages, which are the target cells and phages, can be found out in the corresponding previous wells.

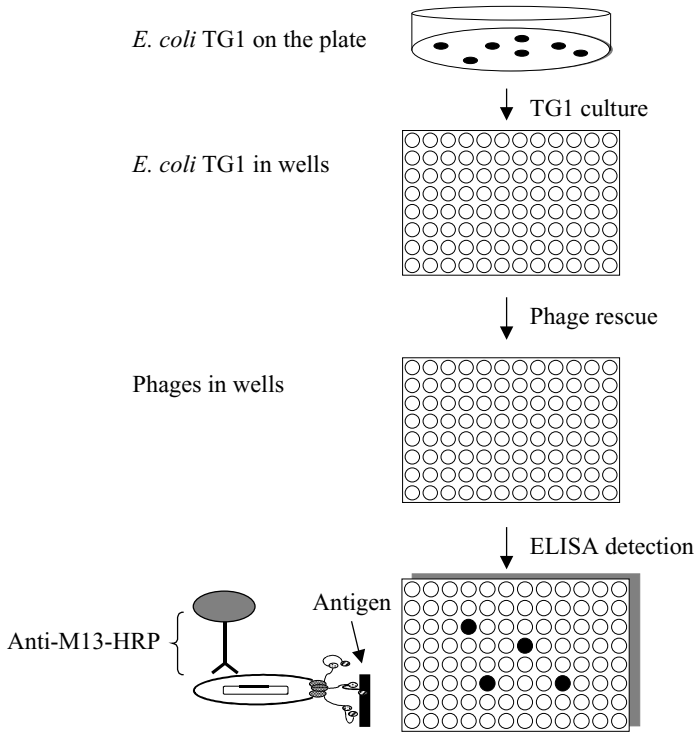

Figure 8. The screening of phages. The *E. coli* TG1 single clones on a Agar plate are transferred to wells so that each well contains only one kind of TG1 cells. Then these single TG1 clones are used to reproduce phages in other corresponding wells. Finally, the reproduced phages are detected with the antigen by ELISA accompanied by the HRP-labeled anti-M13 coat protein antibody. The positions of the positive ELISA signals indicate the corresponding positions of the antigen-specific TG1 clones and phages in previous wells .

1.4.5. Expression and purification of scFv antibodies

So far, the scFv antibody is displayed on the surface of the phage; but in most cases, the soluble scFv antibody without fusing to the phage is required. According to the pCANTAB5E vector structure in Figure 9, the protein synthesis begins at the gene3 signal sequence, goes through scFv and E tag sequences, then meets an amber stop codon. The *E. coli* TG1 host strain has a suppresser tRNA which allows readthrough of the amber stop codon, thus the protein synthesis will move on to make gene3 proteins to propagate phages, this is the reason why TG1 cells are usually used to produce phages. The gene3 protein is assembled at the tip of the phage; and it is just the protein with which the scFv antibody is fused, this is the reason why the scFv antibody is displayed on the surface of the phage.

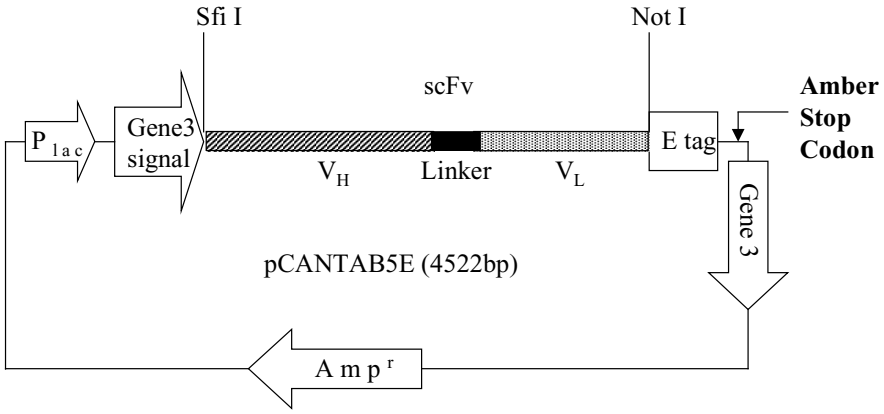

Figure 9. The structure of the pCANTAB5E vector. The scFv gene is inserted in the vector between the SfiI and NotI restriction sites. The E-tag sequence is behind the scFv gene, which introduces a detection marker for the scFv antibody. The Gene3 sequence encodes a phage coat protein which is necessary for the phage propagation; and Gene3 protein is also the protein to which the scFv antibody is fused. The amber stop codon is between the E-tag and the gene3 sequences; it controls whether the phage or the soluble antibody is produced. If the translation passes through the amber stop codon, Gene3 protein fused with scFv antibody is produced, the phage will be propagated; If the translation stops here, only the soluble scFv antibody with E-tag is produced.

Therefore, the *E. coli* HB2151 strain which does not produce the tRNA suppressor is employed for the expression of the soluble scFv antibody. As shown in Figure 10, the switch to the soluble scFv antibody production is accomplished by infecting the HB2151 cells with the phage. Since HB2151 is a nonsuppressor strain, the amber stop codon will be recognized; after the induction with IPTG, the protein synthesis will be stopped before the gene3 protein, thus only soluble scFv antibodies are produced. The soluble scFv antibody can be purified by many methods such as chromatography.

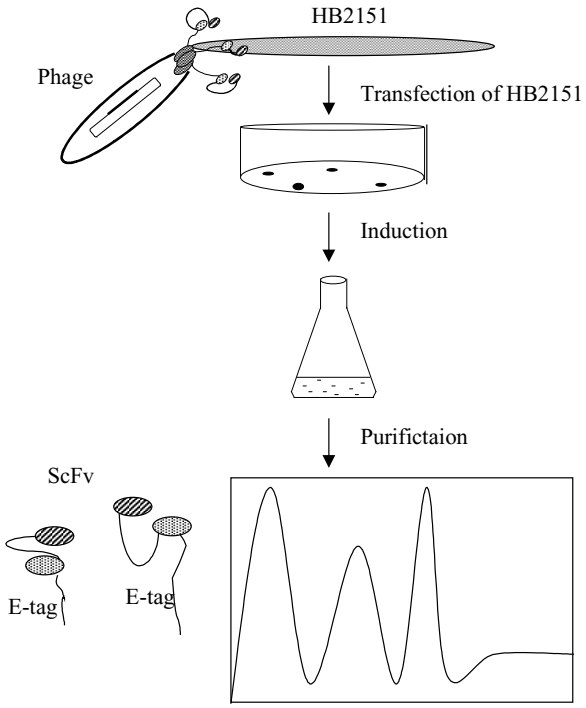

Figure 10: The expression and purification of scFv antibodies. The *E. coli* HB2151 cells are transfected by a phage in order to obtain the antibody gene carried by the phage. The transfected HB2151 cells grow on the Agar plate with ampicillin. A single clone on the Agar plate is transferred to a flask to express the soluble scFv antibodies with the E-tag. The scFv antibody is finally purified by chromatography.

1.4.6. Tetramerization of scFv

Comparing to the traditional antibody technique, the phage display recombinant antibody system has an unique advantage of manipulating scFv antibodies (Ryn and Nam, 2000). For example, DNA shuffling allows the recombination of DNA fragments from different scFv genes to form a new hetero-antibody in order to have the higher affinity (Osbourn et al., 1996) and stability (Jung et al, 1999), or to humanize a murine antibody (Beiboer et al., 2000).

Since the scFv antibody has only one binding site to the antigen, the functional affinity is not as good as that of a monoclonal antibody which binds to the antigen on two sites. Therefore, another manipulation of scFv antibody is to engineer the multivalent miniantibodies (scFv fragments linked with hinges and oligomerization domains) in order to improve their functional affinities. By this manipulation, the dimeric miniantibody, the tetrameric

miniantibody and the bispecific miniantibody can be formed (Plückthun and Pack, 1997). In this study, we focused our work on the tetramerization of scFv.

The structure of the tetravalent scFv in Figure 11 shows that the oligomerization domains (p53 domains) associate with each other to connect four scFv monomers together, which means that the scFv tetramer would automatically form if the scFv monomer had the hinge/p53 sequence. Therefore, the key point of the tetramerization is to add the hinge/p53 sequence behind the scFv monomer.

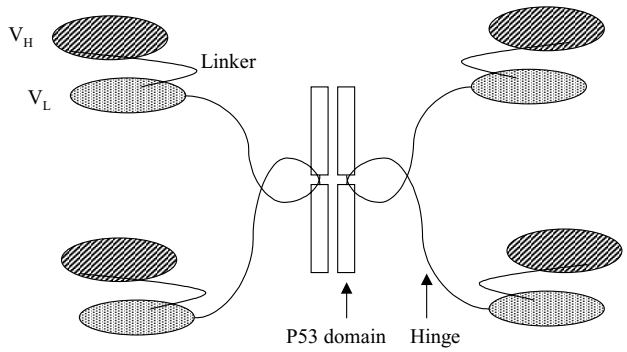

Figure 11. The schematic diagram of a tetravalent scFv miniantibody. Four scFv monomers are forming a tetravalent complex. The upper hinge is from human IgG3, the self-associating peptide is the oligomerization domain of human p53. (Adapted from Plückthun and Pack, 1997).

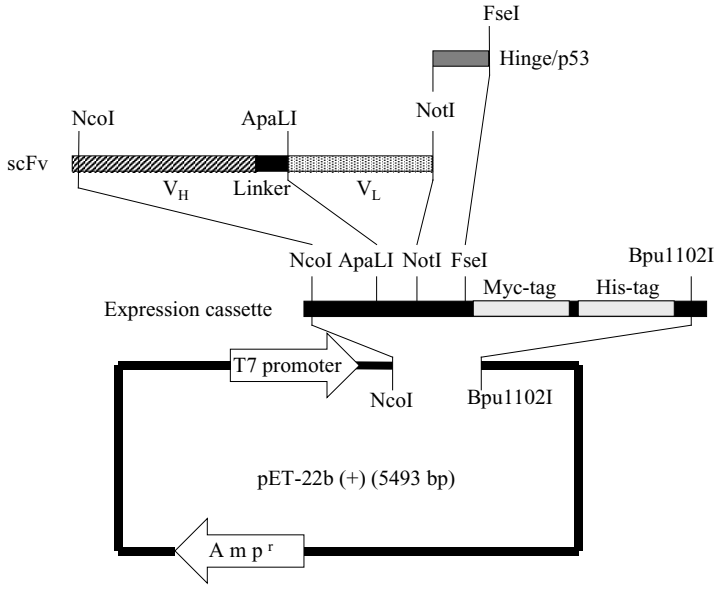

Figure 12. The vector construction for the tetravalent antibody. The starting source is the pET22b(+) vector. The first step was to insert the expression cassette into the pET22b(+) vector at the NcoI and Bpu1102I sites; the second step is to insert the scFv gene into the vector at the NcoI and NotI sites; the third step is to insert the hinge/p53 sequence behind the scFv gene at the NotI and FseI sites.

In principle, the addition of the hinge/p53 sequence is just a simple cloning step, but since we have two requirements, it becomes a little bit complicated. First, we want to use the pET22b(+) vector to substitute the pCANTAB5E vector in order to express the scFv antibody in *E. coli* BL21(DE3) strain which is much more widely used in the laboratory than the HB2151 strain; secondly, we want to introduce the myc tag for the detection of the scFv antibody and the His tag for the purification of the scFv antibody. Thus the whole cloning strategy includes three steps, as shown in Figure 12, the first step is to insert the expression

cassette into the pET22b(+) vector to introduce the necessary restriction sites, the myc tag and the His tag; the second step is to insert the scFv gene into the vector; the third step is to insert the hinge/p53 sequence.

1.5. Aim of this work

A reliable diagnosis of Alzheimer's disease (AD) usually is not established until postmortem studies of the brains are performed because there are no diagnostic tests that are totally reliable. As there is no effective therapy, the brain biopsy is not advised because it is too invasive.

Studies have now found a two-fold increase of the concentration of Tau in the cerebrospinal fluid (CSF) of AD-patients compared to controls by the enzyme-linked immunosorbent assay (ELISA) (Arai et al.,1995; Hock et al., 1995; Jensen et al., 1995; Mori et al., 1995; Motter et al., 1995; Munroe et al., 1995; Nitsch et al., 1995; Vigo-Pelfrey et al., 1995). Although error bars are large in these measurements and there is no clear distinction between other neuronal degenerative diseases, it would be an important step forward, if one would generate specific antibodies against Alzheimer Tau (e.g., against phosphorylation sites) with high affinities, that could be used to establish an ELISA assay, that would be really specific for AD-Tau and more sensitive than the existing ones (Trojanowski et al., 1997).

Since Tau in CSF may be in different states of phosphorylation, truncation or conformation which would be detected if novel antibodies were available. Therefore, in this study, we have used the phage display recombinant antibody technique to generate antibodies against Tau protein and phosphorylated Tau protein; likewise, we have generated recombinant antibodies against the microtubule affinity regulating kinase (MARK) as diagnostic tools. The recombinant antibodies against Tau were used to analyze the human brain Tau from AD patients. Finally, Tau and MARK expressions in eucaryotic cells were analyzed with the recombinant antibodies.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemicals were purchased at analytical grade from following companies:

Amersham-Buchler	Merck
Amersham Pharmacia Biotech	New England Biolabs
AppliChem	Pharma-Waldhof
Biomol	Pierce
Bio-Rad	Promega
Boehringer-Mannheim	Qiagen
Fluka	Riedel-de-Haen
Gerbu	Serva
InViTaq	Sigma
Kodak	

2.1.2. Proteins

2.1.2.1. Enzymes

Alkaline phosphatase	Boehringer-Mannheim
Restriction endonucleases	New England Biolabs
T4 Ligase	Amersham Pharmacia Biotech
Taq Polymerase	InViTaq

2.1.2.2. Antibodies

Anti-M13 coat protein-HRP antibody	1mg/ml	Amersham Pharmacia Biotech
Anti-mouse-HRP antibody	0.8mg/ml	Dianova
Anti-rabbit-HRP antibody	0.8mg/ml	Dianova
Anti-mouse-FITC antibody	1.3mg/ml	Dianova
Anti-rabbit-FITC antibody	1.5mg/ml	Dianova

Anti-rat-AMCA antibody	1.5mg/ml	Dianova
Anti-E tag-HRP antibody	1mg/ml	Amersham Pharmacia Biotech
Anti-HA tag polyclonal antibody	1mg/ml	Mo Be Tec
Anti-myc tag antibody	1.1mg/ml	Invitrogene
Anti-myc tag-HPR antibody	1.1mg/ml	Invitrogene
AT8 antibody	0.5mg/ml	Innogentics S. A.
AT180 antibody	0.5 mg/ml	Innogentic S. A.
Tau-1 antibody	1mg/ml	Purified in our lab.
YL1/2 antibody	1mg/ml	Serotec Ltd.

HRP is the horseradish peroxidase; FITC, AMCA are fluorescence dyes. They are coupled to the antibodies.

2.1.2.3. Tau proteins

All *E. coli* clones or Baculovirus containing genes of the recombinant human Tau and Tau constructs or mutants were generously given by Dr. Jacek Biernat from our lab (Biernat et al., 1992), and the proteins were purified in our lab by my colleagues or by myself according to the heat-denaturing method (Weingarten et al., 1975; Herzog and Weber, 1978; Sandoval and Weber, 1980; Gustke et al., 1994).

Tau constructs used in this study were Tau40, Tau40 with a 10-histidine-tag at the N-terminal (10-Histidine-Tau40), HTau23, K5, K13, K17, K19; Tau mutants used in this study were AP11, AP17, AP25, Tau23A231 and Tau40A235; they were mutated at the different Thr-Pro and Ser-Pro sites, where Thr or Pro was mutated to Ala (see 6.3. in the Appendix). Phosphorylated Tau40 and AP17 were purified from Baculovirus-Sf9 cell system which was established by Dr. Jacek Biernat in our lab (Biernat and Mandelkow, 1999). Phosphorylated K17, K19, AP11, AP25, Tau23A231 and Tau40A235 were prepared by an in vitro method using the mouse brain extract (Zheng-Fischhöfer, 1998).

Native Tau proteins from Alzheimer patient brains and healthy control brains were purified by Dr. Martin von Bergen from our lab following the Davies method (Greenberg and Davies, 1990). Two phosphorylated peptides, which consist of parts of the third repeat sequence, were purchased from Eurogentic. One is termed “S320p/S324p”, which has the sequence of ^{316}SKVTS(p)KCGS(p)LGNI^{328}, with two phosphorylated sites at Ser320 and Ser324; the other is named “ S320p”, which has the sequence of ^{316}SKVTS(p)KCGSLGNI^{328}, with only one phosphorylated site at Ser320.

2.1.2.4. MARK proteins.

The *E. coli* clones containing the recombinant microtubule affinity regulating kinase (MARK) genes were cloned by Dr.Gerard Drewes from our lab, and the protein was purified in our lab by Mr. Thomas Timm or by myself. Three MARK isoforms were used in this study, MARK1, MARK2 and MARK3 (Drewes et al., 1997).

2.1.2.5. Other proteins

Bovine serum albumin (BSA) was purchased from Gerbu. SDS-PAGE marker proteins were purchased from Sigma. Milk powder was bought from a supermarket, then was extracted by acetone and dried in air.

2.1.3. DNA

2.1.3.1. Primers for scFv clonings

Random primer (hexamer)	Random sequences
Light chain primer	Unknown sequence
Heavy chain primer 1	Unknown sequence
Heavy chain primer 2	Unknown sequence
Linker primer	Unknown sequence
RS primer mix	Unknown sequence

All above primers were purchased from Amersham Pharmacia Biotech

2.1.3.2. Primers for DNA sequencing

S1 primer	5' CAA CGT GAA AAA ATT ATT ATT CGC 3'
S3 primer	5' GGT TCA GGC GGA GGT GGC TCT GG 3'
S4 primer	5'CCA GAG CCA CCT CCG CCT GAA CC 3'
S6 primer	5’ GTA AAT GAA TTT TCT GTA TGA GG 3'
LMB3 primer	5 CAG GAA ACA GCT ATG AC 3’
T7 promoter primer	5‘ TAATACGACTCACTATAGGG 3'
T7 terminator primer	5’ GCT AGT TAT TGC TCA GCG G 3’

All above primers were purchased from MWG.

2.1.3.3. Oligo-nucleotides for the expression cassette construction

Mcs-1	5’ CC AAC TGG CGG CCG CAG GCC GGC CAG AAC AAA AAC TCA TCT CAG AAG AGG ATC TGA ATA GCG CCG 3’
Mcs-2	5’ TAAT GGT ACG GCC CAG CCG GCC ATG GCC CAG GTG CTG CAC TCG AGT GTG CAC AGG TCC AAC TGG CGG CCGC 3’
Mcs-3	5’ TT GCT CAG CGG TGG CAG CAG CCA ACT CAG CTT CCT TTC GGG CTT TTC AGA GCC TCA GGT CTC AGT GG 3’
Mcs-4	5’ GCC TCA GGT CTC AGT GGT GGT GGT GGT GGT GGT GGT GGT GGT CGA CGG CGC TAT TCA GAT CCT 3’

All above oligo-nucleotides were purchased from MWG.

2.1.3.4. Primers for the hinge/p53 fragment amplification

P53-1 primer	5’ GCG GCC GCA ACC CCG CTG GGT GAC ACC ACC 3’
P53-2 primer	5’ GTC GAC TGG CCG GCC CGG CGC GCC TCC GCT 3’

These two primers were purchased from MWG.

2.1.3.5. Plasmids and vectors

pCANTAB5E	Had been digested by SfiI and NotI enzymes, and dephosphorylated.	Amersham Pharmacia Biotech
pET22b(+)	The vector for the tetravalent scFv construction	Novagen
PMSTetp53His	Contains the hinge/p53 domain which was used to form tetravalent scFv antibodies	The gift from Prof. A. Plückthun (University Zurich, Switzerland)

2.1.4. Bacteria

TG1	for phage production	Amersham Pharmacia Biotech
HB2151	for soluble scFv expression	Amersham Pharmacia Biotech
Xl-2-Blue	for all cloning steps	Strategene
Bl21 (DE3)	for tetravalent antibody expression	Strategene

2.1.5. Cells

CHO-H62	Chinese hamster ovary (CHO) H62 cell line. It is the control CHO cell line transfected with the blank pEU1 vector
CHO-E34	CHO cell line overexpressing Tau40
CHO-MARK2	Inducible MARK2-CHO cell line, which express MARK2 only if the inductor Doxcycline is present

All above cell lines were constructed in our lab.

2.1.6. Equipment and accessories

2.1.6.1. Centrifugation

Cold centrifuge J2-21M/E and corresponding rotors	Beckmann
Table centrifuge 5402	Eppendorf
Table centrifuge 5403 and corresponding rotors	Eppendorf
Table centrifuge 5415C	Eppendorf

2.1.6.2. Chromatography

FPLC system and corresponding accessories	Amersham Pharmacia Biotech
HiTrap anti-E tag affinity column	Amersham phamacia biotech
Ni-NTA-agarose	Qiagen
Protein G, GammaBind® plus sepharose	Amersham Pharmacia Biotech
Sephacryl S-400 HR	Amersham Pharmacia Biotech

2.1.6.3. Electrophoresis

Electrophoresis power supply	EMBL
Gel electrophoresis system for protein separation	Höfer
Horizontal electrophoresis chamber for DNA/RNA separation	Amersham Pharmacia Biotech
Gel photo processor	Mitsubishi

2.1.6.4. Immunofluoresence microscopy

Axioplan fluorescence microscopy	Zeiss, Jena, Germany
CCD camera	Visicam, Visitron, Puchheim, Germany

2.1.6.5.Other equipment and accessories

ELISA reader	Dynatech
French press	Aminco
Speed vac concentrator	Bachofer
Incubator shaker innova 4300	New Brunswick Scientific
Electro-cell-manipulator 600 (BTX)	Invitrogen
Novablot electrophoretic transfer 2117-250	Amersham Pharmacia Biotech
UV/Visible spectrophotometer ultrospec1000	Amersham Pharmacia Biotech

2.1.7. Photographic materials

Developer	Ilford
Fixer	Ilford
Hyperfilm ECL	Amersham Life Science

2.1.8. Plastic wares

Agar solid media dishes for *E. coli* culture	NUNC
Cell culture flasks and dishes	NUNC
Marxsorp^{TM} plates for ELISA	NUNC
Microspin empty columns	Amersham Pharmacia Biotech

2.1.9. Software

Omiga	for DNA analysis	Oxford Molecular Ltd.
MetaMorph	for immunofluorescence imaging	Universal Imaging Corp.

2.1.10. Solutions

Permafluor aqueous mounting medium	Purchased from Immunotech
6x DNA loading buffer	70%(w/v) Saccharose, 0.1M Na_2EDTA, 0.25%(w/v) Bromphenolblue, pH7.5
ECL Western blotting detection reagents	Purchased from Amersham Phamacia Biotech
ELISA coating buffer	0.14% (w/v) Na_2CO_3, 0.3% $NaHCO_3$, pH9.6
ELISA substrate reaction solution	1.15% citric acid·H_2O,1.58% (w/v) $Na_2HPO_4 \cdot 2H_2O$, 0.05% (w/v) o-Phenylenediamin, 0.02% H_2O_2 (freshly added), pH4.5
Hirokawa I buffer	80mM Na-PIPES, 1mM $MgCl_2 \cdot 6H_2O$, 1mM Na-EGTA, 4% PEG6000, pH6.9
KAc (3M)	3M potassium acetate, pH5.0
10x NotI digestion buffer	100mM Tris/HCl, 100mM $MgCl_2$, 10mM DTT, 0.34% (w/v) Triton X-100, pH8.0
PBS buffer	8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in one liter H_2O, pH7.4
PBST buffer	PBS containing 0.1% Tween20
5% milk/PBS solution	PBS containing 5% milk powder
5% milk/PBST solution	PBST containing 5% milk powder
PEG/NaCl solution	20% polyethylene glycol 8000, 2.5M NaCl
2x SDS-PAGE loading buffer	160mM Tris/HCl, 10% (v/v) glycerol, 10% (w/v) SDS, 0.01% (w/v) Bromphenolblue, 2% (v/v) ß-ME, pH6.8

1x SDS-PAGE running buffer	25mM Tris, 50mM glycine, 3.5mM SDS, pH7.2
10x SfiI digestion buffer	100mM Tris/HCl, 500mM NaCl, 100mM $MgCl_2$, 10mM DTT, pH8.0
Spot-membrane regeneration buffer I	8M urea, 1%SDS, 10mM ß-ME in H_2O
Spot-membrane regeneration buffer II	10% acetic acid, 50% ethanol in H_2O
TAE buffer	0.89M Tris/HCl, 25mM Na_2-EDTA, pH8.0
TE buffer	10mM Tris/HCl, 0.1mM EDTA, pH8.0
1x TES buffer	0.5M Tris/HCl, 0.5mM EDTA, pH7.4
Urea lysis buffer	100mM Tris/HCL, 150mM NaCl, 8M Urea, pH7.5
Western blotting buffer	48mM Tris, 39mM glycine, 20% (v/v) methanol, 0.0375% (w/v) SDS, pH7.5

2.1.11 Media

2.1.11.1. Cell culture media

Basal ISCOVE medium	Seromed®, Biochrom KG, Berlin, Germany
Fetal calf serum (FCS)	Sigma
Geneticin 418	Sigma
HAM's F12 medium (with L-Glutamine)	Gibcol BRL, Life Technologies
1x Trypsin	Gibcol BRL, Life Technologies
Penicillin/streptomycin (100x)	Sigma
Puromycin	Sigma

Basal ISCOVE medium	90%	for Tau-1 hybridoma cells
FCS	10%	
Penicillin/streptomycin (100x)	1%	

HAM's F12 medium (with L-Glutamine)	90%	for CHO-E34 and -H62 cells
FCS	10%	
Geneticin 418	600µg/ml	

HAM's F12 medium (with L-Glutamine)	90%	for CHO-MARK2 cells
FCS	10%	
Geneticin 418	600µg/ml	
Puromycin	2.5µg/ml	

2.1.11.2. *E. coli* culture media

LB	10g tryptone, 5g yeast extract, 5g NaCl in 1 liter H_2O
TSS	10g PEG3350, 5% (v/v) DMSO, 50mM $MgCl_2$, in 100ml LB medium, pH6.5
2xYT	16g tryptone, 10g yeast extract, 5g NaCl in 1 liter H_2O.
2xYT-A	2xYT containing 100µg/ml ampicillin
2xYT-AG	2xYT medium containing 100µg/ml ampicillin and 2% glucose.
2xYT-AK	2xYT containing 100µg/ml ampicillin and 50µg/ml kanamycin.
2xYT-G	2xYT medium containing 2% glucose.

TSS medium was sterilized by 0.22µm filter. Others were sterilized by autoclave.

2.1.11.3. *E. coli* culture solid Agar-medium plates (Agar plates)

LB plate	15g Agar in 1 liter LB medium
LB-50C plate	LB plate containing 50µg/ml carbenicillin
Minimal plate	12g Na_2HPO_4, 6g KH_2PO_4, 2g NH_4Cl, 15g agar in 1 litter H_2O. Cooled to 50^oC then added 2mM $MgCl_2$, 2mM $CaCl_2$, 2mM thiamine hydrochloride, 0.2% glucose. pH7.4
TYE plate	16g tryptone, 10g yeast extract, 0.5g NaCl, 15g agar in 1 liter H_2O. Cooled to 50^oC then added ampicillin to 100µg/ml and glucose to 2%.
SOBAG plates	20g tryptone, 5g yeast extract, 5g NaCl, 15g agar in 1 litter H_2O. Cooled to 50^oC then added $MgCl_2$ to 10mM, ampicillin to 100µg/ml and glucose to 2%.
SOBAG-N plates	SOBAG plate containing 100µg/ml nalidixic acid.

All media were sterilized by autoclave.

2.2. General methods

Many of the following methods are from laboratory manuals (for example, Ausubel et al., 1988; Sambrook et al, 1989), and many of them were slightly modified in our lab.

2.2.1. Cell biological methods

2.2.1.1. Culture of Tau-1 hybridoma cells

Hybridoma cells that produce the Tau-1 monoclonal antibody were cultured in basal ISCOVE media with 7.5% CO_2 atmosphere at 37^oC. The cell density was 10^6/ml; they grew by suspension in the medium, and secreted the Tau-1 antibody into the medium. The medium was changed by spinning down cells at 1000xg for 10 minutes, removing the old medium and

resuspending the cell pellet into fresh medium. For the first two weeks, cells could be diluted 1:2 every three days; after two weeks, since cells grew slowly, the medium was only changed, but the medium volume was not enlarged. The old medium was kept at 4°C up to two weeks for the purification of Tau-1 antibody.

2.2.1.2. Culture of CHO-H62 and CHO-E34 cells

CHO-H62 and CHO-E34 cells were cultured in HAM's media (containing 600µg/ml Geneticin418) with 5% CO_2 atmosphere at 37°C. Since the cells adhered to the ground surface of the flask, the medium was changed simply by removing the old medium and adding the fresh medium. The cells grew very rapidly, if necessary, they were divided into new flasks. For dividing the cells, the old medium was removed; PBS was added to wash the cells and PBS was removed; 1xTrypsin was added and incubated at 37°C for 5 minutes; the fresh medium was directly added and cells from the flask surface were scraped off; the cells were divided into 3-10 new flasks.

2.2.1.3. Culture of CHO-MARK2 cells and the induction of MARK2

CHO-MARK2-inducible cells were cultured in HAM's media (containing 600µg/ml Geneticin418 and 2.5µg/ml Puromycin) with 5% CO_2 atmosphere at 37°C. All handling steps were the same as that in 2.2.1.2.. For the induction of MARK2, the cells were put into fresh medium; then Doxcyclin was added to a concentration of 0.2µg/ml, with further incubation for 1-2 days (Drewes et al, 1997). After one day, cells produced a detectable amount of MARK2.

2.2.1.4. Immunofluoresence (IF) and the light microscopy

1. Culture cells on cover slip.
2. Remove the old medium, add Hirokawa I buffer, incubate at 37°C for 5 minutes, then remove the Hirokawa I buffer.
3. Fixe the cells with cold methanol at -20°C for 5 minutes, then remove the methanol, immediately wash the cells 3 times with PBS.

4. Block the cells with 5% milk/PBS at 37°C for 30 minutes, then shortly wash the cells 3 times with PBS.
5. Dilute the primary antibody in 5% milk/PBS, incubate with the cells at 37°C for 45 minutes, then wash the cells 3 times with PBS.
6. Dilute the fluorescent dye-labeled secondary antibody in 5% milk/PBS, incubate with the cells at 37°C for 45 minutes, then wash the cells 3 times with PBS.
7. Inversely place the cover slip on the permaflour aqueous mounting medium on the microscopy glass slide. Keep the slide at 4°C overnight and protect it from light.
8. The cells are examined using an Axioplan fluorescence microscope. Images are taken with a cooled CCD camera and processed with the MetaMorph software.

2.2.2. Microbiological methods

2.2.2.1. Culture and storage of *E.coli*

E. coli cells were incubated overnight at 37°C with shaking at 200-250rpm in 5ml medium. The overnight culture was supplemented with glycerol to 20% and stored at –80°C. The *E.coli* cells were also cultured on Agar plates overnight at 37°C or 30°C. Agar plates could be kept at 4°C for up to 2 weeks.

2.2.2.2. Preparation of log phase *E. coli* cells

The glycerol stock of *E. coli* cells was diluted into the LB, 2xYT or other media at 1:100, incubated at 37°C with shaking at 200rpm until the OD_{600nm} reached values of 0.4-0.6 (about 2-3 hours). This represented the log phase *E. coli* culture.

2.2.2.3. Preparation of the competent *E. coli* cells for the heat-shock transformation

1. *E. coli* cells were streaked onto a minimal plate and grew overnight at 37°C.
2. One colony was transferred to 5ml 2xYT medium and incubated overnight at 37°C with shaking at 250rpm.

3. 100ml of 2xYT medium was inoculated with 1ml of the overnight culture. Incubated at 37°C with shaking at 250rpm until the culture reached an OD_{600nm} of 0.4-0.5.
4. The cells were sedimented at 2,500g for 15 minutes at 4°C, then gently resuspended in 10ml ice-cold TSS medium and placed on ice. The cells were used for transformation within 2-3 hours.

2.2.2.4. Preparation of the electro-competent *E. coli* cells for the electroporation

1. 50ml LB medium was inoculated with a single colony from the LB plate, incubated overnight at 37°C with shaking at 200rpm.
2. 50ml of the overnight culture was added to 1 liter LB medium, incubated at 37°C with shaking at 200rpm until the OD_{600nm} reached 0.5-0.6 (about 2 hours).
3. The culture was cooled at 0°C for 30 minutes, centrifuged at 4,000g for 15 minutes to pellet the cells. The pellet was sequentially washed with 1 liter cold H_2O , 500ml cold H_2O , 40ml cold 10% glycerol. Each wash step was performed by resuspending the cells, centrifuging to pellet cells, and discarding the supernatant.
4. The cells were finally resuspended in 2ml cold 10% glycerol, and 50µl aliquots were stored at -80°C.

2.2.2.5. Precipitation of M13 phages

The overnight culture for the phage rescue was centrifuged at 4,000g for 30 minutes to pellet the cells. The supernatant was supplemented with 1/5 volume of PEG/NaCl solution, kept on ice for at least 1 hour. Then the supernatant was spun at 10,000g for 15 minutes to precipitate phages. The phage pellet was resuspended in 1ml 2xYT medium or PBS, stored at 4°C up to 2 weeks; alternatively, glycerol was added to 15-20% and stored at -80°C (Ridar et al., 19996).

2.2.2.6. Counting of the number of the transformed, infected or transfected *E. coli* cells

The transformed, infected or transfected *E. coli* cells were diluted by ratios of $1:10^3$, $1:10^4$ and $1:10^5$. 100µl of the diluted cells were plated on SOBAG plates, incubated overnight at 37°C. The number of colonies on SOBAG plates was counted to estimate the number of the original

cells. The number of the re-infected TG1 cells also reflected the number of phages because a phage could only infect one TG1 cell.

2.2.2.7. Induction of scFv antibody expression in *E. coli* cells

E. coli HB2151 or BL21(DE3) cells were inoculated in 50ml 2xYT-AG medium and incubated overnight at 30°C. The overnight culture was transferred to 1 liter 2xYT-A medium, incubated at 30°C with shaking at 250 rpm until OD_{600nm} reached 0.9. Then IPTG-b was added to a final concentration of 1mM, and incubation was continued for at least 3 hours at 30°C with shaking at 250rpm.

2.2.2.8. Growth of phages from the Griffin.1 library

1. The whole bacterial library stock (about $1x10^{10}$ clones with a diversity of 10^9) was inoculated with 500ml 2xYT–AG medium and incubated with shaking at 37°C until the OD_{600nm} reached 0.5 (about 1.5-2 hours).
2. 25ml of this culture (about 10^{10} clones) was infected with M13KO7 helper phage by adding it in a ratio of 1:20, the infection was achieved without shaking at 37°C in a water bath for 30 minutes.
3. The infected cells were spun at 3,300g for 10 minutes. The pellet was resuspended gently in 500ml 2xYT-AK medium, and incubated with shaking at 30°C overnight.
4. The culture was spun at 10,000g for 20 minutes. The cells were discarded. 100ml of PEG/NaCl solution was added to the supernatant, and left for 1 hour or more at 4°C.
5. The supernatant was spun at 11,000g for 20 minutes. The supernatant was aspirated off, the phage pellet was resuspended in 5ml PBS, respun at 11,000g for 10 minutes.
6. The supernatant (containing phages) was supplemented by glycerol to 15%, and 500µl aliquots were stored at -80°C.

2.2.3. Molecular biological methods

2.2.3.1. Electrophoresis of DNA

DNA samples were added 6x DNA loading buffer, run in 0.7-2% agarose gels in TAE buffer. The voltage was 100V, running time was about 30-60 minutes. Gels were stained with ethidiumbromide (4 µg/ml in H_2O) for 20 minutes, destained with H_2O for 10 minutes. Pictures were taken with the gel photo processor system.

2.2.3.2. Precipitation of DNA.

The DNA sample was supplemented with 1/10 volume of 3M KAc and 2.5 volumes of cold 100% ethanol, incubated at -20°C for at least 30 minutes. The DNA sample was centrifuged at 14,000g for 10 minutes at 4°C. The supernatant was removed and the pellet was washed by 50µl cold 70% ethanol. The DNA sample was centrifuged again to remove 70% ethanol, the DNA pellet was dried in air for 5 minutes, and then was resuspended in 10-50µl TE buffer or H_2O.

2.2.3.3. Quantification of DNA

Gel quantification was carried out to measure the amount of DNA. The DNA was run in an agarose gel with two different volumes, parallel to a standard sample containing a known amount of DNA. The gel was stained and photographed. The intensities of DNA bands were compared, the rough amount of DNA could therefore be estimated.

Another method to measure the DNA amount was carried out by a photometer. The DNA was diluted in H_2O, the absorption was measured at 260nm. The DNA concentration was:
OD_{260nm} x dilution folds x 50µg/ml.

2.2.3.4. Preparation of plasmids

All plasmid mini-preparations were carried out with the NucleoSpin® Plus Kit (Macherey-Nagel) following the user manual. All plasmid midi-preparations were carried out with the Nucleobond® AX Kit (Macherey-Nagel) following the user manual.

2.2.3.5. Restriction digestion of DNA

Components were mixed in a 500µl tube according to the table on the right side. The mix was incubated at 37°C for 1 hour, then directly supplemented with 6xDNA loading buffer and run on an agarose gel.

10x Buffer	1µl
Restrict endonuclease	5-10 Units
DNA	0.1-10µg
H_2O	xµl
---	---
Total volume	10µl

2.2.3.6. Isolation of DNA from agarose gels

The DNA was excised from the agarose gel and the gel size was minimized, it was isolated from the gel pieces by the Nucleotrap Kit (Macherey-Nagel) following the user manual.

2.2.3.7. Ligation reaction

The components in the table on the right side was mixed in a 500µl tube. The mix was incubated at 16°C overnight. The molar ratio between the digested vector and the digested insert was around 1:5.

10x Buffer	1µl
ligase (5U/µl)	1µl
Digested vector	200ng
Digested insert	200ng
H_2O	xµl
---	---
Total volume	10µl

2.2.3.8. Heat-shock transformation

Heat-shock transformations for ultracompetent XL2-Blue were achieved following the instruction manual of Stratagene from where the XL2-Blue was purchased.

Heat-shock transformation for competent *E. coli* TG1 cells was carried out as follows:

1. Plasmid (or the ligation mix) was added to 1ml ice-cold competent TG1 cells which was freshly prepared following the procedures in 2.2.2.3.
2. The solution was swirled gently to mix and placed on ice for 45 minutes.
3. The tube was incubated in a 42°C water bath for 2 minutes, then chilled on ice.

2.2.3.9. Electroporation

Electroporation is a high efficient transformation method. The plasmid (or the ligation mix) must be salt-free. If not, it was precipitated with ethanol and the pellet was washed twice with 70% ethanol to remove all salts. The DNA pellet was dried in air and resuspended into 2µl H_2O. 1µl of salt-free DNA was mixed well with 40µl cold electro-competent *E. coli* cells (see 2.2.2.4.). Electroporation was achieved using the electro-cell-manipulator under the conditions of 2,500V voltage and 129 Ohm resistance.

2.2.3.10. Polymerase chain reaction (PCR)

The PCR was performed following the user manual of InViTaq from where the Taq-polymerase was purchased. The PCR reaction system and the temperature cycler program are summarized as follows:

10x Buffer (including $MgCl_2$)	1-10µl
dNTP	0.2-2mM
Taq polymerase	1-5 units
Forward primer	1-5µM
Backward primer	1-5µM
H_2O	xµl
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Total volume	10-100µl

Temperature cycle program:

Pre-heat:	96°C,	15 seconds	
denaturing:	96°C,	15-30 seconds	} 30 cycles
Annealing:	50-60°C,	30-60 seconds	} 30 cycles
Elongation:	72°C,	30-60 seconds	} 30 cycles
Completion:	72°C,	5-10 minutes	

2.2.3.11. Sequencing of DNA

DNA sequencing reactions were performed using the fluorescent dye labeling method. The reaction mix and the temperature cycle program are summarized in the following table. After the reaction, the reaction mix was supplemented with ethanol to final concentration of 70% in order to precipitate DNA products The sample was incubated on ice for 10 minutes, centrifuged at 14,000g at 4°C for 30 minutes. The pellet was dried in air for 10 minutes. The sequencing of the sample was carried out in the Inst. für Zellbiochemie (UKE, Hamburg University) with an automatic sequencer. The analysis of the sequences were performed with the Omiga software package in our lab.

Purified DNA (1µg/µl)	1µl
Sequencing primer (10pmol/µl)	0.5µl
"Big-dye" reaction mix	2.5µl
Diluent solution	5.5µl
dH_2O	0.5µl
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Total volume	10µl

Temperature cycle program:

96°C, 30 seconds
50°C, 15 seconds
60°C, 4 minutes
} 25 cycles

2.2.4. Biochemical methods

2.2.4.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed following a modified protocol in our lab (Laemmli, 1970; Matsudaira and Burgess, 1978). The stacking gel was 4%, and the separating gel was 10% or 15%. Protein samples were 1:1 diluted in 2xSDS-PAGE loading buffer, and heated for 2 minutes at 95°C. The electrophoresis was carried out at 150V and maximal 35mA in SDS-PAGE running buffer. Molecular weight marker proteins were ß-Galaktosidase (116KD), phosphorylase B (97KD), bovine serum albumin (66KD), ovalbumin (45KD), glycerinaldehyde-3-phosphate-dehydrogenase (36KD), carbonic anhydrase (29KD), lactoglobulin (18KD) and cytochrom C (14KD).

2.2.4.2. Western blotting with antibodies

Western blotting is performed following a modified method in our lab (Towbin et al., 1979):

1. The SDS polyacrylamide gel was washed with Western blotting buffer for 10 minutes.
2. The protein was electro-transferred from the gel to the PVDF membranes (Millipore) with the current of 1mA/cm^2 for 30-60 minutes.
3. The membrane was blocked for 1 hour at 37°C with 5% milk/PBST.
4. The primary antibody was diluted into 5% milk/PBST and incubated with the PVDF membrane for 1 hour at 37°C. Then the membrane was washed 3 times with PBST.
5. The secondary antibody was diluted into 5% milk/PBST and incubated with the PVDF membrane for 1 hour at 37°C. Then the membrane was washed 3 times with PBST.
6. The substrate reaction was carried out with ECL Western blotting detection reagents. This generates the chemilluminescence that is detected by the Hyperfilm.

2.2.4.3. Western blotting with phages

The procedures were nearly the same as above. The main difference was that the precipitated phages (1:50-1:200 diluted) was employed as the primary antibody, and the anti-M13 coat protein-HRP antibody (1:5000 diluted) was the secondary antibody.

2.2.4.4. Spot-membrane immunoblot

Peptide spot membranes comprising the sequence of Tau or MARK1 in 15mer peptides were provided by Dr. R. Frank (GBF, Braunschweig, Germany, Tegge et al., 1995). The membrane could be used for immunoblots using either antibodies or phages.

1. The membrane was wetted 3 times with PBST.
2. 5% milk/PBST was applied to block free sites on the membrane for 1 hour at 37°C, then the membrane was briefly washed with PBST.
3. The primary antibody (or phages) were diluted in 5% milk/PBST and incubated with the membrane at 37°C for 1 hour. The membrane was then sequentially washed 3 times with PBST containing 0.5M NaCl and 3 times with PBST.
4. The secondary antibody was diluted in 5% milk/PBST and incubated with the membrane at 37°C for 1 hour. The membrane was then sequentially washed 3 times with PBST containing 0.5M NaCl and 3 times with PBST.
5. The substrate reaction was carried out with ECL detection reagents and Hyperfilm.
6. The regeneration of the membrane was achieved by sequentially washing the membrane with H_2O, the regeneration buffer I (containing Urea and SDS), the regeneration buffer II (containing HAc) and 100% ethanol. Each solution was used 3 times, each time was 10 minutes. Finally, the membrane was dried in air and stored at -20°C.

2.2.4.5. ELISA

1. The antigen was diluted in the ELISA coating buffer to a concentration of 2µg/ml.
2. The well in a 96-well-plate (Marxsorp™, NUNC) was coated with 100µl of the antigen solution at room temperature for 1 hour. The antigen solution was then discarded, and the well was washed 3 times with 100µl PBS.

(The peptide antigen was dissolved in H_2O, and an aliquot of 100µl was applied to the well. Then the well was incubated at 37°C in an air-incubator to dry the peptide solution overnight).

3. The well was blocked with 150µl of 5% milk/PBS for 1 hour at room temperature. Then 5% milk/PBS was discarded, and the well was washed 3 times with 150µl PBS.
4. The primary antibody (or phages) was diluted in 5% milk/PBS, an aliquot of 100µl was put in the antigen coated well and incubated for 1 hour at room temperature. Then the primary antibody was discarded, and the well was washed 3 times with PBST.
   (If the competition ELISA was performed, the competitor was involved in the 5% milk/PBS solution).
5. The secondary antibody was diluted in 5% milk/PBS, an aliquot of 100µl was placed in the well and incubated at room temperature for 1 hour. Then the secondary antibody was removed, and the well was washed 3 times with PBST and 3 times with PBS.
6. 100µl ELISA substrate solution was added to the well, left at room temperature for 1-5 minutes, then the substrate reaction was stopped with 50µl of 1N H_2SO_4.
7. The reading was taken by subtracting the OD_{490nm} from the OD_{410nm}.value.

2.2.4.6. Purification of Tau-1 monoclonal antibody

The medium from the hybridoma cell culture was filtrated and supplemented with 1/20 volume of 1M Tris/HCl (pH7.5) buffer. It was then applied to 5ml Protein G column which was pre-equilibrated with 50mM Tris/HCl (pH 7.5). The column was washed with 25ml Tris/HCl (pH 7.5). Tau-1 monoclonal antibody was eluted with 15ml of 25mM glycine (pH 2.75), and immediately neutralized with 7.5ml of 1M Tris/HCl (pH7.5). The eluted Tau-1 antibody was concentrated, glycerol was added to 50% and stored at -20°C.

2.2.4.7. Preparation of the periplasmic extract

10ml of the bacterial culture induced with IPTG-b were centrifuged at 4,000g for 30 minutes. The cell pellet was resuspended in 0.5ml of ice-cold 1xTES buffer (containing EDTA and 0.5M sucrose), then 0.75ml of ice-cold 1/5xTES buffer was added and vortexed, followed by incubation on ice for 30 minutes. Centrifugation was carried out at 10,000g for 10 minutes to

remove the pellet. The supernatant (periplasmic extract) was directly taken for scFv antibody purification or stored at −20°C until needed.

2.3. Phage display recombinant antibody system

2.3.1. Conversion of Tau-1 monoclonal antibody into scFv antibody

2.3.1.1. Purification of total RNA and mRNA

The glassware were made RNase-free by baking at 250°C for 3 hours, the plasticware were made RNase-free by autoclaving at 120°C for 30 minutes. Total RNA purification was carried out with the Totally RNA™ kit (Ambion) which provided a modified phenol extraction method (Chomczynski and Sacchi, 1987). The messenger RNA (mRNA) was purified from the total RNA with Oligotex™ kit (Qiagen) which was based on the binding of mRNA polyA tail to oligo-dT beads. The total RNA and mRNA were stored at -80°C.

2.3.1.2. Preparation of V_H and V_L by RT-PCR

37°C 1 hour	mRNA (100-800ng)	xµl
	Primed First-strand Mix (including reverse transcriptase, Random primer, dNTP and buffer)	11µl
	DTT solution (200mM)	1µl
	H_2O	xµl
	Total volume	33µl

The 500µl tube for reverse transcription (RT) was made RNase-free by autoclaving at 120°C for 30 minutes. Shortly before use, mRNA was heated to 65°C for 10 minutes and cooled immediately on ice. RT was performed following Amersham Pharmacia Biotech user manual using its reagents (summarized above). RT for V_H and V_L were achieved in separate tubes. After RT was finished, the tubes were heated to 95°C for 5 minutes to inactive the reverse transcriptase. PCR amplification was performed according to the following protocols, the cycle program was: 94°C, 30 seconds; 55°C, 1 minute; 72°C, 1 minute. 30 cycles.

For V_L:	
First strand reaction mix	33µl
Heavy primer 1	2µl
Heavy primer 2	2µl
Taq-polymerase (InViTaq)	1µl
H_2O	62l
Final volume	100µl

For V_L:	
First strand reaction mix	33µl
Light primer mix	2µl
Taq-polymerase (InViTaq)	1µl
H_2O	64µl
Final volume	100µl

After RT-PCR, all products were run in an 1.5% agarose gel. The 340bp (V_H) and 325bp (V_L) DNA bands were excised using clean scalpels. The gel pieces were placed into the microspin empty columns and centrifuged at 735g for 2 minutes. The flow-through contained V_H or V_L. They were concentrated to at least 3ng/µl by ethanol precipitation.

2.3.1.3. Assembly of scFv gene by PCR

10x Buffer (InViTaq)	5µl
dNTP (InViTaq, 12.5mM each)	4µl
Taq polymerase(InViTaq, 5U/µl)	1µl
V_H (50ng)	xµl
V_L (50ng)	xµl
H_2O	xµl
Final volume	50µl

Program:
94°C, 30 seconds
64°C, 4 minute
} 7 cycles

Usually the scFv DNA amount was very low, the second PCR was necessary to amplify the scFv gene as follows:

Assembly product from above	50µl
10x Buffer (InViTaq)	5µl
dNTP(InViTaq, 12.5mM each)	1.6µl
Taq polymerase (InViTaq, 5U/µl)	1µl
RS (restriction site) primer	4µl
H_2O	38.4µl
Final volume	100µl

Program:
94°C, 30 seconds
55°C, 1 minute
72°C, 1 minute
} 30 cycles

After the second PCR, scFv was purified by a microspin column which was based on gel-filtration to separate scFv from primers, unassembled V_H and V_L. as follows:

1. The microspin column was filled with 750µl Sephacryl S-400 HR resin, the storage solution was removed by centrifuging at 735g for 1 minute.
2. The column was equilibrated twice by adding 200µl TE buffer and spinning as above.
3. 100µl PCR product was applied to the column and centrifuged at 735g for 2 minutes. The flow-through contained the purified scFv.

After the microspin column purification, the scFv was checked by agarose electrophoresis and concentrated to at least 4µg/µl by ethanol precipitation.

2.3.1.4. Restriction digestion and ligation

SfiI digestion:

scFv (300ng)	up to 70µl	Overlay with 50µl mineral oil,
SfiI buffer (10x)	8.5µl	50°C, 4 hours
SfiI (20U)	xµl	
H_2O	xµl	
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Final volume	85µl	

Not I digestion:

SfiI digestion product (from above)	85µl	37°C, 4 hours
Not I (40U)	xµl	
3M NaCl	3.6µl	
Not I buffer	1.5µl	
H_2O	xµl	
---	---	
total volume	100µl	

After digestion, the digested scFv DNA was purified by the microspin column (see 2.3.1.3.). 250ng digested pCANTAB5E vector and 150ng digested scFv (molar ratio 1:5) were mixed in the 50µl reaction system according to the user manual from Amersham Pharmacia Biotech where the ligase was purchased. The ligation was performed overnight at 16°C After ligation, the mix was heated to 70°C for 10 minutes to inactivate the ligase.

2.3.1.5. Transformation and the phage rescue

50µl of the ligation mix was added to 1ml freshly prepared competent *E.coli*. TG1 cells to perform the heat-shock transformation. 100µl of the transformed cells were supplemented with glycerol to 15% and stored at -80°C. The rest or the transformed cells were used to produce phage (phage rescue) according to the following procedures:

1. 900µl transformed cells incubated with 9.1ml 2xYT-G medium at 37°C for 1 hour.
2. Ampicillin was added to a final concentration of 100µg/ml, and $4x10^{10}$ pfu helper phage M13K07 was added followed by incubation for 1 hour with shaking at 37°C.
3. The cells were pelleted by centrifugation, resuspended in 10ml 2xYT-AK, and grown at 37°C overnight.
4. The overnight culture was centrifuged to remove the cells, and the supernatant with phages was added to 2ml PEG/NaCl solution to precipitate phages (see 2.2.2.5.).

2.3.1.6. Selection of phages

1. Ni-NTA-agarose was packed in a small empty column, the bed volume of the gel was about 200µl. The column was equilibrated with PBS. 200µg of 10-Histidine-Tau40 protein was applied on the column at a very low flow rate. After application, the column was washed three times with 3x 1ml PBS.
2. 0.5-1ml phages (precipitated and resuspended in PBS) were applied to the column. The column was washed with 2ml PBS and 2ml PBS containing 1M NaCl.
3. The bound phages were eluted twice with 0.5ml of 300mM imidazol in PBS.
4. 10ml log phase *E. coli* TG1 cells were re-infected with the eluted phages for 1 hour at 37°C with shaking at 250 rpm.
5. The new phages were rescued (see steps 2 to 4 in 2.3.1.5.).

2.3.1.7. Screening of phages

1. The Log Phase *E. coli* TG1 cells were re-infected by phages, plated on SOBAG plates and incubated overnight at 30°C.
2. Single colonies were placed into 400µl 2xYT-AG medium in a well of the 96-well-plate (the TG plate) and grown at 30°C overnight.

3. 40µl of overnight culture were transferred to 400µl 2xYT-AG containing $2x10^8$ pfu M13KO7 in corresponding wells in another new plate (the Phage plate) and grown for 2 hours at 37°C with shaking at 150rpm. (The remaining TG1 cells in the TG plate were supplemented with glycerol to 15% and stored at -80°C).
4. The Phage plate were centrifuged at 1,500g for 20 minutes at room temperature. The supernatant was carefully removed, the cells were resuspended in 2xYT-AK medium and grown with shaking at 250rpm overnight at 37°C.
5. The Phage plate was centrifuged as described above, 30µl supernatants with phages were carefully transferred to corresponding wells in a plate coated with Tau40 to perform ELISA (the rest of phages could be stored at 4°C up to 7 days).

2.3.1.8. Transfection of *E. coli* HB2151 cell by phages

1. A tube was filled with 400 µl log phase HB2151 cells.
2. 2µl phage solution was added to infect the cells at 37°C for 30 minutes with shaking.
3. The HB2151 cells were plated on SOBAG-N plates and incubated overnight at 30°C.
4. Single colonies on SOBAG-N plates were picked up into 1ml 2xYT-AG medium and grown overnight at 37°C.
5. 100µl overnight culture was transferred to 1ml 2xYT-AG (the rest cells were supplemented with glycerol to 15% and stored at -80°C), incubated at 30°C with shaking at 250rpm until OD_{600nm} reached 0.9. The cells were centrifuged at 1,500g for 20 minutes at room temperature, the supernatant was carefully removed, the pellet was resuspended into 1ml freshly prepared 2xYT-AI (containing inductor IPTG-b) and incubated for at least 3 hours at 30°C with shaking at 250rpm.
6. The cells were centrifuged as described above, the supernatant was carefully transferred to a Tau-coated plate to perform ELISA, the secondary antibody was the anti-E tag antibody.

2.3.1.9. Localization of the scFv antibody

The *E. coli* HB2151 clone was induced in 50ml medium (see 2.2.2.7.). The culture was centrifuged at 1,500g for 20 minutes at room temperature. The supernatant were removed and centrifuged again at 10,000g for 15 minutes at 4°C to completely remove cells, and finally

filtered through a 0.45µm filter and stored at -20°C. The cell pellet was extracted with TES buffer to slightly open the cell wall to get the periplasmic extract (see 2.2.4.7). The periplasma-free cells were resuspended in 5ml TES buffer, and completely opened by the French press; the cell debris was sedimented by centrifugation at 10,000g for 10 minutes, the supernatant contained the intracellular soluble antibodies, i.e. the whole cell extract. The supernatant, the periplasmic extract and the whole cell extract were assayed by ELISA using the anti-E tag-HRP antibody as the secondary antibody, to verify where the scFv antibody was concentrated.

2.3.1.10. Purification of the scFv antibody

The *E. coli* HB2151 cells were cultured in 1 liter medium and induced by IPTG-b to produce the scFv antibody (see 2.2.2.7.). The periplasma was extracted by 20ml 1xTES and 30ml 1/5xTES buffer (see 2.2.4.7.). The periplasmic extract was filtrated, and adjusted to pH7-8. The periplasmic extract was applied on the HiTrap Anti-E tag affinity column equilibrated with PBS at a flow rate of 5ml/minute. The column was washed with 5 volumes of PBS. The scFv antibody was eluted with 1M glycine (pH 3.0), and immediately neutralized by adding 1/10 volume of 1M Tris buffer (pH8.2). The scFv antibody was concentrated, supplemented with glycerol to 50% and stored at -20°C.

2.3.2. Selection of scFv antibodies from the Griffin.1 library

2.3.2.1. Selection of phages with immunotube

1. 10-30mg/ml of the target protein (antigen) was coated on immunotube (NUNC Marxsorp^{TM}) in 4ml ELISA coating buffer at room temperature overnight.
2. The immunotube was blocked with 5ml 5% milk/PBS for 2 hours at room temperature.
3. 10^{10}-10^{13} phages in 4ml 5% milk/PBS were incubated in the immunotube for at least 3 hours at room temperature.
4. The phage solution was removed. The immunotube was washed 10 times with PBST, and then 10 times with PBS (20 times wash with PBST and then 20 times wash with PBS for further rounds of selection), 5ml (the full volume of the immunotube) buffer was used each time.

5. Phages were eluted with 1ml freshly prepared 100mM triethylamine (pH 11) and immediately neutralized with 0.5 ml 1M Tris/HCl (pH7.5). The immunotube was also filled with 200µl 1M Tris/HCl (pH7.5) to neutralize the remaining phages on the tube.
6. 500µl eluted phages was put into 10ml log phase *E. coli* TG1 cells to re-infect the cells. The immunotube was also filled with 4ml log phase TG1 cells to allow the re-infection of the cells by the remaining phages on the tube. Both re-infections are carried out by incubation for 30 minutes without shaking in a 37°C water bath.
7. The re-infected cells were pooled, centrifuged at 4,000g for 10 minutes to remove the supernatant, and plated on TYE plate for incubation overnight at 30°C.
8. Colonies were scraped off the plates into 2xYT medium. 1% of the cells were put into 100ml 2XYT-AG medium and grown at 37°C till OD_{600nm} reached 0.5. (The rest of the scraped cells were supplemented glycerol to 15% and stored at -80°C).
9. 10 ml (about $4x10^8$ bacterial cells) culture was mixed with $8x10^{10}$ helper phage M13K07 (the ratio of the TG1 cells to the helper phages was 1:20) to allow the infection of the TG1 cells for 30 min at 37°C in a water bath without shaking.
10. The cells were spun at 4,000g for 10 minutes, the supernatant was discarded, the cell pellet was resuspended in 50ml 2xYT-AK and cultured at 30°C overnight.
11. The cells were spun at 4,000g for 30 minutes, the supernatant with phages was added 8ml PEG/NaCl solution to precipitate the phages (see 2.2.2.5.).

2.3.2.2. Selection of phages with spot-membrane

1. The spot-membrane immunoblot using the phages was performed, and the ECL detection was carried out (see 2.2.4.4.). The target spot was found from the film, and its position on the spot-membrane was carefully marked.
2. 2µl log phase TG1 cells were directly added to the target spot, the membrane was put into a small container with wet paper towels and incubated at 37°C for 30 minutes.
3. The target spot was gently touched with a tip, and then the tip was put into 5ml 2XYT-AG medium and incubated overnight at 37°C.
4. 1ml overnight culture was used to rescue phages (see the steps 8-11 in 2.3.2.1.), the rest cells were supplemented with glycerol to 15% and stored at -80°C.
5. The spot-membrane was regenerated immediately (see step 6 in 2.2.4.4.).

2.3.2.3. Selection of phages with peptide

1. The peptide stock solution (10mg/ml, in DMSO) was diluted into H_2O to a final concentration of 2µg/ml. 100µl were placed into the well in a 96-well-plate (NUNC Marxsorp^{TM}) and incubated at 37°C in the air-incubator to dry it overnight.
2. The wells were blocked with 5% milk/PBS.
3. The phage (1:10 diluted in 5% milk/PBS) was added to the well and incubated at room temperature for 2 hours.
4. The wells were washed 10 times with 100µl PBS, and 10 times with 100µl PBST.
5. 100µl of log phase *E. coli* TG1 cells were added into the well to be re-infected at 37°C for 30 minutes. The cells were plated on TYE plates to incubate overnight at 30°C.
6. The colonies on the plates were used to rescue phages (see the steps 8-11 in 2.3.2.1.).

2.3.2.4. Selection of MARK1-specific phages with competition

1. The NUNC Marxsorp^{TM} plate well was coated with 100µl of 5µg/ml MARK1 in ELISA coating buffer overnight at room temperature.
2. The well was blocked with 150µl 5% milk/PBS.
3. The phages (1:10 diluted in 90µl 5% milk/PBS) were incubated in the well at room temperature for 2 hours. The well was washed 3 times with PBS.
4. MARK2 solution (1µg in 100µl 5% milk/PBS) was incubated in the well at room temperature for 2 hours. The well was washed 3 times with PBS.
5. MARK3 solution (1µg in 100µl 5% milk/PBS) was incubated in the well at room temperature for 2 hours. The well was washed 20 times with PBS.
6. 100µl log phase *E. coli* TG1 cells were added into the well to be re-infected at 37°C for 30 minutes. The cells were plated on TYE plates and incubated overnight at 30°C.
7. The colonies on the plates were used to rescue phages (see the steps 8 to 11 in 2.3.2.2.).

2.3.2.5. Purification of the scFv antibody

The scFv antibody from the Griffin.1 library has a His tag instead of an E tag, therefore Ni–NTA-agarose was used for the chromatography. The *E.coli* HB2151 cells were induced

(see 2.2.2.7.) and the periplasmic extract was prepared (see 2.2.4.7.). The periplasmic extract was dialyzed against PBS to remove EDTA which could destroy Ni-NTA-agarose. The dialyzed periplasmic extract was applied to the Ni-NTA-agarose column pre-equilibrated with PBS. The column was washed with 2 column volumes of PBS. The scFv antibody was eluted with the 0-300mM imidazol gradient. The scFv antibody was concentrated and dialyzed against PBS, supplemented with glycerol to 50% and stored at -20°C.

2.4. Tetramerization of the scFv antibody

2.4.1. Construction of the hinge/p53 fragment and the expression-cassette

2.4.1.1. Amplification of the hinge/p53 fragment

10x Buffer (InViTaq)	5µl
dNTP(InViTaq, 12.5mM each)	2µl
Taq polymerase(InViTaq, 5U/µl)	1µl
PMSTetp53His plasmids (1ng/µl)	1µl
P53-1 primer (50pmol/µl)	1µl
P53-2 primer (50pmol/µl)	1µl
H_2O	20µl
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Final volume	50µl

Program:

96°C, 15 seconds
55°C, 30 seconds
72°C, 30 seconds
} 30 cycle

72°C, 10 minutes

The hinge/p53 fragment was amplified from the PMSTetp53His plasmid. The PCR components and the cycler program are summarized in the above tables. After PCR, the product was run in a 2% agarose gel, the 70bp product was excised, extracted from the gel and concentrated by ethanol precipitation.

2.4.1.2. Construction of the expression-cassette

Four oligo-nucleotides, Mcs-1 to mcs-4, were used to construct the expression cassette. The first PCR was carried out to generate the middle fragment (113bp) of the expression-cassette. The PCR reaction mix and program were as follows. The PCR product was run in a 2% agarose gel, the 113bp band was excised, extracted from the gel and concentrated by ethanol precipitation.

10x Buffer (InViTaq)	5µl
dNTP(InViTaq, 12.5mM each)	2µl
Taq polymerase(InViTaq, 5U/µl)	1µl
Mcs-1 (50pmol/µl)	1µl
Mcs-2 (50pmol/µl)	1µl
H_2O	20µl
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Final volume	50µl

Program:
96°C, 1 minute
65°C, 2 minute
72°C, 10 minutes } 1 cycle
96°C, 30 seconds
55°C, 1 minute
72°C, 1 minute } 30 cycles

The second PCR was carried out to generate the whole expression-cassette (219bp). The PCR reaction mix and program were as follows.

10x Buffer (InViTaq)	5µl
dNTP (InViTaq, 12.5mM each)	2µl
Taq polymerase(InViTaq, 5U/µl)	1µl
Mcs-2 (50pmol/µl)	1µl
Mcs-3 (50pmol/µl)	1µl
Middle fragment (10ng/µl)	5µl
H_2O	15µl
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Final volume	50µl

Program:
96°C, 30 seconds
60°C, 1 minute
72°C, 1 minutes } 10 cycle
96°C, 30 seconds
72°C, 1 minute } 20 cycles

The PCR product was run in a 2% agarose gel, the 219bp band was excised, extracted from the gel and concentrated by ethanol precipitation.

2.4.2.. Construction of the pET22b(+)ExpcastP53 vector

2.4.2.1. Insertion of the expression-cassette into the pET22b(+) vector

The expression-cassette and pET22b(+) plasmids were digested with NcoI and Bpu1102. They were applied to a 2% agarose gel. The digested products were excised, extracted and concentrated by ethanol precipitation. The ligation was carried out overnight at 16°C. The ligation mix was precipitated with ethanol, washed twice with 70% ethanol, resuspended into 2µl H_2O and used to perform electroporation to transform *E. coli* BL21(DE3) cells. Transformed cells were plated and grown on LB-50C plates at 37°C overnight. Colonies were picked into LB medium containing 50µg/ml carbenicillin and incubated overnight at 37°C. Plasmid mini-preparation and a set of restriction digestions were done to check the plasmids.

Finally the candidate clones were sequenced to confirm the right clones. The new vector was called “pET22b(+)Expcast”.

2.4.2.2. Insertion of the scFv-Tau13-24 gene into the pET22b(+)Expcast vector

The plasmid of the scFv-Tau13-24 clone, was digested with NcoI and NotI restriction enzymes to obtain the scFv gene. The pET22b(+)Expcast plasmid was digested with the same enzymes. After the digestion, the products were run in a 1% agarose gel. The digested scFv–Tau13-24 gene was around 700bp, the digested pET22b(+)Expcast were around 5,500bp. The isolation of the two DNA species from the agarose gel, ethanol precipitation, ligation and electroporation were carried out as usual (see 2.2.3.). Several clones were picked from the LB-50C plate and grown in LB medium containing 50µg/ml carbenicillin overnight at 37°C. The plasmids were prepared, and defined with a set of restriction digestions. The new plasmid was called “scFv-Tau13-24-pET22b(+)Expcast”.

2.4.2.3. Insertion of the hinge/p53 fragment into scFv-Tau13-24-pET22b(+)Expcast vector

The hinge/p53 fragment and the scFv-pET22bExpcast plasmid were digested with NcoI and FseI. They were run in a 2% and 1% agarose gels, respectively. The DNA at 70bp (digested hinge/p53 fragment) and 6,000bp (digested scFv-pET22b(+)Expcast) were excised, and isolated from the gel. The ethanol precipitation, ligation, electroporation and plasmid mini-preparation were achieved (see 2.2.3.). The new plasmid was defined with a set of restriction digestions, it was called “scFv-pET22b(+)ExpcastP53”.

2.4.3. Construction of the scFv gene into the pET22b(+)ExpcastP53 vector

In order to use the newly constructed vector to make any tetravalent scFv antibody, the blank vector was prepared. The scFv-Tau13-24-pET22b(+)ExpcastP53 plasmid was digested with NcoI and NotI and dephosphorylated by alkaline phosphatase. The digested plasmid was run in a 1% agarose gel, the vector without the scFv-Tau13-14 gene was excised and extracted from the gel. The vector amount was measured spectroscopically and stored at -20°C. This vector was called “pET22b(+)ExpcastP53”.

The construction of a scFv gene into the pET22b(+)ExpcastP53 vector is just a business as usual. The scFv DNA was digested with NcoI and NotI and then run in a 1% agarose gel. The digested products were excised, extracted from the gel and concentrated by the ethanol precipitated. The digested scFv DNA and the digested pET22b(+)ExpcastP53 vector were mixed to carry out ligation overnight at 16°C. The ligation mix was electroporated to transform *E. coli* BL21(DE3) cells. Several transformed colonies were picked from the LB-50C plate, and grown in LB medium containing 50µg/ml carbenicillin overnight at 37°C. The plasmids were prepared and defined with a set of restriction digestions.

2.4.4. Expression and purification of the tetravalent scFv antibody

1. *E. coli* BL21(DE3) containing the scFv-pET22b(+)ExpcastP52 plasmid was induced (see 2.2.2.4.). The induction time was over 16 hours (overnight).
2. The cells were pelleted by centrifugation at 5,000g for 15 minutes. The pellet was resuspended in the urea lysis buffer, and incubated at 4°C with agitation overnight.
3. The cells was centrifuged at 10,000g for 15 minutes and the supernatant was filtrated.
4. The extract was applied to the Ni-NTA-agarose column pre-equilibrated with the urea lysis buffer.
5. The column was washed with 2 column volumes of the urea lysis buffer, followed by 2 column volumes of urea lysis buffer containing 30mM imidazol.
6. The tetravalent scFv antibody was eluted with 2 column volumes of urea lysis buffer containing 100mM imidazol.
7. The elution was sequentially dialyzed against PBS containing 4M, 2M, 1M, 0.5M and 0M urea. Each dialysis lasted 2 hours or more.
8. The elution was carefully concentrated avoiding to have the precipitation, added glycerol to 50% and store at -20°C.

3. Results

3.1. Conversion of Tau-1 antibody into scFv antibody

Tau-1 monoclonal antibody was usually purified from the culture of the hybridoma cells. Using the phage display recombinant antibody technique, we converted the production of Tau-1 monoclonal antibody into *E. coli*.

3.1.1. Purification of total RNA and mRNA

In order to get the source for converting Tau-1 monoclonal antibody into scFv antibody, the total RNA and mRNA were purified from the Tau-1 hybridoma cells. From 10^8 cells, 100µg total RNA was purified by a modified phenol extraction method; from the 100µg total RNA, 800ng mRNA was purified with the Oligo-dT beads (see 2.3.1.1.).

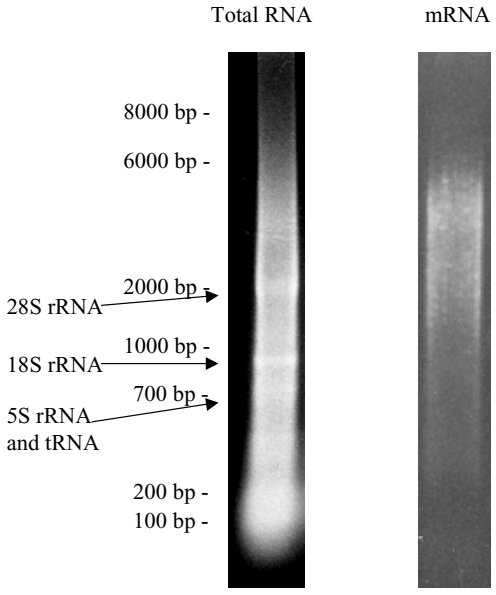

Figure 13. Total RNA and mRNA purification. The total RNA (2µg) appeared as a smear with 2 clearly visible bands around 900bp and 2000bp, corresponding to the 18S and 28S ribosomal RNA, respectively. There was also a diffuse band around 700bp due to the tRNA and 5S ribosomal RNA. There was a large number of small fragments around 100bp due to the degradation although the protection was very well done. The purified mRNA (100ng) was a smear between 200bp-6000bp.

After the native agarose gel electrophoresis, the total RNA showed a smear along the whole lane with two clearly visible bands around 900bp and 1000bp (Figure 13), which corresponded to the 18S and 28S ribosomal RNA, respectively (Hipfel et al., 1998; Monstein et al., 1998). The purified mRNA appeared as a smear along the lane due to the fact that the mRNA species have different lengths. The mRNA smear was mainly between 200bp-6000bp,

which correspond to proteins in the range of 50-2000 amino acids. The purified mRNA were the templates for the synthesis of the cDNA.

3.1.2. Preparation of V_H and V_L by RT-PCR

The reverse transcription (RT) and the polymerase chain reaction (PCR) were performed for the synthesis of V_H and V_L (see 2.3.1.2.). The RT was achieved to get the cDNA library from the purified mRNA (200ng). The PCR was the amplification for the antibody V_H gene and V_L gene in this cDNA library, which was achieved by using the primer 1 and primer 2 for the V_H and the light primer mix for the V_L.

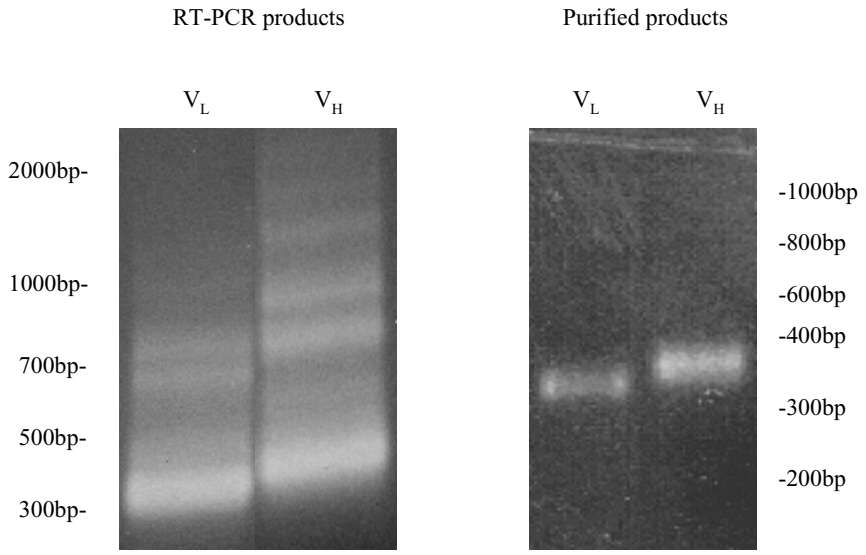

Figure 14. V_H and V_L synthesis. The RT-PCR products were not pure, the main products were around 300-400bp. After isolation from the gels, the purified V_L and V_H showed the sizes of 350bp (V_H) and 325bp (V_L), respectively.

The RT-PCR products were run in a 1.5% agarose gel, the major bands appeared around 300bp (Figure 14), they were the synthesized V_H and V_L (De Jaeger et al., 1997; Krebber et al., 1997). The gels containing the 300bp-bands were excised, the DNA was isolated from the gel, and finally was concentrated by ethanol precipitation. The amount of the purified V_H (350bp) and V_L (325bp) were measured by agarose gel quantification (see 2.2.3.3.), and then they were used to assembly the scFv gene in the next experiment.

3.1.3. Assembly of scFv

The separated V_H and V_L must be build together to form a complete gene, e.g., scFv gene, for a functional antibody, this synthesis was the so-called "assembly. The equal amounts of V_H and V_L (50ng each) were linked together with the linker primer through a PCR (see 2.3.1.3.). After the PCR, a new product around 750bp appeared (Figure 15), corresponding to the scFv DNA (De Jaeger et al., 1997; Krebber et al., 1997; Frenkel et al., 2000). On the gel, there appeared also other products including unused V_H, V_L and the linker primer. The scFv DNA was purified by gel filtration with the microspin column (see 2.3.1.3.), it appeared as a single band on the agarose gel. The amount of the scFv DNA was estimated by gel quantification (see 2.2.3.3.), and then it was digested with the restriction endonucleases in the next step.

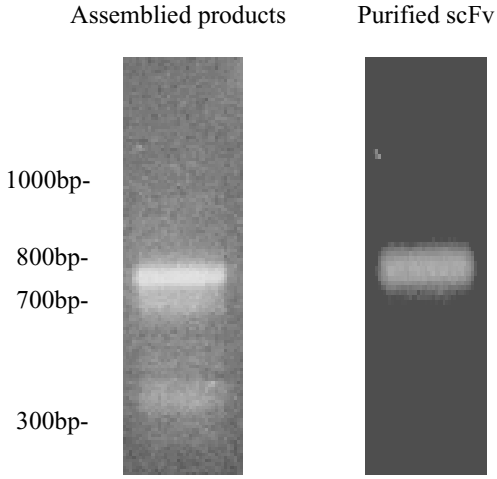

Figure 15. The assembly of the scFv. After the linkage reaction, there was a predominant band around 750bp, indicating the assembled scFv. The bands around 350bp were the unreacted V_H and V_L. The band around 700bp is an unknown by-product. After purification, scFv showed a single band at 750bp.

3.1.4. Restriction digestion and ligation

The scFv antibody will be displayed by the phage only if scFv DNA was recombined into pCANTAB5E vector. The scFv DNA contained SfiI (5' end) and NotI (3' end) restriction sites which could be used to insert it into pCANTAB5E vector. The purchased pCANTAB5E vector had been digested by SfiI and NotI enzymes; 300ng of the scFv DNA was digested with the same enzymes too (see 2.3.1.4.). After the digestion, the scFv DNA was purified by the gel filtration with the microspin column (see 2.3.1.3), concentrated by ethanol precipitation and quantified by a agarose gel analysis (see 2.2.3.).

250ng pCANTAB5E vector and 150ng digested scFv (molar ratio about 1:5) were used to achieve the ligation reaction at 16°C overnight (see 2.2.3.7.). After ligation, an aliquot was analyzed by the agarose gel electrophoresis to control the ligation reaction (Figure 16). If the vector shows a slight shift compared with the unreacted vector, the ligation was successful.

The mix could therefore be used for the transformation of *E. coli* TG1 cells to generate the TG1 cell library containing scFv genes.

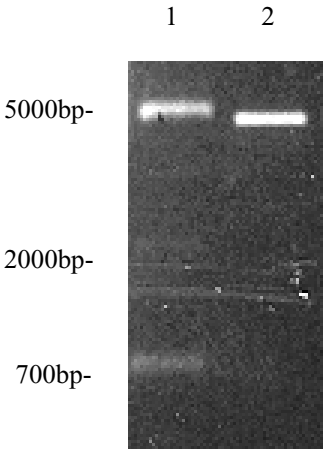

Figure 16. Ligation reaction. Lane 1: the ligation mix; lane 2: pCANTAB5E vector alone. After ligation, the pCANTAB5E vector showed a clear shift compared with the unligated vector. In addition, there was unreacted scFv around 750bp, and a by-product around 2000bp which is unknown.

3.1.5. Transformation and phage rescue

The transformation of TG1 cells with the ligation mix made an *E. coli* library which contained scFv genes. 1ml freshly prepared competent *E. coli* TG1 cells were transformed with the ligation mix (50µl) using the heat-shock method (see 2.2.3.8.). The total number of transformed TG1 cells was counted (see 2.2.4.6.), and it was about 3,000. This cell library was called "Hybri-Tau1 TG1 library", which meant that this *E. coli* library contained the scFv genes from the Tau-1 hybridoma cells.

The next step was to make the phage library from the Hybri-Tau1 TG1 library, which is called "phage rescue". First, the Hybri-Tau1 TG1 library was infected by the M13KO7 helper phages to obtain the necessary wild-type gene3 for the phage production, the ratio of TG1 cells to the helper phages was 1:10 (see 2.3.1.5.); then the infected TG1 cells grew overnight to produce phages and secret them into the culture medium. The phages were precipitated by the PEG/NaCl solution (see2.2.2.5.), and resuspended in 1ml fresh 2xYT medium, this phage library was called "Hybri–Tau1 phage library".

From the number of transformed TG1 cells, we knew that there were about 3,000 different individual phages carrying antibodies in this library. In principle, there is at least one clone in this library displaying the Tau-specific scFv antibody because it was originally constructed from the mRNA out of the Tau1-Hybridoma cells that produces Tau-1 monoclonal antibody.

The next step is to find out the Tau-specific clone among this library, it is the so-called "selection".

3.1.6. Selection of phages

For the Hybri-Tau1 phage library, the Histag-Tau40-Ni-NTA-agarose affinity column was employed to perform four rounds of selection in order to enrich the phages which carried the scFv antibody against Tau40 (see 2.3.1.6.). The number of the selected phages in each round was counted by calculating the number of the TG1 colonies on Agar plates (see 2.2.2.6.). The immuno-activity of the phages to Tau was detected with ELISA: Tau40 was coated on the well, the precipitated phages (1:100 diluted) was incubated to the coated Tau40, the secondary anti-M13 coat protein-HRP antibody (1:10,000 diluted) recognized the bound phages (Dente et al., 1994).

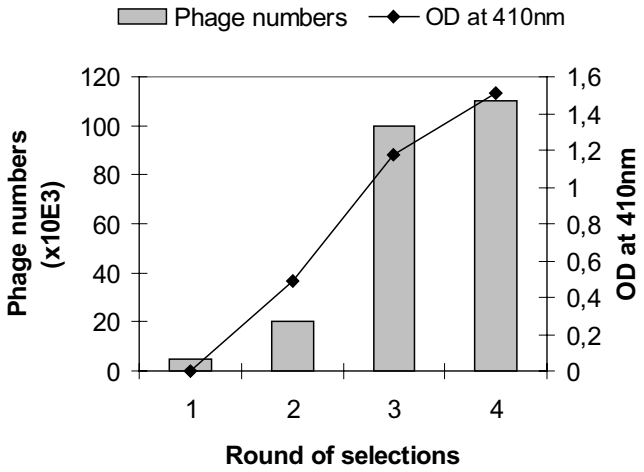

Figure 17. Enrichment of the phages. The number of selected phages increased from 5,000 to 100,000; the ELISA signal against Tau40 increased from 0.1 to 1.5; both the phage numbers and the ELISA signals highly increased after three rounds of selection.

Following the round of selection, both the number of the selected phages and the ELISA signal increased (Figure 17), indicating that the phages specific for Tau40 were enriched (Coia et al., 1997; Krebber et al., 1997). Changes occurred significantly in the first three rounds. After each round, the number of selected phages increased 4-5 fold and the ELISA signals increased 3-4 fold. Especially the phages selected from the second round showed a strong ELISA signal around 0.5, compared with the first round which had no ELISA signals. The third round and fourth round had the similar amount of the selected phages but the slight

increase of the ELISA signals. After four rounds of selection, we got an enriched phage library which showed the high positive signals against Tau40; this phage library was called "Hybri-Tau1/Tau40 phage library", which meant that this phage library was selected by Tau40 from the Hybri–Tau1 phage library. This library contained many different antibodies (i.e. the polyclonal scFv antibodies), therefore it had to be screened to find out the single clone (i.e. the monoclonal scFv antibody).

3.1.7. Screening of phages

The Hybri-Tau1/Tau40 phage library was screened in order to get the single Tau-specific clone (see 2.3.1.7.), briefly, the single TG1 cell clones from this library were cultured to produce the monoclonal phages, and the ELISA was used to detect which phage bound to Tau. The ELISA used Tau40 as the coated antigen, the phage supernatant (1:3 diluted) was the primary antibody, the anti-M13 coat protein-HRP antibody (1:5,000 diluted) was the secondary antibody.

Clones	OD_{410nm}–BSA	OD_{410nm}-Tau40	ΔOD_{410nm}
C7	0.075	1.075	1.0
E3	0.153	1.228	1.075
F10	0.104	1.192	1.086
G4	0.085	0.817	0.732
E4	0.086	0.349	0.265
Other 88 clones	0.6-0.9	1.0-1.1	0.2-0.4

Table 1. The screening of the Hybri-Tau1/Tau40 library. OD_{410nm}–BSA and OD_{410nm}–Tau40 represented the OD values at 410nm when the antigen for ELISA was BSA or Tau40. ΔOD_{410nm} was the value of subtracting the OD_{410nm}–BSA from the OD_{410nm}-Tau40. The names of the clones such as C7, E3, F10, G4 and E4 were corresponding to the positions on the 96-well plate. There were 93 clones screened in total. Four clones showed the specific ELISA signals; one clone showed the low specific signal; 88 clones had high signal against BSA which were unspecific signals.

From 93 selected individual phages (Table 1), 4 single (monoclonal) phages, i. e., C7, E3, F10 and G4 showed specific ELISA signals against Tau40, their signal differed between BSA and Tau40 more than 0.7. The single phage of E4 showed a low affinity to Tau40 because its ELISA signal between BSA and Tau40 differed only by about 0.3, it could be a candidate

which had the low affinity for Tau40. Most clones (88 clones) showed just the slight ELISA signal difference between BSA and Tau40, therefore these clones were not specific for Tau40. If these 88 phages were 1:5 diluted, they showed no ELISA signal against BSA or Tau40, indicating that they just bound unspecifically. The Tau40-specific monoclonal phages, C7, E3, F10 and G4, were then used to switch their scFv genes to the *E. coli* HB2151 cells which is a protein expression system.

3.1.8. Transfection of *E.coli* HB2151 cells by phages

It is necessary not only to get the phages against Tau40, but also the soluble scFv antibody protein. Therefore, the phages with the scFv gene were used to transfect *E.coli* HB2151 cells which function as a protein expression system (in contrast, the *E.coli* TG1 cell strain is a phage propagation system). Among the five Tau40-specific clones, E4 clone had a low affinity and was not worked on further. The remaining four Tau40-specific phages (e.g., C7, E3, F10 and G4) were switched to HB2151 cells by transfecting the log phase HB2151 cells (see 2.3.1.8.). The transfected HB2151 cells were induced for three hours, then ELISA was used to detect which HB2151 clone produced the soluble scFv antibody. Tau40 was the coated antigen for the ELISA, the culture medium (containing soluble scFv antibody, 1:3 diluted) as the primary antibody, and the anti-E tag antibody (1:5,000) as the secondary antibody to recognize the E tag in the scFv antibody.

Clones	OD_{410nm}–BSA	OD_{410nm}-Tau40	ΔOD_{410nm}
C7-HB2151	0.113	1.454	1.339
E3-HB2151	0.190	1.467	1.277
F10-HB2151	0.569	1.452	0.833
G4-HB2151	0.421	1.464	1.043

Table 2. ELISA detection of the soluble scFv in the transfected HB2151 cells. Clones listed here represented the transfected HB2151 cells. OD_{410nm}–BSA and OD_{410nm}–Tau40 represented the OD values at 410nm when the antigen for ELISA was BSA or Tau40. ΔOD_{410nm} was the value of subtracting the OD_{410nm}–BSA from the OD_{410nm}-Tau40. All clones showed similar ELISA signals against Tau40, but C7-HB2151 and E3-HB2151 had low background signals against BSA, whereas the backgrounds of F10 and G4 were relatively high.

The C7-HB2151 clone showed the highest ELISA signal difference compared with other clones under the same conditions (Table 2), followed by the E3-HB2151 clone. Although F10

and G4 showed the specificity for Tau40, their background signals against BSA were high, and therefore they were not as specific as C7-HB2151 and E3-HB2151 clones. Since the ELISA detected the soluble scFv antibody in the culture medium and there appeared positive signals, we knew that the scFv antibodies were presented in the culture medium. But the soluble scFv antibody may also localize in other places of *E. coli* HB2151, e.g. the cytoplasma, the periplasma or the supernatant. So for the purification of the antibody, it is necessary to determine the localization of the major fraction of the scFv antibody in *E. coli*.

3.1.9. Localization of the scFv antibody

In order to know where the active soluble scFv is localized, the different samples were prepared using the C7-HB2151 clone (see 2.3.1.9.). The cells were induced with 1mM IPTG–b for 3 hours, the culture was spun to get the supernatant. The cell pellet was incubated with TES buffer containing EDTA and 0.5M sucrose, which slightly damaged the cell outer membrane by osmotic shock to release periplasma (Power et al., 1992), then the cells were spun to get the supernatant, i.e. the periplasmic extract (see 2.2.4.7.). The periplasma-free cell pellet was then completely broken up by the French press to get the scFv from inside the cells. Finally, ELISA was employed to detect which fraction had the highest positive signal. For the ELISA, the three fractions (all were 1:3 diluted) were incubated with the coated Tau40, and the anti-E tag antibody (1:5,000) which recognized the E tag in the scFv antibody was used as the secondary antibody.

Fractions	OD_{410nm}– BSA	OD_{410nm}- Tau40	ΔOD_{410nm}	Volumes (ml)	ΔOD_{410nm} x Volume
Supernatant	0.029	0.194	0.165	50	8.25
Periplasmic extract	0.054	1.510	1.456	6.25	9.1
Cell extract	0.046	0.062	0.016	1	0.016

Table 3. ELISA detection of the scFv locations. OD_{410nm}–BSA and OD_{410nm}–Tau40 represented the OD values at 410nm when the antigen for ELISA was BSA or Tau40. ΔOD_{410nm} was the value of subtracting the OD_{410nm}–BSA from the OD_{410nm}-Tau40. The amount of scFv was simply counted by ΔOD_{410nm} x Volume. The most prominent ELISA signal was from the periplasmic extract. By this measurement, 50% of the active soluble scFv antibody was in the periplasmic extract and 50% in the supernatant.

The ELISA showed that the periplasmic extract had the most specific and strongest signals; the supernatant had also a very high signals; and the signal of the whole cell extract was negligible (Table 3). So all together , the supernatant contained about 50% of the active soluble scFv because of its large volume; the periplasmic extract contained the other 50%. Both the supernatant and the periplasmic extract could be used to purify the soluble scFv; the whole cell extract were useless and therefore discarded.

3.1.10. Purification of the scFv antibody

In principle, the supernatant should have been usable to purify scFv because it had a large fraction of the active scFv (see Table 3). However, the HiTrap anti-E tag affinity column requires that the scFv concentration is higher than 1µg/ml, in practice, the scFv concentration in the supernatant was too low to purify it on the column so that only the periplasmic extract was used for the purification.

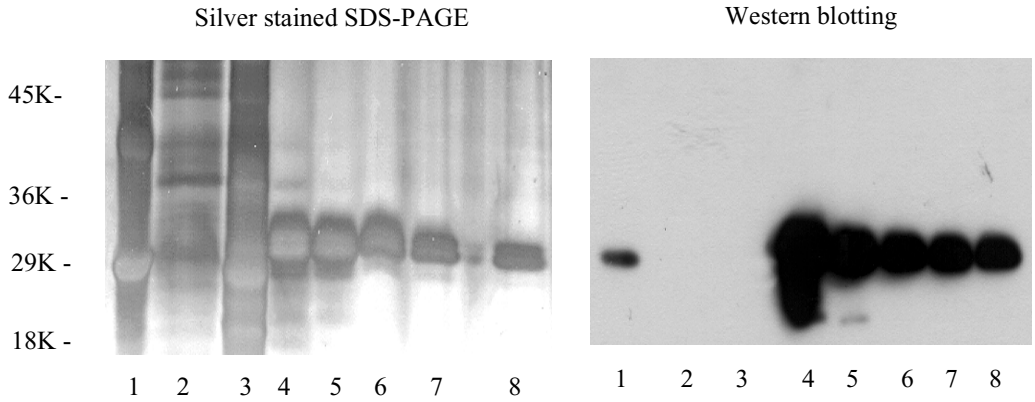

Figure 18. Purification of the C7-scFv antibody. 10% SDS-PAGE was silver-stained, Western blotting was performed with the anti-E tag antibody. Lane 1: the periplasmic extract; lane 2: flow-through from the HighTrap anti-E tag affinity column; lane 3: wash of the column; lane 4 to 8: eluted fractions 1 to 5. About 1µg protein was in each line.

The C7-HB2151 clone was used as the example to purify the scFv antibody. The C7-scFv antibody was successfully purified from the periplasmic extract by chromatography on the HiTrap anti-E tag affinity column which was eluted with 1M glycine, pH3.0. (see 2.3.1.10.). The C7-scFv antibody fractions were tested with 10% SDS-PAGE and Western blotting. By the silver-stained SDS-PAGE, the C7-scFv antibody showed one main band around 30KD, indicating a high degree of purity (Figure 18). By Western blotting, the same band was detected with the anti-E tag antibody, confirming that it was the scFv antibody as designed.

The western blotting also showed that the scFv was enriched by purification, none scFv antibody was lost in the flow-through and wash fractions. The purified C7-scFv antibody was analyzed further for its properties such as the epitope, the amino acid sequence and the recognition of Alzheimer Tau in the following experiments.

3.1.11. Epitope identification of the scFv-Tau193-204 antibody

Tau40-spot-membrane that comprises the whole Tau40 sequence in 15mer peptides, was used to identify the epitope of the purified C7-scFv antibody (0.3mg/ml) by immunoblot (see 2.2.4.4.; Frank et al., 1996; Bocher et al., 1997). The C7-scFv antibody (1:100 diluted) was applied to the Tau40-spot-membrane as the primary antibody; the secondary anti-E tag-HRP antibody (1:2,500 diluted) was used to detected the E tag in the C7-scFv antibody.

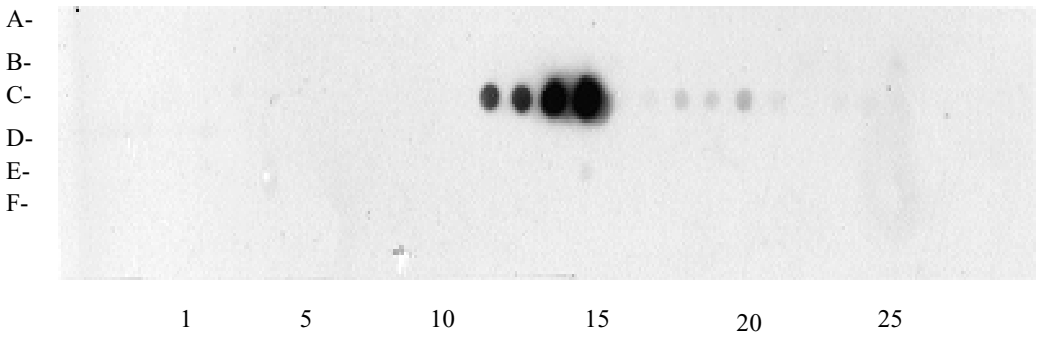

Figure 19. Epitope identification of the C7-scFv antibody with the Tau40-spot membrane that comprises the Tau40 sequence in 15mer peptides. The letters on the Y-axis showed lines of spots, numbers on the X-axis indicated the spot numbers in each line, the beginning peptide of Tau40 is on the upper left corner. The spots of C14 and C15 (the 14th and 15th spots in line C) were weakly recognized, whereas C16 and C17 (the 16th and 17th spots in line C) were strongly recognized. Some very weak signals appeared on the spots of C18, C19 and C20 (the 18th, 19th and 20th spots in line C).

The scFv reacted with four peptides. Two of them (C14 and C15) were strongly recognized (Figure 19). According to the sequences of the peptides C14 and C15 (Table 4), the minimal sequence for a strong signal is ^{193}**DRSGYSSPGSPG**^{204}. This sequence therefore represented the epitope of the scFv. The location of this epitope is exactly the epitope of the Tau-1 monoclonal antibody, which one purifies from Tau-1 hybridoma cells. And this is expected as the Tau-1 hybridoma cells were the source for the mRNA. This scFv antibody was named "scFv-Tau193-204".

Since the initial part, ^{193}DRSGYS^{198} was also presented in the weakly-reacting peptides C12 and C13, this hexapeptide represented the core of the epitope, with another strong contribution from the following sequence ^{199}SPGSPG^{204}; consistent with this, the non-reactive neighboring peptides C11 (residues 181-195, the 11th spot in line C) and C16 (residues 196-210) interrupted the main determinant. Interestingly, the strongest reaction was seen with a sequence that contained two Ser-Pro motifs spaced by one Gly. This configuration is reminiscent of a collagen helix where successive Ser residues would be aligned on one side of the helix and could therefore contribute to an interacting surface (for a comparison with the epitope of the phosphorylation-dependent antibody AT-8, see 4.1.7. in discussions). We also noted the weak reaction with peptides C18, C19 and C20 corresponding to residues 202-216, residues 205-219, and residues 208-222, respectively. This region is also rich in Pro residues and contained two closely spaced Thr-Pro motifs (Thr212 and Thr214) which could be phosphorylated by proline-dependent kinases and were recognized by phosphorylation-dependent antibody (Biernat et al., 1992; Roder et al., 1997; Ikura et al., 1998; Zheng-Fischhöfer et al., 1998).

Spots	Peptide sequences	Signal intensities
C11	^{181}TPPSSGEPPKSGDRS^{195}	No signal
C12	^{184}SSGEPPKSGDRSGYS^{198}	Weak signal
C13	^{187}EPPKSGDRSGYSSPG^{201}	Weak signal
C14	^{190}KSG**DRSGYSSPGSPG**^{204}	Strong signal
C15	^{193}**DRSGYSSPGSPG**TPG^{207}	Strong signal
C16	^{196}GYSSPGSPGTPGSRS^{210}	No signal
C18	^{202}SPGTPGSRSRTPSLP^{216}	Very weak signal
C19	^{205}TPGSRSRTPSLPTPP^{219}	Very weak signal
C20	^{208}SRSRTPSLPTPPTRE^{222}	Very weak signal

Table 4. Peptides recognized by the scFv on the Tau40-spot membrane. Names of the spots corresponded to the spot positions on the membrane, for example, C11 is the 11th spot in line C. The C12 and C13 had relative weak signals, C14 and C15 had about 5-fold higher signals. C18, C19 and C20 had very weak signals.

3.1.12. Determination of the amino acid sequences of the scFv-Tau193-204 antibody

The scFv gene in the pCANATAB5E vector was sequenced in order to determine the amino acid sequence of the scFv-Tau193-204 antibody (see 2.2.3.11.). The V_H and V_L DNA were

sequenced separately. Among the sequencing primers (see 2.1.3.2.), S1 was the forward primer and S4 was the backward primer for the V_H; likewise, S3 and S6 were the forward primer and the backward primer for the V_L.

The C7 clone and the E3 clone had similar sequences, with the minor differences at the two ends of scFv antibody (Figure 20). Both sequences contained typical complementarity determining regions (CDRs) which form the binding site to the epitope (see how to identify the CDRs in 4.1.7 in discussions). The E3 clone had the same CDRs as the C7 clone, indicating that E3-scFv antibody binds to the same epitope, i.e. ^{193}DRSGYSSPGSPG^{204}, therefore they show the same antibody specificity although they have some amino acid differences.

C7: MA**Y**V**Q**LQQSGAELVRSGASVKLSCTASGFNIKDYYIQWVKQRPEQG
E3: MA**H**V**K**LQQSGAELVRSGASVKLSCTASGFNIKDYYIQWVKQRPEQG
V_H
C7: LEWIGWIDPENGDSDSVPKFQGKATMTVDTSSNTAYLQLSSLTSEDT
E3: LEWIGWIDPENGDSDSVPKFQGKATMTVDTSSNTAYLQLSSLTSEDT

C7: AVYYCNRGWGYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSP
E3: AVYYCNRGWGYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSP
Linker
C7: LTF**L**VTIGQPASISCKSSQSLLNSDGKTYLSWLLQRPGQSPKRLIYLVS
E3: LTF**S**VTIGQPASISCKSSQSLLNSDGKTYLSWLLQRPGQSPKRLIYLVS
V_L
C7: KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFG
E3: KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFG

C7:GGT**K**LE**M**KRAAAGAPVPYPDPLEPR
E3:GGT**N**LE**L**KRAAAGAPVPYPDPLEPR
E-tag

Figure 20. Amino acid sequences of scFv-Tau193-204 (C7 and E3 clones). Wavy underlined domains are the complementarity determining regions (CDRs); the double underlined fragment is the linker. V_H is before the linker, V_L is behind the linker. The underlined fatprinted letters indicate amino acids at both ends that differed between the C7 clone and the E3 clone. The single underlined fragment is the E-tag.

3.1.13. Analysis of Alzheimer Tau by the scFv-Tau193-204 antibody

Western blotting was employed to analyze Tau samples using the purified scFv–Tau193-204 antibody (0.3mg/ml) and Tau-1 monoclonal antibody (1mg/ml). Tau was purified from autopsy tissue of control human brains (control-brain–HTau)and Alzheimer brains (AD-brain-HTau) by Dr. Martin von Bergern from our lab following the Davies method (Greenberg and Davies, 1990). While the scFv-Tau193-204 antibody (1:50 diluted) was the primary antibody, the secondary antibody was anti-E tag-HRP antibody (1:5,000 diluted); when the Tau-1 antibody (1:2,000 diluted) was used, the secondary antibody was anti-mouse-HRP (1:5,000 diluted).

The western blotting pattern with scFv-Tau193-204 antibody was nearly the same as that of Tau-1 monoclonal antibody (Figure 21). Both antibodies recognized the control-brain-HTau very strongly because it was mostly dephosphorylated; but they recognized AD-brain-HTau weakly because it was hyper-phosphorylated. These results showed as expected that the scFv–Tau193-204 and Tau-1 antibodies had the same dependence on phosphorylation, i. e., they reacted only when the residues in the region of the epitope were dephosphorylated (Biernat et al., 1992; Ledesma et al., 1995; Takashima et al., 1998). The results also proved that the recombinant antibody could be used for the recombinant Tau proteins, as well as the native Tau proteins.

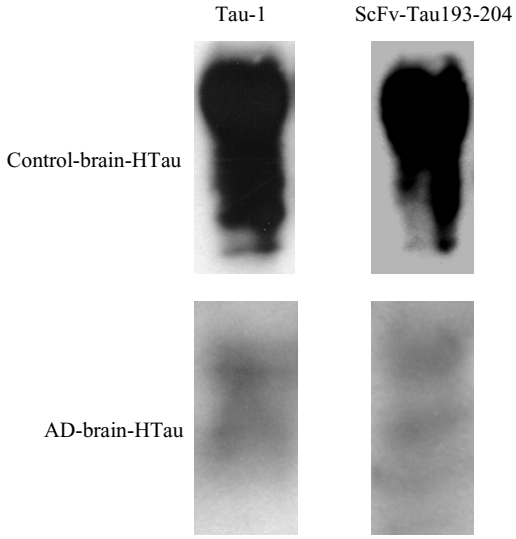

Figure 21. Western blotting analysis of the control-brain-HTau and the AD-brain-HTau with scFv-Tau193-204 and Tau-1 antibodies. Both antibodies recognized control-brain–HTau very strongly; but they recognized AD-brain-HTau very weakly, only a few diffuse bands were visible in the AD-brain-HTau.

3.2. ScFv antibodies from the Griffin.1 library

In order to get more recombinant antibodies against other sites of Tau protein, the Griffin. 1 library was used for the selection of antibodies against Tau proteins. The Griffin.1 library was

kindly given by Prof. G. Winter (University of Cambridge, U. K.). This was a ready-to-go library, i. e. cells in this library already contained scFv antibody genes. This library contained 10^9 scFv candidates and all scFv genes were in the phagemid vector of pHEN2 (Griffiths et al., 1994; Nissim et al., 1994). Additionally, the six–Histidine-tag and the myc-tag were constructed behind the scFv gene instead of the E-tag.

The phage selection was based on the affinity binding between the immobilized antigen and the antibody on the tip of the phage (see 1.4.3. in introduction). Since proteins could be physically absorbed by plastics, the immunotube and the 96-well-plate were used as tools for phage selection. The Tau40-spot-membrane, containing the covalently bound peptides, was also used as a tool for phage selection in this study.

3.2.1. Selection of phages with immunotube

Two Tau proteins (antigens) that were initially cloned by Dr. Jacek Biernat were coated on the immnotubes for phage selections from the Griffin.1 library. One was the hyper-phosphorylated Tau40 protein, the largest isoform of Tau, termed Tau40p. It was purified from the Sf9 cells transfected with Tau40 by the Baculovirus-sf9 cell system (Biernat and Mandelkow, 1999). The other antigen, the phosphorylated K19, containing only three repeats, termed K19p, was in vitro phosphorylated by the mouse brain extract which contain many kinds of kinase activities (Zheng-Fischhöfer et al., 1998). Selections were done with the antigen-coated immunotubes (see 2.3.2.1.). Tau40p (or K19p) was coated on the immunotube at the concentration of 10mg/ml. The Griffin.1 phage library was incubated in the immunotube for three hours to allow the binding of the target phages to Tau40p (or K19p). The immunotube was washed 10–20 times with PBST to remove the unspecific phages. The bound phages were eluted by triethylamine (pH11) and used to re-infect the log phase TG1 cells. The re–infected TG1 cells were used to perform the phage rescue overnight to get the new phage library. Half of the new phages was used to do the next round of selection, the other half was used for ELISA detection and others.

The number of the selected phages in each round was counted by calculating the number of the TG1 colonies on Agar plates (see the protocol in 2.2.4.6.). The immunor-activity of the phages to Tau40p (or K19p) was detected with ELISA. The ELISA used Tau40p (or K19p) as

the coated antigen, the precipitated phages (1:100 diluted) and the anti-M13 coat protein-HRP antibody (1:10,000 diluted) were the primary and secondary antibody, respectively.

During the selection, the numbers of the selected phages and the specific ELISA signals against the antigen increased in both cases, showing that the phages against the antigens were enriched (Figure 22). After three rounds of selection, the enriched phage library showed the high ELISA signal against Tau40p or K19p.

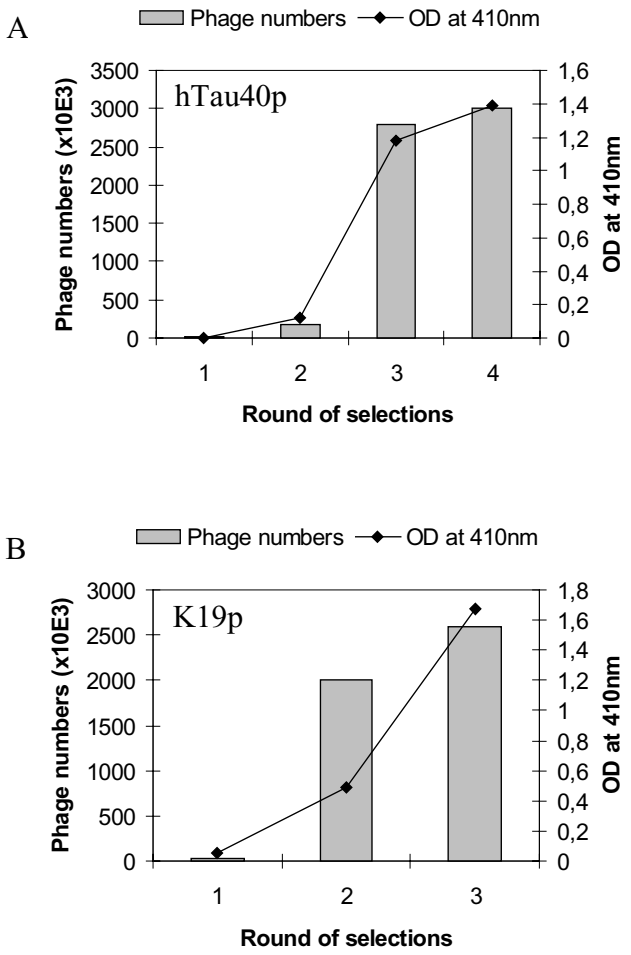

Figure 22. Enrichment of phages during selections. **A**: Four rounds of selection using hyper-phosphorylated Tau40 as antigen. The 2nd round gave about 200,000 phages and an ELISA reading of ~0.1. The 3rd and 4th rounds gave ELISA signals of ~1.3, phage counts of 2.8-3.0x10^6. **B**: Three rounds of selection using in vitro phosphorylated K19 as antigen. The phage increased from 35,000 (the 1st round) to 2.6x10^6 (the 3rd round). The ELISA reading. was 0.05, 0.5 and 1.6, respectively.

After selection by Tau40p in four rounds, the phage library was called "Griffin/Tau40p phage library"; likewise, after selection by K19p in three rounds, the phage library was called "Griffin/K19p phage library". The rate of phage enrichment was different in the two cases. The phages of the Griffin/Tau40p phage library were enriched slowly; there was no significant change between the first and the second round. Both the phage count and the ELISA signal rapidly increased between the second and the third round. The Griffin/K19p phage library was enriched rapidly, only one round of selection gave already a high ELISA signal, which was similar to the Hybri-Tau-1/Tau40 phage library (see 3.1.6.).

In principle, the Griffin/Tau40p phage library contained phages against not only the phosphorylated sites, but also the dephosphorylated sites of Tau40 due to the fact that Tau40p had the sites which could never be phosphorylated and the sites which could not be fully phosphorylated (Kenessey and Yen, 1993; Ikura et al., 1998). The ELISA results confirmed this assumption (Table 5). Some phages bound to Tau40 were not competed away by Tau40p, but strongly competed by Tau40, indicating that these phages are Tau40-specific, i.e. they were phosphorylation-independent; The phages bound to Tau40p were slightly competed by Tau40, but strongly competed by Tau40p, indicating that these phages were Tau40p-specific, i.e. they were phosphorylation-dependent. The signal for Tau40p (1.912) was much higher than the signal for Tau40 (0.764), indicating that the main phage population of the library was phosphorylation-dependent; and due to the same reason, the Tau40p-specific phage could only be slightly competed by Tau40 (signal was reduced from 1.912 to 1.762).

Antigen	OD_{410nm}		
BSA	0.065		
Tau40	0.764	0.714 (involved 100ng Tau40**p** as the competitor)	0.236 (involved 100ng Tau40 as the competitor)
Tau40**p**	1.912	0.718 (involved 100ng Tau40**p** as the competitor	1.762 (involved 100ng Tau40 as the competitor)

Table 5. ELISA detection of the Griffin/Tau40p phage library. Phages had specific signals against Tau40 and Tau40**p**. The Tau40-specific phages and Tau40**p**-specific phages could be slightly competed away by Tau40**p** and Tau40, respectively; but strongly competed away by Tau40 and Tau40**p**, respectively.

Since the phosphorylation-dependent phages were the main population in the Griffin/Tau40p phage library, they could be simply obtained by screening. In order to obtain the minor phosphorylation-independent antibodies, the Tau40-spot-membrane was used as the selection tool to enrich them in the following experiment.

The above results could be also true for the Griffin/K19p phage library, and more interestingly, the repeat domain in K19p contained several phosphorylated sites near each other, this opens a chance to obtain the antibody against the double-phosphorylated-sites of Tau (Lee et al., 1988a and 1988b; Hoffmann et al., 1997). We tested this phage library by ELISA with different antigens, the results showed that the phages recognized Tau40, Tau40p and S320p/S324p peptide (Table 6). In particular, the ELISA signal against the peptide highly

decreased if the S320p/S324p peptide was involved as a competitor, whereas K19 had no influence, indicating that some phages specifically recognized this double-phosphorylated site. After comparing the signal against S320p/S324p peptide (0.464) with the signal against K19p (2.213), we concluded that the S320p/S324p-specific phages were the minor population in this library, it is better to enrich them by a further round of selection with the S320p/S324p peptide.

Antigens	OD_{410nm}		
BSA	0.082		
K19	1.685		
K19p	2.213		
S320p/S324p peptide	0.464	0.106 (involved 100ng S320p/S324p peptide as the competitor)	0.455 (involved 100ng K19 protein as the competitor)

Table 6. ELISA detection of the Griffin/K19p phage library. Phages had a specific signal against K19, K19p and the S320p/S324p peptide. The supplemented soluble S320p/S324p peptide significantly competed the phages bound to the immobilized peptide, after competition, the signal was nearly background.

3.2.2. Selection of phages with Tau40-spot-membrane

The Griffin/Tau40p phage library was first tested by the Tau40-spot-membrane (see 2.2.4.4.). The phages (1:200) were used as the primary antibody, the secondary anti-M13 coat protein-HRP antibody (1:5,000 diluted) recognized the bound phages. After ECL detection, the membrane showed many highlighted spots with different intensities, distributed over the whole membrane (Figure 23). It proved that, in the Griffin/Tau40p phage library, there were many different phages against dephosphorylated sites of Tau40, with epitopes distributed from the N- to the C–terminal region of Tau.

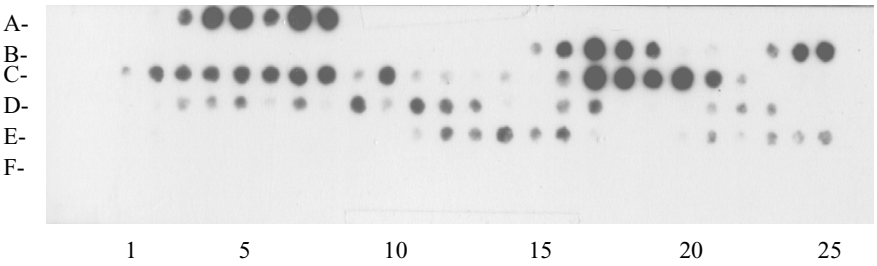

Figure 23. Detection of the Griffin/Tau40p phage library by the Tau40-spot-membrane. The letters on the Y-axis indicated the lines of spots, the numbers on the X-axis indicated the spot numbers in each line; the spot A1 (the first spot in line A) was the beginning peptide. Most of the strong signals occurred in line A, line B and line C; signals in line D and line E were relatively weak.

Therefore, the Griffin/Tau40p phage library was used to select phages against the dephosphorylated sites of Tau by the Tau40-spot-membrane (see 2.3.2.2.), briefly, 2µl log phase TG1 cells were added to the target spot on the membrane to allow the re-infection, then the re-infected TG1 cells were recovered from the membrane and used to rescue the target phages. Since the signal intensity of the spot in Figure 23 reflected the amount and affinity of the phage, the phages which had a strong signal would give a better chance to yield a good scFv antibody. Thus the phages that bound to the spot A8 (the spot No. 8 in the line A) were chosen to re-infect TG1 cells, and then a new phage library, which was called "Griffin/spot A8 phage library", was generated. ELISA detection showed that it was a Tau40–specific phage library, did not recognize the phosphorylated Tau. Thus, the next step was to screen this library and obtain the single phosphorylation-independent clones.

3.2.3. Selection of phages with S320p/S324p peptide

In order to enrich the phages against the S320p/S324p peptide. The selection with the same peptide-coated plastic well was carried out for the Griffin/K19p phage library (see 2.3.2.3.). About 10,000 clones were obtained after the peptide selection, and they were then used to rescue phages. The new phage library showed a high and specific ELISA signal against the S320p/S324p peptide, it was called "Griffin/SpSp phage library". ELISA detection showed that the signal of this library against the S320p/S324p peptide was improved 5-fold (OD_{410nm} value was increased from 0.4 to 2.3), it was the same high increase as the signal against K19, indicating that the phages were enriched enough for screening.

3.2.4. Screening of phages

The Griffin/Tau40p phage library, the Griffin/SpSp phage library and the Griffin/spot A8 phage library were screened (see 2.3.1.7.). The ELISA was used to detect whether the single phage was positive to the antigen. For ELISA detection, the phage supernatant (1:3 diluted) was the primary antibody, the anti-M13 coat protein-HRP antibody (1:5,000 diluted) was the secondary antibody.

For the screening of the Griffin/Tau40p phage library, the Tau40 and Tau40p were used as the coated antigens for ELISA. Among 95 individuals, 10 phages showed positive ELISA signals against Tau40p with different specificities (Table 7), one clone showed a very high ELISA signal, two clones (A2 and B2) had the middle specificities, seven clones had low affinities to Tau40p. Other 85 phages were unspecific phages.

Clone number	OD_{410nm}-Tau40	OD_{410nm}-Tau40p	ΔOD_{410nm}
1	0.1	1.0	0.9
2 (A2 and B2)	0.05-0.1	0.5-0.6	0.4-0.5
7	0.05-0.1	0.2-0.3	0.1-0.2
85	0.05-0.1	0.1	0

Table 7. Screening of the Griffin/Tau40p phage library. OD_{410nm}–Tau40 and OD_{410nm}–Tau40p represented the OD values at 410nm when the antigen for ELISA was Tau40 or Tau40p. ΔOD_{410nm} was the value of subtracting the OD_{410nm}–Tau40 from the OD_{410nm}-Tau40p. Names of A2 and B2 were derived from the positions in the 96-well-plate used in the screening.

For the screening of the Griffin/SpSp phage library, the different Tau constructs, which were all cloned by Dr. Jacek Biernat, were employed as coated antigen for the ELISA detection. Considering that the S320p/S324p peptide and K19p had both the dephosphorylated and phosphorylated sites, the ELISA results were classified into six groups (Table 8).

Groups	SpSp	R4	R1/3	R3	Other	unspecific
Numbers of clones	22	14	1 (A1)	1 (C11)	1 (A3)	9
OD_{410nm}-BSA	0.1-0.2					
OD_{410nm}-K19	0.1-0.2	**2.0-2.5**	**2.3**	**2.2**	**2.5**	0.1-0.2
OD_{410nm}-SpSp	**0.5-2.7**	0.1-0.2	0.1	**0.7**	**2.2**	0.1-0.2
OD_{410nm}-K19p	**2.0-2.5**	**2.0-2.5**	**2.8**	**2.6**	**2.4**	0.1-0.2
OD_{410nm}-K5	0.1-0.2					
OD_{410nm}-K13	0.1-0.2	**0.8-2.5**	0.05	0.2	**1.4**	0.1-0.2

Table 8. Screening of the Griffin/SpSp phage library. OD_{410nm}-BSA, -K19, -SpSp, –K19p, -K5 and -K13 represented the OD values at 410nm when antigens for ELISA were BSA, K19, S320p/S324p peptide, K19p, K5 or K13. The names of A1, C11 and A3 were derived from the positions in the 96-well-plates used in screening.

The SpSp group (22 clones) were against the S320p/S324p peptide because they recognized only phosphorylated antigens; The R4 group (14 clones) were against the dephosphorylated fourth repeat of Tau because the Tau construct K13 contained only the fourth repeat (Figure

24). The R1/3 group (one clone, termed A1) was against the dephosphorylated first or third repeat of Tau because it did not recognize K13 containing the fourth repeat; The R3 group (one clone, termed C11) was against the dephosphorylated third repeat of Tau because K13 (containing the fourth repeat) was not recognized, whereas the S320p/S324p peptide (containing dephosphorylated sites in the third repeat) was recognized. The A3 clone showed ambiguous results which were difficult to interpret and were not pursued further. Other nine clones were unspecific.

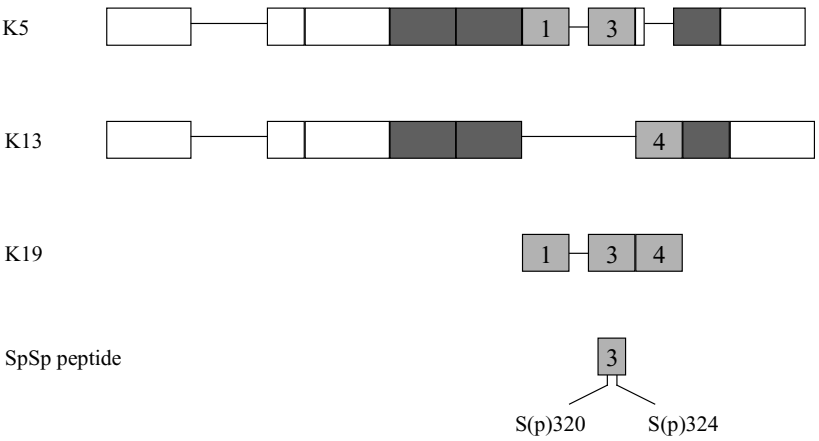

Figure 24. Diagrams of Tau constructs and the SpSp peptide. The Tau construct K5, K13 and K19 have the different repeats. K5 has only a very small part of the fourth repeat, whereas K13 has the whole fourth repeat. The SpSp peptide has two phosphorylated sites at Ser320 and Ser324. (All diagrams are adapted from Dr. Jacek Biernat).

For the Griffin/spot A8 phage library, the ELISA detection used the mouse-Tau40 (Kampers et al., 1999) and the human-Tau40 as the antigens. 60 clones were screened, 17 of them were found to recognized only the human Tau40, these were the human-Tau-specific clones; other 43 clones recognized neither the mouse-Tau nor the human-Tau40, they were unspecific clones.

3.2.5. Transfection of HB2151 *E. coli* cells by phages

In order to express scFv proteins in *E. coli* instead of phages, scFv genes were switched from the TG1 cell system (for phage production) to the HB2151 cell system (for expression of the scFv protein) by using the phages to transfect the log phase HB2151 cells (see 2.3.1.8.). The transfected HB2151 cells were induced by IPTG-b , and ELISA was used to detect which HB2151 clone produced the soluble scFv antibody. The ELISA used the culture medium (containing the soluble scFv antibody, 1:3 diluted) as the primary antibody, and the anti-myc

tag-HRP antibody (1:2,500 diluted), that recognized the myc−tag in the scFv antibody (Evan et al., 1985), as the secondary antibody.

The positive phages from the screenings were taken to transfect HB2151 cells (Table 9). ELISA detection for the soluble scFv was done with antigen pairs, one antigen as the negative control, the other as the positive control. For the Griffin/Tau40p phage library, Griffin/SpSp phage library and Griffin/spot A8 phage library, the Tau40−Tau40p, K19−S320p/S324p peptide, and mouse Tau−human Tau40 pairs were used, respectively. The soluble scFv were detected both from the medium and the periplasmic extract. If one of these two fractions gave a positive signal, the clone was regarded as positive. The positive clones were then induced and their periplasmic extracts were used to purify the soluble scFv proteins.

Libraries	Griffin/Tau40p	Griffin/SpSp		Griffin/spot A8
Number of phages used to transfect HB2151	10 (all positive clones)	22 (all SpSp group)	1 (D1, from R4 group)	17 (all positive clones)
Number of transfected HB2151 clones	10	22	1	17
Positive HB2151 clones which produced the soluble scFv	2 (A2 and B2)	17	1 (D1)	2 (B1 and D6)

Table 9. Transfection of HB2151 cells. Names of A2, B2, D1, B1 and D6 were derived from the corresponding position in the 96-well-plates used in screening. After transfection, all phages generated their corresponding HB2151 clones. But only some of the transfected HB2151 clones (e. g., the positive clones) produced soluble scFv protein.

3.2.6. Expression and purification of the scFv antibody

Purification employed the Ni-NTA-agarose column chromatography (see 2.3.2.5.), because the scFv antibody from the Griffin.1 library has the six-Histidine-tag (Skerra et al., 1991) instead of the E-tag. The A2 clone (from the Griffin/Tau40p phage library) was chosen as an example for performing the chromatography. Only one protein peak resulted from the gradient elution at about 100mM imidazol concentration. The Coomassie stained SDS-PAGE showed that the first half of the elution was not pure; whereas the second half of the elution was nearly pure and showed a single band (Figure 25). The impurity was due to the reason that the one-step procedure was not sufficient for the purification to homogeneity of the scFv

antibody, because several proteins from *E. coli* cells bound to immobilized metal-ions (Müller et al., 1998).

Western blotting showed that all scFv antibodies bound to the Ni-NTA-agarose, there were no scFv in the flow-through and the wash. The scFv was around 30KD as expected. The purified soluble scFv proteins were then used for the further characterizations such as the epitope determination, and the analysis of the Alzheimer Tau.

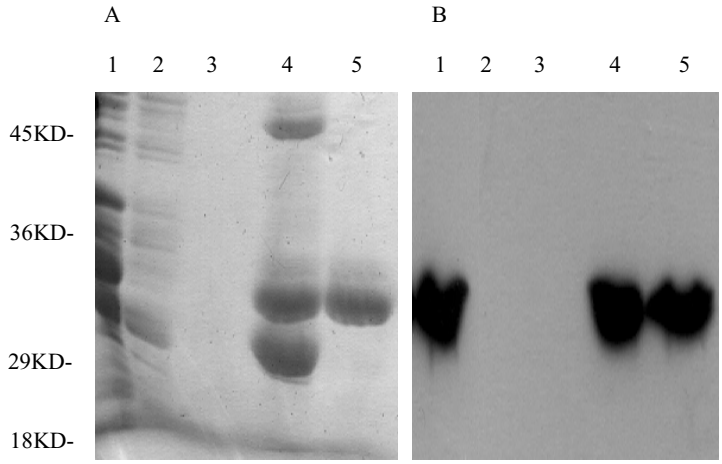

Figure 25. Purification of scFv (A2). A: the Coomassie stained SDS-PAGE; B: Western blotting using the anti-myc antibody. Lane 1: the periplasmic extract; lane 2: the flow-through from the column; lane 3: the wash of the column; lane 4: the elution fraction 1; lane 5: the elution fraction 2.

3.2.7. Identification of epitopes

3.2.7.1. Epitope identification of the scFv-TauS235p antibody

It was known that A2 clone (from the Griffin/Tau40p phage library) was phosphorylation–dependent because it recognized only the phosphorylated Tau40 (see Table 6). In order to find the exact epitope of this scFv antibody, a set of phosphorylated Tau constructs and mutants were chosen to react with this scFv antibody.

Phosphorylated Tau40, Tau23 and AP17, termed “Tau40p”, “Tau23p” and “AP17p”, respectively, were purified from the Baculovirus-Sf9 cell system (Biernat and Mandelkow, 1999). The phosphorylated K17, AP11 and AP25, termed “K17p”, “AP11p” and “AP25p”,

respectively, were phosphorylated by the in vitro method using the mouse brain extract containing kinase activities (Zheng-Fischhöfer et al., 1998). All of these *E. coli* clones or Baculovirus containing the recombinant human Tau construct and mutant genes were generously given by Dr. Jacek Biernat from our lab (Biernat et al., 1992), and the proteins were purified in our lab by our colleagues or by myself, the in-vitro-phosphorylation of the protein was carried out in our lab by myself.

Constructs	Characteristics of constructs	Signal	Deduction of the epitope location
Tau40p	Phosphorylated at 21 sites.	+	In these 21 sites.
Tau23p	Without E45-A103 fragment.	+	Not on Ser46, Thr50, Thr69 and Ser293. Could be in other 17 sites.
AP17p	all SP and TP sites were mutated, sites in repeats were phosphorylated.	-	Not in the repeats, could be in the SP and TP sites.
AP11p	The six SP motifs mutated, the TP sites were phosphorylated.	-	Not in the TP sites, could be in the SP sites.
K17p	Without Ser396, 404 and 422. Ser199, 202 and 235 sites were phosphorylated	+	Not on Ser396, 404 and 422. Must be among Ser199, 202 and 235.
AP25p	Ser199 and Ser202 were mutated.	+	At the phosphorylated Ser235.

Table 10. Epitope identification of A2-scFv by Western blotting. Constructs were blotted to the PVDF membrane, the purified A2-scFv was used as the first antibody. The + and - symbols represented the positive or negative Western blotting signals.

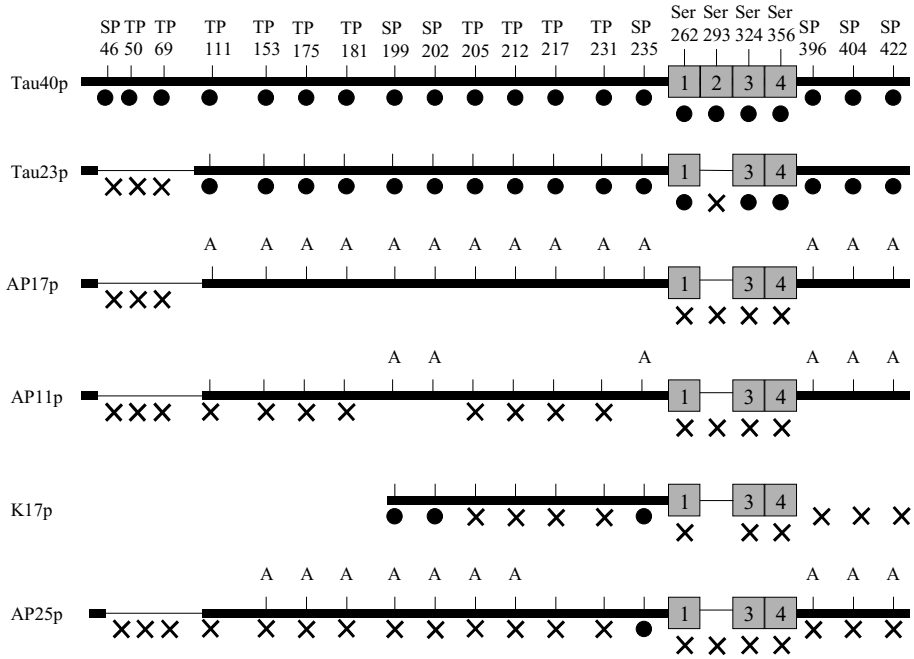

Figure 26. Diagram of the binding of A2-scFv antibody to different Tau constructs. The dark points indicate the scFv antibody binding sites. The crosses indicated the sites to which the scFv antibody could not bind.

Five different phosphorylated Tau constructs, Tau40p, Tau23p, AP17p, AP11p, K17p and AP25p were employed to perform the western blotting with the purified A2-scFv antibody (0.5mg/ml). The A2-scFv antibody (1:200 diluted) was the primary antibody, the secondary antibody was anti-myc tag-HRP antibody (1:2000 diluted) which recognized the myc tag in the scFv antibody. The positive signals were obtained by Tau40p, Tau23p, K17p and AP25p. According to the deductions illustrated in Table 10 and Figure 26, the epitope of A2-scFv was centered around phosphorylated Ser235.

Two further Tau mutants, Tau23A231 (Thr231 mutated into Ala) and Tau40A235 (Ser235 mutated into Ala) were used for the western blotting in order to ensure the above deduction, and also to test if Thr231 had an influence on the interaction between Ser235 and the scFv. The phosphorylated Tau23A231 and Tau40A235, termed Tau23A231p and Tau40A235p, respectively, were in vitro phosphorylated by the mouse brain extract containing kinase activities ((Zheng-Fischhöfer, 1998).

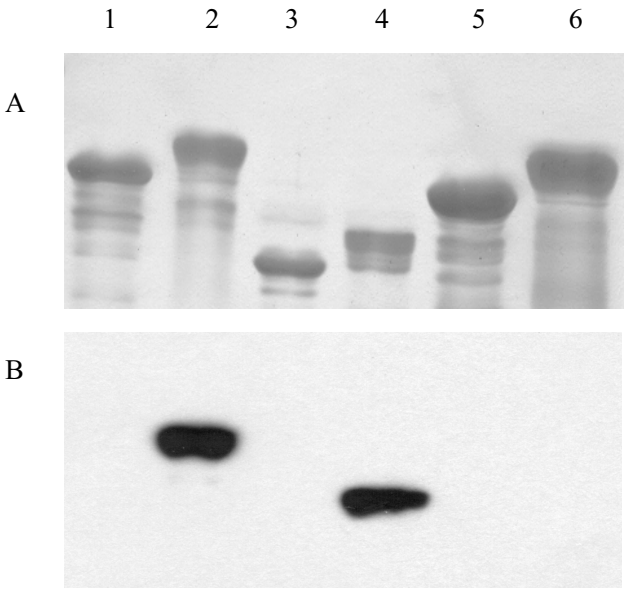

Figure 27. Epitope determination of A2-scFv. Western blotting was used to determine the exact epitope of A2-scFv (from the Griffin/Tau40p library). Lane 1: Tau40; lane 2: Tau40p; lane 3: Tau23A231; lane 4: Tau23A231p; lane 5: Tau40A235; lane 6: Tau40A235p. Tau40p and Tau23A231p showed signals, but Tau40A235p did not show the signal. which meant that mutation of Ser235 to Alanine did not allow the formation of the epitope after the phosphorylation.

In six samples, only Tau40p and Tau23A231p showed signals (Figure 27). The scFv did not recognize Tau40A235p whose Ser was mutated to Ala; this meant that the epitope of the scFv was lost, and therefore phosphorylated Ser235 was part of the epitope of the scFv. The results

also showed that Thr231 was not recognized, it was not involved in the epitope although it is very close to Ser235. This clone was therefore named "scFv-Tau235p". The B2 clone (the other clone from Griffin/Tau40p phage library) turned out to have the same epitope as the A2 clone.

3.2.7.2. Epitope identification of the scFv-Tau349-360 antibody

The D1 clone was against the fourth repeat of Tau40 because it was from R4 group of the Griffin/SpSp phage library. In order to identify its exact epitope, Tau40-spot-membrane was used to perform an immunoblot (see 2.2.4.4.) with purified D1-scFv antibody (0.5mg/ml). The D1-scFv antibody (1:100 diluted) was used as the primary antibody, the secondary anti-myc tag-HRP antibody (1:2,000 diluted) was used to detect the myc tag in the primary scFv antibody.

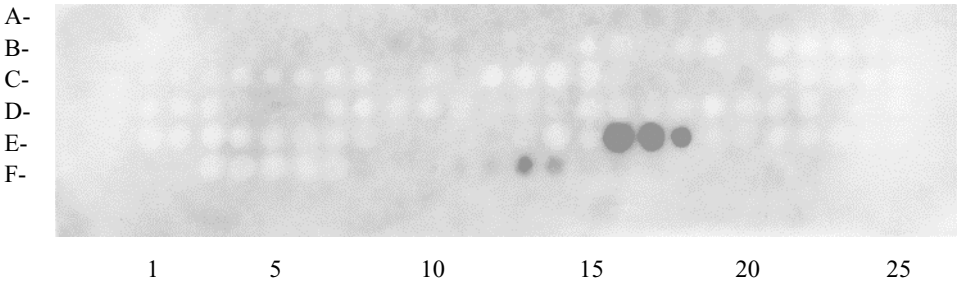

Figure 28. Epitope identification of D1-scFv by the Tau40-spot-membrane. The letters on Y-axis represented lines of spots, the numbers on X-axis represented numbers of spots in each line. After the detection, the spots of E16, E17 and E18 (the spots No. 16, 17 and 18 in line E) showed specific signals; the spots of F14 and F15 (the spots No. 14 and 15 in line F) showed much weaker signals.

Spots	Peptide sequence	signal intensity
E16	^{346}FKD**RVQSKIGSLDNI**^{360}	Strong
E17	^{349}**RVQSKIGSLDNI**THV^{363}	Strong
E18	^{352}SKIGSLDNITHVPGG^{366}	weak
F13	^{412}SSTGSIDMVDSPQLA^{426}	Very weak
F14	^{415}GSIDMVDSPQLATLA^{429}	Very weak

Table 11. Peptides recognized by the D1-scFv antibody. Names of spots were derived from their positions on the membrane, for example, E18 was the 18th spot in line E. E18 had a relative weak signal, whereas E16 and E17 had about 3-fold stronger signals. The spots of F13 and F14 showed very weak signals due to cross-reactions.

The result showed that D1-scFv antibody strongly recognized three peptides in line E, and weakly recognized another two peptides in line F (Figure 28). The weak signals in line F were probably from cross-reactions. The weaker signal on E18 was due to the peptide lack of a part of epitope; thus signals on E16 and E17 reflected the real epitope. According to peptide sequences (Table 11), the epitope of the D1 clone is ^{349}**RVQSKIGSLDNI**^{360}. This clone was named "scFv-Tau349-360".

3.2.7.3. Epitope identification of the scFv-TauS320p/S324p antibody

The A10 clone was from the SpSp group of Griffin/SpSp phage library (named due to the position on the 96-well-plate in the screening). It was known that the A10-scFv antibody recognized the S320p/S324p peptide (see Table 8 in 3.2.3.), but we did not know that it recognized either the doubly-phosphorylated site or the single-phosphorylated site. Therefore, its exact epitope was identified by ELISA. The S320p and S320p/S324p peptides were purchased from the company Eurogentic, they were coated on the well by drying overnight (see 2.2.4.5.), the purified A10-scFv antibody (0.5mg/ml) was 1:100 diluted and used to incubate with the antigenic coated-peptide, the secondary antibody was the anti-myc-HRP (1:2,500 diluted) antibody.

The ELISA results showed that the A10-scFv antibody recognized the S320p/S324p peptide and could be competed with the same peptide. On the other hand, the S320p peptide (only phosphorylated at Ser320) was not recognized at all, and this peptide could not compete the bound scFv (Table 12). Therefore A10 clone recognized the doubly–phosphorylated S320p/S324p site and was named "scFv-Tau320p/S324p".

Coated peptide	Peptide for competition (involved in the solution)	OD_{410nm}
S320p peptide	None	0.029
S320p/S324p peptide	None	1.074
S320p/S324p peptide	S320p peptide (100ng)	1.043
S320p/S324p peptide	S320p/S324p peptide (100ng)	0.206

Table 12. Epitope identification of A10-scFv by ELISA. The peptides coated on the plate by drying method (see 2.2.4.5.). Peptides contained in the solution were used for the competition.

3.2.7.4. Epitope identification of the scFv-Tau13-24 antibody

Tau-spot-membrane immunoblot (see 2.2.4.4.) was used to identify B1 clone (from Griffin/A-8 phage library). The immunoblot showed that the clone recognized the peptides in line A, the strongest signals appeared at A4 and A5, the signals on A3 and A6 were relative weaker (Figure 29). According to the peptide sequences (Table 13), the epitope of B1 clone is **^{13}DHAGTYGLGDRK^{24}**. It corresponded to human Tau 13-24 sequence and was therefore named "scFv-Tau13-24".

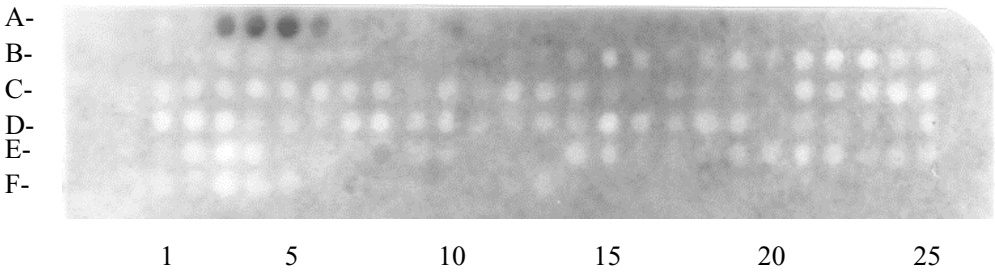

Figure 29. Epitope identification of B1 clone. The letters on Y-axis represented lines of spots, the numbers on X-axis indicated the numbers of spots in each line. The B1-scFv recognized A3 to A6 spots (the 3rd to 6th spots in line A).

Spots	Peptide sequences	Signal intensity
A3	^{7}EFEVMEDHAGTYGLG^{21}	Weak
A4	^{10}VME**DHAGTYGLGDRK**^{24}	Strong
A5	^{13}**DHAGTYGLGDRK**DQG^{27}	Strong
A6	^{16}GTYGLGDRKDQGGYT^{30}	Very weak

Table 13. Peptides recognized by B1 clone on Tau40-spot-membrane. Names of spots were derived from their positions on the membrane, i.e., A3 to A6.are the 3rd to 6th spots in line A. A3 had a relative weak signal, A4 and A5 had about 3-fold stronger signals, the very weak signal on A6 was due to a cross-reaction.

3.2.8. Determination of the amino acid sequence of the scFv-TauS235p antibody

The scFv-Tau235p gene was sequenced (see 2.2.3.11.) in order to determine the amino acid sequence. The V_H gene and the V_L gene were sequenced separately. Among the sequencing primers (see 2.1.3.2.), LMB3 was the forward primer for the V_H gene, S4 was the backward primer for V_H; likewise, S3 and S6 were the forward and backward primers for the V_L gene, respectively.

From the amino acid sequence of the scFv-TauS235p antibody (Figure 30), we could find two cysteins in the V_H and two in the V_L, as is similar to the scFv-Tau193-204 antibody (see Figure 20 in 3.1.12.). The linkers are also similar in these two antibodies. The typical complementarity determining regions (CDRs, see how to identify the CDRs in 4.1.7 in discussions) are also found in the scFv-TauS235p antibody, and they are very different in these two antibodies; on the contrary, the other domain (except for the CDRs) have some similarities between them.

MAQVQLVQSGAEVKKPGATVKISCKVSGYTFTDYYMHWVQQAPGKGLEW
V_H
MGLVDPEDGETIYAEKFQGRVTITADTSTDTAYMELSSLRSEDTAVYYCARV

GSPPNDNWGQGTLVTVSSGGGGSGGGGSGGSALQSVLTQPPSASGTPGQRVT
Linker V_L
ISCSGSSSNIGSNYVYWYQQLPGTAPKLLIYRNNQRPSGVPDRFSGSKSGTSAS

LAISGLRSEDEADYYCAAWDDSLPSFVFGGGTKLTVLGA | AAAHHHHHHGA
The end of V_L His tag
AEQKLISEEDLNGAA
myc tag

Figure 30. Amino acid sequence of scFv-Tau235p. The double-underlined sequence is the linker, the single-underlined sequences are complementarity determining regions (CDRs), the His tag is dot-underlined and the myc tag is wave-underlined. V_H is before the linker and V_L is behind the linker.

3.2.9. Analysis of Alzheimer Tau by the scFv-TauS235p antibody

Western blotting was employed to investigate brain Tau samples by purified scFv–TauS235p antibody (0.5mg/ml) and AT180 monoclonal antibody (0.5mg/ml). Tau samples were purified from the autopsy tissue of control human brains (control-brain–HTau) and Alzheimer brains (AD-brain-HTau) by Dr. Martin von Bergern from our lab following Davies method (Greenberg and Davies, 1990). While the scFv-TauS235p antibody (1:200 diluted) was the primary antibody, the secondary antibody was anti-myc tag-HRP antibody (1:2000 diluted) which recognized the myc tag in the scFv-TauS235p antibody; while AT180 antibody (1:2000 diluted) was used, the secondary antibody was anti-mouse-HRP (1:5000 diluted).

Two antibodies showed similar patterns, they did not recognize the control–brain–HTau, but they did recognize the AD-brain-HTau (Figure 31). The similarity was due to that both of

them recognized the phosphorylated-Ser235 site (Goedert et al., 1994; Anderton et al., 1998; Godemann et al., 1999; Meijer et al., 2000). But there was one clear difference between the two patterns, the result of the scFv-TauS235p antibody lacked the bands in the 40-50KD range, where the result of the AT180 antibody showed strong signals. This could be due to the fact that Tau fragments between 40-50KD were phosphorylated at both the Thr231 and Ser235 sites so that AT180 showed signals because it recognized these doubly-phosphorylated sites; but the scFv-TauS235p had no signal because it recognized the phosphorylated Ser235 site only.

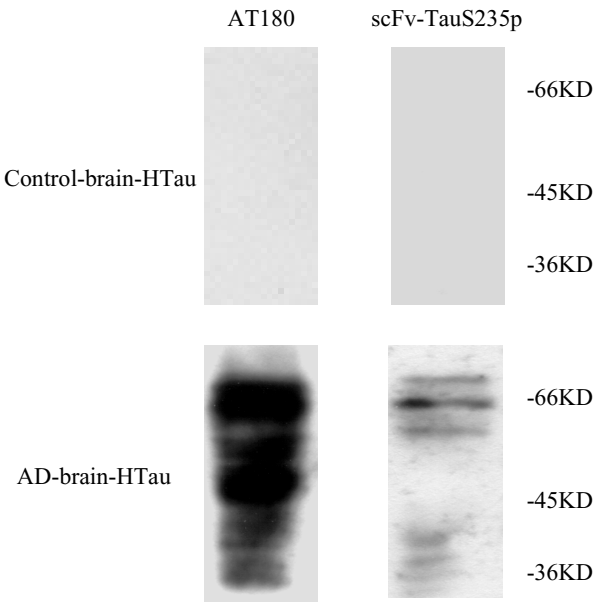

Figure 31. Brain Tau probed with scFv-TauS235p and AT180. 5µg of the control-brain-HTau or AD-brain-HTau was applied for the western blotting. Both antibodies recognized the AD-brain-HTau, but not the control-brain-HTau. The scFv did not recognize the bands of AD-brain-HTau in the 40–50KD range, presumably because they are double-phosphorylated at Thr231 and S235.

The purified scFv-TauS320p/S324p antibody (1mg/ml, working dilution was 1:50) was used to detect the control-/AD-brain-HTau and the recombinant Tau by Western blotting. It recognized the control-brain-HTau, the AD-brain-HTau and the phosphorylated recombinant Tau40 (Figure 32). AD-brain-HTau was hyper-phosphorylated at many sites, since the scFv–TauS320p/S324p recognized it (Figure 32A), therefore it must be phosphorylated at the Ser320 and Ser324 site. The signals were smeared out between 29-66KD, which represented different Tau isoforms and degraded fragments. The phosphorylated recombinant Tau appeared to have the same properties as the AD-brain-HTau, its signal also smeared in the same range. The control-brain-HTau was usually assumed to be dephosphorylated. But the result showed a signal band at 40KD in the control-brain-HTau sample. In contrast, the dephosphorylated recombinant Tau, which was purified from *E. coli*, was not recognized. There are two explanations for the signal at 40KD, one is that it was the cross-reaction, because the control-brain-HTau purified from the human brain might mix with other proteins

that reacted with the scFv-TauS320p/S324p antibody; the other explanation is that the normal-brain HTau was phosphorylated at the Ser320 and S324 site.

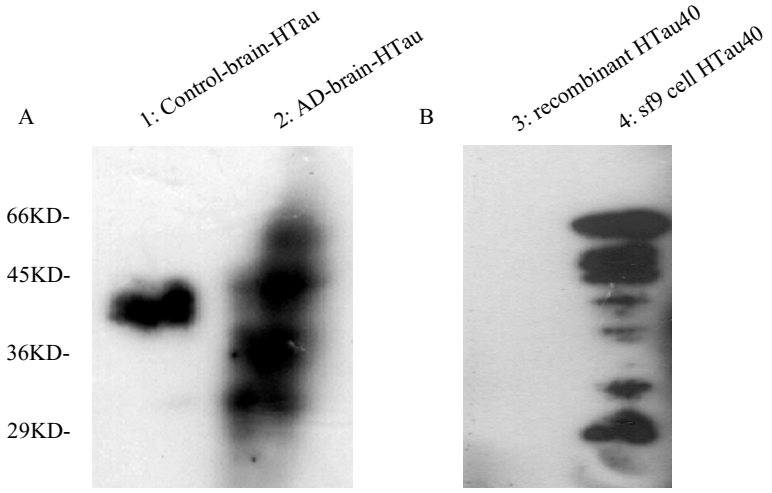

Figure 32. Western blotting detection of the brain Tau (A) and the recombinant Tau (B) with the scFv-TauS320p/S324p antibody. Lane 1: control-brain-HTau (5µg); lane 2: AD-brain-HTau (5µg); lane3: the dephosphorylated recombinant HTau40 (2µg); lane4: the phosphorylated recombinant HTau40 (purified from Sf9 cells). There was a band at about 40KD in the control-brain-HTau, but there was no signal in the dephosphorylated recombinant Tau. The AD-brain-HTau showed a smear from 29KD to 66KD, the phosphorylated recombinant Tau40 had also the smear in the same range.

3.2.10. Analysis of Tau expression in CHO cells with the scFv-TauS235p antibody

The purified scFv-TauS235p antibody (1mg/ml) was used to detect Tau40 in CHO-E34 cells (e.g. Tau40 overexpressing cells) by immunofluorescence (IF, see 2.2.1.4.). To visualize Tau40, the primary antibody was the scFv–TauS235p antibody (1:50 diluted); the secondary antibody was the anti-myc tag antibody (1:200 diluted) which recognized the myc tag in the scFv antibody; the third antibody was the anti-mouse-FITC antibody (1:200 diluted). To visualize the microtubule network, the YL1/2 antibody (1:100 diluted) which recognized Tubulin was the primary antibody; the secondary antibody was the anti-rat-AMCA (1:100 diluted).

As illustrated in Figure 33, the YL1/2 antibody recognizing tubulin revealed the microtubule network both in interphase (extended network) and mitosis (mitotic spindle). A comparison of tubulin staining and Tau40 staining showed that scFv–TauS235p only stained the mitotic cells, but did not stain the interphase cells at all. This result indicated that Ser235 became

phosphorylated during mitosis and dephosphorylated during interphase. This result is consistent with the result which had been found in our lab with AT180 monoclonal antibody (Pruess et al., 1995; Preuss and Mandelkow, 1998; Illenberger et al., 1998).

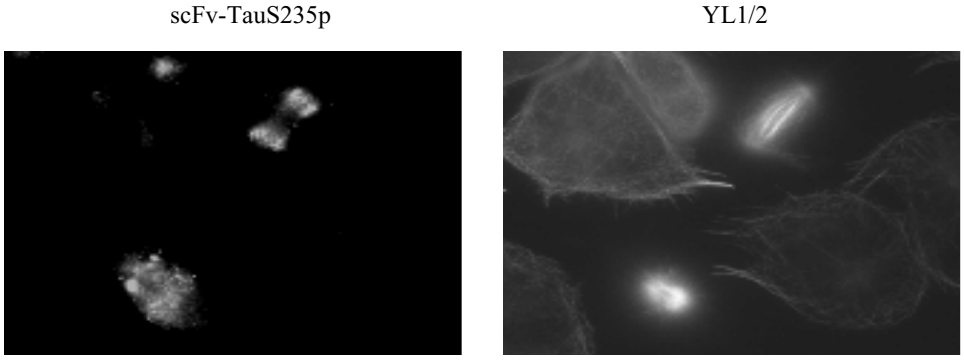

Figure 33. Tau expression probed in CHO cells with scFv-TauS235p by immunofluorescence. The microtubule network was stained with YL1/2 antibody, Tau40 was stained with scFv-Tau235p. The field on the right contained several interphase cells and two mitotic cells (note mitotic spindles), the field on the left illustrated that Tau40 was stained only in the two mitotic cells.

3.3. Tetramerization of scFv antibody

We could not ignore one unsatisfactory fact that many *E. coli* HB2151 cells did not produce soluble active scFv antibody (at least, we could not detect them by ELISA. see 3.2.4. in results), this meant some valuable scFv in current system would get lost. There are two possible reasons for this problem. The first, the yield of expressed scFv was too low to detect. The second, the expressed scFv had too low affinity to bind to the antigen so that it could not be detect by immunoassay such as the ELISA. The scFv expressed in *E. coli* HB2151 cells is a monovalent antibody, i. e., it has only one binding site to the antigen. As we know, a monoclonal antibody has two binding sites to the antigen. Thus, the functional affinity of scFv was not as good as a monoclonal antibody. In order to improve the yield and the functional affinity of scFv, a new expression system was created by the following procedures.

The design of the new system is based on the idea of forming a tetravalent scFv antibody to improve its functional affinity. The key experiment is to add the hinge/p53 fragment behind the scFv, by which the scFv will self-associate to form a tetramer. Due to the restriction site conflict, another sequence (so-called the expression−cassette) is added before adding the hinge/p53 fragment in order to introduce the necessary restriction sites

3.3.1. Preparation of the hinge/p53 fragment and the expression-cassette

3.3.1.1. Preparation of the hinge/p53 fragment

The hinge/p53 fragment was derived from the PMSTetp53His plasmid which was generously provided from Prof. A. Plückthun (Plückthun and Pack, 1997), with modifications for the restriction sites at two ends. It was practically prepared by PCR with the primers containing the modified sites (see 2.4.1.1.). On the agarose gel, the hinge/p53 fragment appeared as a 195bp band (Figure 34). After isolation from the agarose gel, the hinge/p53 fragment was then used to construct the tetravalent scFv system in later steps.

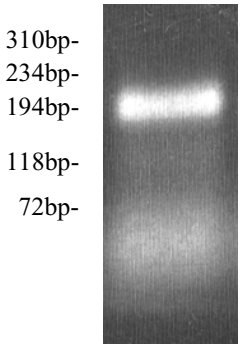

Figure 34. Amplification of the hinge/p53 fragment. The main band around 195bp was the hinge/p53 fragment, the small fragment around 70bp was the unreacted primers

3.3.1.2. Construction of expression-cassette

Expression-cassette was constructed with four oligo-nucleotides by two PCR steps (see 2.4.1.2.). The first PCR used two oligo-nucleotides, mcs-1 and mcs-4, to make the middle fragment of the expression-cassette; after PCR, a 113bp middle fragment was obtained (Figure 35), and the product was isolated from the gel. The second PCR employed another two oligo-nucleotides, mcs-2 and mcs-3, using 113bp fragment as the template, to make the whole expression-cassette.; after PCR, a 219bp fragment was obtained, and isolated from the gel. The 219bp fragment was called “expression-cassette”, which would be ligated into pET22b(+) vector in the next step.

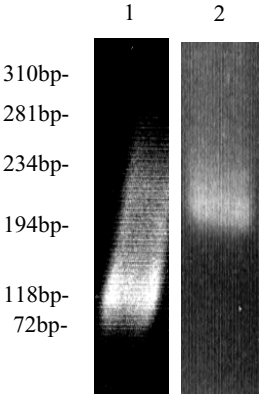

Figure 35. Construction of the expression-cassette. 1: Products made with mcs-1 and mcs-4 primers by the first PCR; 2: Products made with mcs-2 and mcs-3 primers by the second PCR. The desired products were at 113bp and 219bp, respectively. Other products were un-reacted oligo-nucleotides or by-products.

3.3.2. Vector construction for tetravalent scFv antibody

The first step was to insert the expression-cassette into pET-22b(+) vector in order to introduce proper restriction sites (see 2.4.2.1.). The ultracompetent XL2-Blue was transformed by the ligation mix with the heat-shock method. Two clones appeared to contain constructed vectors. The plasmids from these two clones were sequenced, one of them had the correct sequence of expression-cassette, which was exactly the same as designed (Figure 36). This vector was called "pET22b(+)Expcast".

5' T AAT GGT ACG GCC CAG CCG GCCATGGCC CAG GTG CTG CAC TCG AGT GTG CAC AGG TCC AAC TGG CGG CCG CAG GCC GGC CAG AAC AAA AAC TCA TCT CAG AAG AGG ATC TGA ATA GCG CCG TCG ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACT GAG ACC TGA GGC TCT GAA AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA CCGCTGAGC AA 3'

Figure 36. The nucleotide sequence of expression cassette. The sequences which are double underlined are NcoI and Bpu1102I restriction sites, respectively.

The second step was to insert the scFv-Tau13-24 gene into pET22b(+)Expcast vector (see 2.4.2.2.). The competent *E. coli* BL21(DE3) cells were transformed by the ligation mix with the electroporation method. Numerous clones appeared to contain new vectors. Three of them were picked to analyze their plasmids, all had the correct vector. This vector was named "scFv-Tau13-24-pET22b(+)Expcast".

The final step was to insert the hinge/p53 fragment into scFv-Tau13-24-pET22b(+)Expcast vector (see 2.4.2.3.). The competent *E. coli* BL21(DE3) cells were transformed by the ligation mix with the electroporation method. Six clones were tested, and one clone turned out to be the correct one. Thus the vector for tetravalent scFv antibody was obtained, it was called "scFv-Tau13-14-pET22b(+)ExpcastP53". In order to use this vector for other scFv, it was digested with NcoI and NotI restrict enzymes to remove scFv-Tau13-14 gene, the digested vector was called "pET22b(+)ExpcastP53".

3.3.3. Expression and purification of the tetravalent scFv-Tau13-24 antibody

In order to express the tetravalent antibody, scFv-Tau13-24-pET22b(+)ExpcastP53 clone was induced with IPTG-b (see 2.2.2.7.). The supernatant, the periplasmic extract and the whole cell extract were detected by ELISA to localize the active soluble antibody (see 2.3.1.9.). We found only a small fraction of active soluble scFv-Tau13-24 antibody in the culture medium, the cell periplasmic extract and the whole cell extract, which meant that the major antibody was insoluble. Therefore the purification of scFv-Tau13-24 tetramer was carried out involving a urea-extraction step and a later step for the refolding of the antibody (see 2.2.4.).

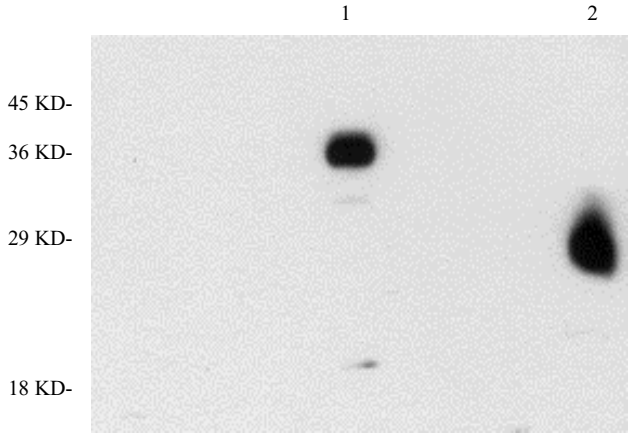

Figure 37. Western blotting analysis of the monovalent and tetravalent scFv-Tau13-24. Lane 1: purified tetramer; lane 2: purified monomer. Western blotting was performed with the anti-myc tag antibody which recognized the myc tag in the antibody. The band, which appeared in lane 1 was not the whole tetramer, it was just one dissociated unit of the tetramer.

The purified scFv-Tau13-24 tetramer was analyzed by Western blotting (Figure 37). Since the tetramer would be dissociated by SDS during the polyacrylamide-gel-electrophoresis (PAGE), the band appeared by Western blotting was just one unit of the tetravalent antibody. However, the results showed that this unit was around 35KD in 10% SDS-PAGE, which was higher than the monovalent scFv-Tau13-24 around 30KD. This expected difference was due to the introduction of the expression-cassette and the hinge/p53 sequences.

3.3.4. Functional affinity improvement of the tetravalent scFv-Tau13-24 antibody

The affinity of a single binding site of the tetravalent antibody to the antigen was not improved, it was the same as the affinity of the monovalent scFv antibody. Since the tetramer

had four binding sites, it kinetically had longer dissociation time, therefore it presented a higher affinity to the antigen, this affinity is called “the functional affinity” or “avidity” (Abraham et al., 1995; Park et al., 1995). The functional affinities of the monovalent scFv-Tau13-24 (0.5mg/ml) and the tetravalent scFv-Tau13-24 antibodies (0.5mg/ml) were compared by ELISA. The monovalent or tetravalent antibody (at different dilutions) incubated with Tau40 coated on the well, the secondary anti-myc tag-HRP (1:2,500 diluted) was used to detect the primary antibody.

The 1:100 diluted tetramer showed comparable signals as the 1:10 diluted monomer (Table 14). Considering that their concentration had 10-fold difference, we concluded that the functional affinity of the tetravalent scFv-Tau13-24 had been improved at least 10 folds.

Dilution	$OD410_{nm}$-monovalent scFv-Tau13-24	$OD410_{nm}$-tetravalent scFv-Tau13-24
1:3	1.85	2.5
1:10	1.075	2.108
1:100	0.593	1.278

Table 14. Comparison between the monovalent and tetravalent scFv–Tau13-24 antibody. The monovalent and tetravalent antibody had the same original concentration (both were 0.5mg/ml). The ELISA signal (1.278) of the tetramer at the dilution of 100 is similar as the signal (1.075) of the monomer at the dilution of 30.

3.4. ScFv antibodies against MARK

Microtubule affinity regulating kinase (MARK) plays an important role in Tau phosphorylation (Trojanowski and Lee, 1995; Johnson and Jenkins, 1996; Drewes et al., 1998; Ebneth et al., 1999; Schneider et al, 1999). It would be helpful if antibodies against MARK are available. At the moment, there is no useful monoclonal antibodies against MARK yet, therefore we tried to select scFv antibodies against MARK from the Griffin.1 library.

3.4.1. Selection with immunotube

Phage selection was performed with the MARK1-coated immunotubes (see 2.3.2.1.). The coating concentration of MARK1 was 10mg/ml, four rounds of selection were carried out.

The number of the selected phages in each round was counted by calculating the number of the colonies on SOBAG plates containing ampicillin (see 2.2.4.6.). The immunore-activity of the phages in each round was detected with ELISA. The MARK1 was coated on the well, the precipitated phages (1:100 diluted) was incubated in the well, the secondary anti-M13 coat protein-HRP antibody (1: 10,000 diluted) detected the binding of the phages.

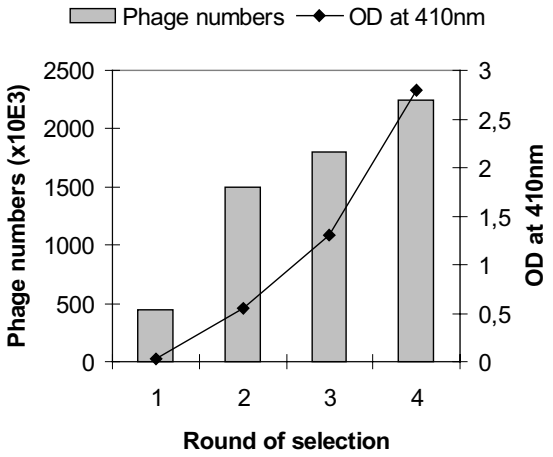

Figure 38. Enrichment of phages during selection. The numbers of selected phages increased from 450,000 to 2,250,000. The ELISA signals against MARK1 increased from close to 0 to 2.9.

The selection cycles showed phage enrichment and increasing ELISA signals (Figure 38). In contrast to the abrupt elevation in selection with Tau40 (see Figure 22A), in this case, the phage quantity and ELISA signals increased rapidly and smoothly, it was similar as the selection with K19p (see Figure 22B).

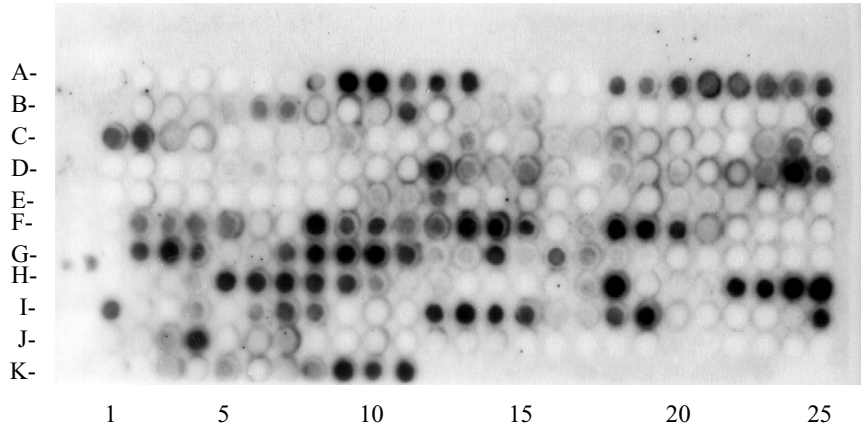

Figure 39. MARK1-spot-membrane detection of Griffin/MARK1 phage library. Letters on Y-axis indicates the spot lines, numbers on X-axis indicates the spot positions in each line, the origin of sequence is at upper left. Signals were distributed all over the membrane with different intensities.

The phages from the fourth round of selection was called “Griffin/MARK1 phage library. In order to explore which kinds and how many kinds of phages were included in this phage library, we used this library to perform immunoblot with the MARK1-spot-membrane (see 2.2.4.4.). MARK1-spot-membrane was similar as Tau40-spot-membrane, it contained 261 of

15mer peptides that comprised the whole MARK1 sequence. The phages were used as the primary antibody (1:500 diluted) to bind to the MARK1-spot-membrane; the secondary anti-M13 coat protein-HRP antibody (1:5,000 diluted) was used to detect the bound phages. The result of immunoblotting (Figure 39) showed that the phages recognized many sites of MARK1 with varying signal intensities. Some sites represented common sequences in all MARK isoforms, some sites were specific for MARK1.

3.4.2.Selection of MARK1-specific phages with the competition method

Some of highlighted spots in Figure 25 corresponded to unique sequences in the MARK1 isoform, which opened a possibility to obtain MARK1-specific phages. We attempted to enrich these phages under competitive conditions, i. e., the competitor was involved when Griffin/MARK1 phage library was incubated to the antigen (MARK1) coated on the plastic surface (see 2.3.2.4.).

After two rounds of competitive selection, the new phage library, which was called "Griffin/MARK1-specific phage library", was tested with MARK1-spot-membrane immunoblot (see 2.2.4.4.). The Griffin/MARK1-specific phage library (1:200 diluted) bound to the MARK1-spot-membrane as the primary antibody, the secondary antibody was the anti-M13 coat protein-HRP (1:5,000 diluted) that detected the bound phages.

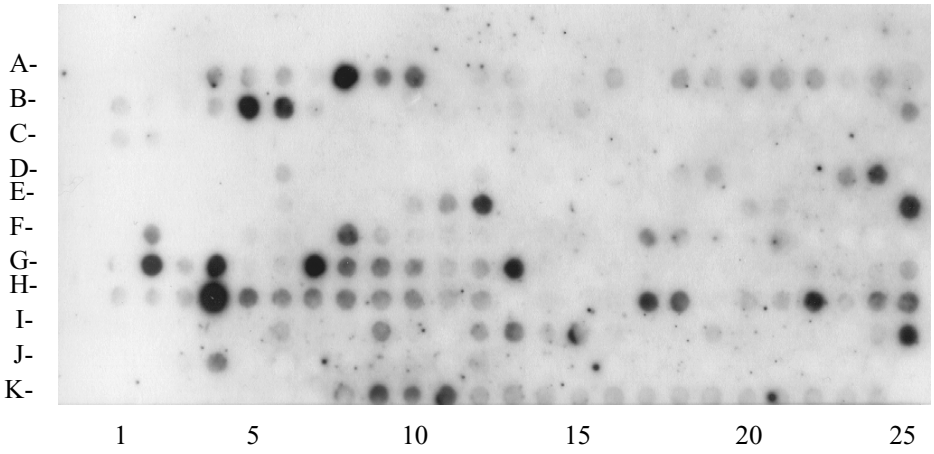

Figure 40. MARK1-spot-membrane detection of Griffin/MARK1-specific phage library. Letters on Y-axis indicate spot lines, numbers on X-axis indicate the spot positions in each line. Compared to the results in Figure 25, highlighted spots in line G and line H were much more pronounced than these in other lines.

The results showed that many spots in line G and line H were recognized (Figure 40). The region of spots H1 to H10 (the first spots to the 10th spot in line H) corresponded to Thr525−Pro567 of MARK1, which was a unique domain only in MARK1 but not in other MARK isoforms. Though signals in the unique domains were not enhanced by the competitive selection comparing Figure 39, the signals of common domains were significantly reduced, namely the MARK1-specific phages were the main population in the library. Thus, in the following screening, we had a better chance to obtain MARK1-specific phages instead of obtaining phages against common sites of all MARK-isoforms.

3.4.3. Screening of phages

In order to have antibodies against MARK common sites and MARK1-specific sites, we screened the Griffin/MARK1 and Griffin/MARK1-specific phage libraries (see 2.3.1.7.). The ELISA was used to detect which phages were specific for the antigen. For ELISA detection, the phage supernatant (1:3 diluted) served as the primary antibody, the anti-M13 coat protein-HRP antibody (1:5,000 diluted) was the secondary antibody.

For the Griffin/MARK phage library, BSA was used as the negative control and MARK1 protein as the antigen for the ELISA. One positive out of 36 was found, it was called “G5 phage” due to its position on the 96-well-plate.

For Griffin/MARK1-specific phage library, MARK1 was used as the antigen, MARK2 and MARK3 as negative controls for ELISA. One MARK1-specific phage was found out of 36 clones, it was called “A11 phage” due to its position on the 96-well-plate. In the next step, we directly used the monoclonal phages to identify the antibody epitopes.

3.4.4. Identification of epitopes

It frequently occurred that clones got lost when they were taken to transfect HB2151 cells. In such cases, the phages could be directly used to identify antibody epitopes instead of the soluble scFv antibody. The MARK1-spot-membrane immunoblots were employed for epitope identifications (see 2.2.4.4.).

3.4.4.1. Epitope identification of the scFv-PanMARK592-603 antibody

The G5 phage (1:300 diluted) was incubated with the MARK1-spot membrane; the secondary anti-M13 coat protein-HRP (1:5,000 diluted) was used to detect if the phage bound to the membrane. The result showed that G5 phage recognized spots of H22 and H23 (Figure 41). The two spots showed similar signal intensities, indicating that both peptides carried the epitope. According to the peptide sequences (Table 15), the epitope of the G5 clone is ^{592}**HSISASTPDRTRFPR**^{603}. This fragment is conserved in all 4 isoforms of MARK, and therefore the G5 clone was named "scFv–PanMARK592-603".

Figure 41. Epitope identification of the G5 clone by MARK1-spot-membrane. Letters on Y-axis indicates the spot lines, numbers on X-axis indicates the spot positions in each line. H22 and H23 (the 22nd and 23rd spots in line H) were recognized, H23 had a little stronger signal intensity than H22, but the difference was not significant

Positions	Peptide sequences	Signal intensity
H22	^{589}PSA**HSISASTPDRTR**^{603}	Strong
H23	^{592}**HSISASTPDRTR**FPR^{606}	Stronger

Table 15. Sequences of peptides recognized by G5 phages. Positions correspond to spots on the membrane. H23 had a little stronger signal intensity than H22.

3.4.4.2. Epitope identification of the scFv-MARK1-535-549 antibody

The A11 phage was also detected by the MARK1-spot membrane immunoblot. The phage (1:300 diluted) was applied to the membrane, the secondary antibody for the detection was the anti-M13 coat protein-HRP antibody (1:5,000 diluted). The result showed that A11 phage strongly recognized H4 spot (Figure 42). Some other spots showed very weak signals too, but

since they were so weak and distributed on so many sites on the membrane, they were unspecific signals due to unknown reasons. Therefore H4 peptide represented the antibody epitope. According to the peptide sequence, the epitope is ^{535}**STGSTVASAGPSARP**^{549}. This sequence is unique for MARK1, it is absent from other MARK forms. A11 clone was named "scFv-MARK1-535-549".

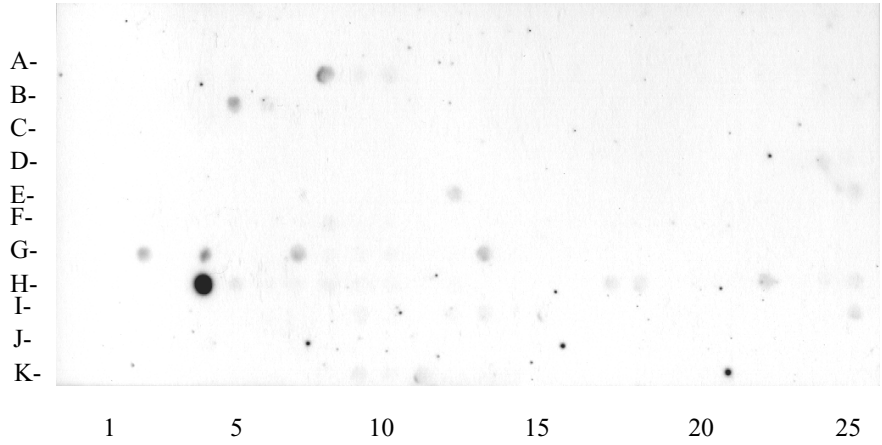

Figure 42. Epitope identification of A11 clone by MARK1-spot-membrane. Letters on Y-axis indicates the spot lines, numbers on X-axis indicates the number of spots in each line. The spot of H4 (the fourth spot in line H) was the only one recognized specifically.

3.4.5. Expression and purification of the tetravalent scFv antibodies against MARK

Since we already had the new expression system (see 3.3) to make tetravalent scFv, which had a better functional affinity. Therefore, instead of switching to HB2151 cell expression system, the antibody genes of scFv-PanMark and scFv-MARK1-535-549 clones were directly constructed into tetravalent scFv system. Briefly, the plasmid was purified from the TG1 cells; the scFv gene was cut out, and then ligated into the pET22b(+)ExpcastP53 vector which would make tetravalent scFv antibody (see 2.4.3.). The correct clone was verified by a set of restriction enzyme digestions.

Expression of the tetravalent scFv antibody in *E. coli* BL21(DE3) cells was induced by IPTG-b (see 2.2.2.7.). After testing the supernatant, the periplasmic extract and the whole cell extract (see 2.3.1.9.), we found nearly no active soluble scFv, this meant that the major antibody was insoluble (similar as the tetravalent scFv-Tau13-24, see 3.3.3.). Therefore, the purification was carried out involving an urea extraction and an antibody refolding steps (see

2.4.4.). After purification, the tetravalent scFv antibodies were used for Western blotting and immunofluorescence for further analysis in the following experiments.

3.4.6. Western blotting detection for MARK by the tetravalent scFv antibodies

Purified tetravalent scFv-PanMARK (0.2mg/ml) and scFv-MARK1-535-549 (0.2mg/ml) antibodies were used for Western blotting. Both tetravalent scFv antibodies were used at the dilution of 1:100, the secondary antibody was anti-myc tag-HRP (1:2,000 diluted).

As expected, scFv-PanMARK recognized all three forms of MARK and did not discriminate between members of the MARK family (Figure 43). On the other hand, it did not recognize many other kinases except maybe a slight crossreaction with cdc2 (Figure 44). This could of course alternatively represent a contamination of MARK in the cdc2 preparation, which has been found in our lab for Tau kinase GSK3ß (Godemann et al, 1999).

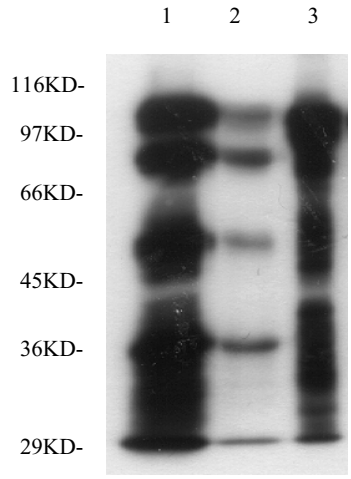

Figure 43. Detection of MARK isoforms by tetravalent scFv-PanMARK. Lane 1:MARK1, (1µg); lane 2: MARK2 (0.1µg); lane 3: MARK3 (0.5µg). The molecular weight of MARK is around 100KD, but since the protein is degraded easily, many smaller fragments are observed.

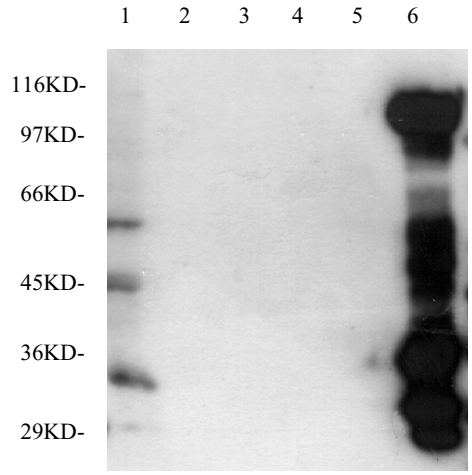

Figure 44. Detection of kinases by tetravalent scFv-PanMARK. Lane 1: cdc2; lane 2: cdk5; lane 3: GSK-3ß. lane 4: MAPK; lane 5: PKA; lane 6: MARK1. Applied amount of each kinase was 0.5µg. PKA was purchased from Boeringer-Mannheim company and others were prepared in our lab. Only MARK1 showed clear signal, cdc2 showed a little signal.

By Western blotting, the tetravalent scFv-MARK1-535-549 antibody strongly recognized recombinant MARK1 only, and did not recognize other recombinant MARK isoforms such as MARK2 and MARK3 (Figure 45), ascertaining that it was a useful MARK1-specific antibody.

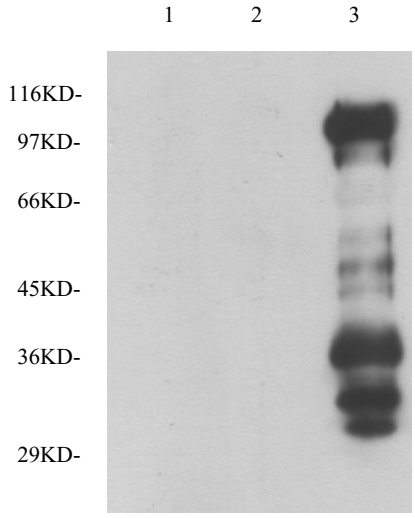

Figure 45. Demonstration of the specificity of the scFv-MARK1-535-549 antibody. Lane 1: MARK3; lane 2: MARK2; lane 3: MARK1. Each MARK was 0.5µg. The lower molecular fragments are degration products of MARK1.

3.4.7. Analysis of MARK in eucaryotic cells by the tetravalent scFv antibody

In order to check if tetravalent scFv antibody could be also used by immunofluorescence (IF), the purified tetravalent scFv-PanMark antibody (1mg/ml) was used to investigate HA-tagged MARK2 in CHO-MARK2 cells. The cells were generated in our lab, they were cultured and induced by doxcycline to express MARK2 (see 2.2.1.3.). Immunofluorescence was carried out following the procedures in 2.2.1.4.. While using the scFv–PanMARK antibody (1:50 diluted) to visualize MARK2, the anti-myc tag antibody (1:200 diluted) and the anti-mouse-FITC antibody (1:200 diluted) were the secondary and the third antibody, respectively. While using the anti-HA tag polyclonal antibody (1:200 diluted) to reveal MARK2, the anti-rabbit-FITC antibody (1:100 diluted) was the second antibody.

The scFv-PanMARK antibody and the anti-HA tag ploYclonal antibody showed similar patterns (Figure 46), indicating that the recombinant antibodies had similar properties as the traditional anti-HA tag antibody. These pattern was also the same as the previous results in our lab (Drewes et al., 1997). We also tested the monovalent scFv-PanMARK antibody by IF, there was no signal visualized, it meant that tetravalent scFv antibody really had much stronger functional affinity than monovalent scFv antibody.

Though the tetravalent scFv-PanMARK antibody could detect the overexpressed MARK2, we did not observe any endogenous MARK in untransfected CHO cells or N2a neuroblastoma cells. It could be due to that the endogenous MARK amount is too low to detect, or the MARK forms in these cells do not have the epitope of the scFv-PanMARK antibody which is against the MARK isoforms from rat (Drewes et al., 1997).

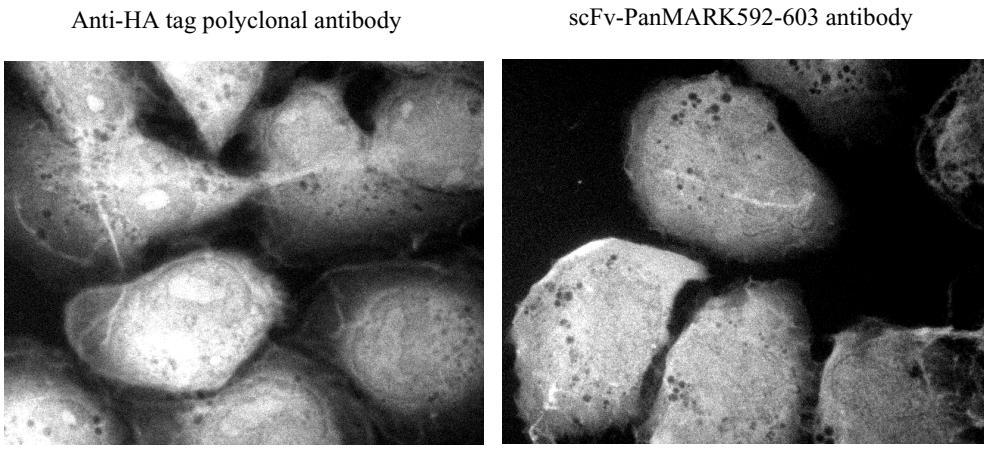

Figure 46. Immunofluoresent investigation of MARK2 in CHO-MARK2 cells by tetravalent scFv-PanMARK592-603 antibody. Cells on the left panel were stained by anti-HA tag polyclonal rabbit antibody; cells in the right side were stained by tetravalent scFv-PanMARK592-603 antibody.

4. Discussion

In this study, we have used the phage display recombinant antibody technique to generate antibodies against Tau protein, phosphorylated Tau protein and MARK for diagnostic analysis. We successfully generated several recombinant antibodies against Tau protein and MARK, and the antibodies were used to detect Tau protein and MARK by western blotting and immunofluorescence.

4.1. Conversion of Tau-1 monoclonal antibody into scFv

4.1.1. Purification of total RNA and mRNA

The mRNA quality is very important for the scFv gene cloning because it is the source of the antibody gene. mRNA can be purified directly from tissues or cells, or indirectly from the total RNA. The direct method is easier than the indirect method, but the purity and yield is not as good. Therefore, for the scFv cloning, the indirect method is recommended.

There are two variants of the total RNA purification: silica-gel-based absorption and phenyl extraction. Silica-gel absorption avoids toxic reagents, easy and fast. The phenyl extraction is a traditional method involving toxic reagents. In practice, the phenyl extraction method always gave better yield and quality of total RNA than silica–gel absorption. In particular, the phenyl extraction is better for the protection of RNA against degradation by RNase because phenyl itself strongly inhibits RNase activity.

4.1.2. Cloning of scFv

Cloning of scFv includes two main steps: reverse transcription–polymerase chain reaction (RT-PCR) and assembly of scFv. RT-PCR is used to get V_H and V_L separately, assembly step is to link V_H and V_L to a complete scFv gene. The heat step shortly before the reverse transcription serves the purpose to destroy the secondary structure of mRNA for efficient primer binding, otherwise the cDNA synthesis may loose some candidates or generate wrong sequences. Primer sequences for V_H and V_L are unknown. Alignment of known antibodies

showed that flanking domains of V_H and V_L are conserved for different antibodies, one may suppose, in principle, that the primer sequences lie in the flanking constant domains of V_H and V_L (Orlandi et al., 1991;Clackson et al., 1991; Marks et al., 1991; Hoogenboom and Winter, 1992; Griffiths et al., 1994; Barbas et al., 1992). There are only a few amino acid differences between different sub-classes of antibodies. Therefore a mixture of primers should ensure getting all candidates.

The DNA amount of V_H and V_L must be equal and more than 50ng each, otherwise the assembly of scFv will not succeed. The Linker primer sequence is unknown, in principle, it contains the complementary part of the 3' end of the V_H and the complementary part of the 5' end of the V_L (Figure 47). The restriction site (RS) primer sequences are unknown, in principle, they are the primer mix for the PCR amplification of scFv, the forward primer has the SfiI restriction site and the complementary part of the 5' end of the V_H, the backward primer has the NotI restriction site and the complementary part of the 3' end of the V_L.

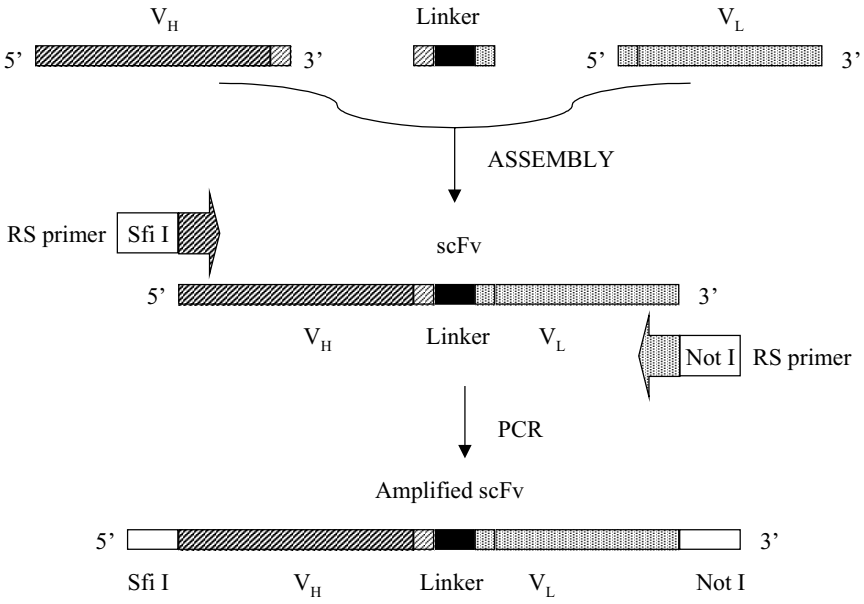

Figure 47. Assembly of scFv. Assembly of scFv contains two steps, the first step is to build V_H and V_L to scFv, it is achieved by PCR using the linker. The second step is to amplify scFv and add restriction sites at two ends of scFv, it is achieved by PCR using RS primers.

4.1.3. Phage display antibody library construction

The construction of the phage display library begins from the scFv restriction digestion, and is followed by ligation, transformation and phage rescue steps. The antibodies can be displayed

as gene3 fusion either by cloning it directly within the phage genome (Parmley and Smith, 1988; Cwirla et al., 1990; McCafferty et al., 1990, 1991; Clackson et al., 1991; Swimmer et al., 1992) or by cloning it into gene3 present within a phagemid plasmid (Bass et al., 1990; Barbas et al., 1991; Brietling et al., 1991;Hoogenboom et al., 1991; Marks et al., 1991; Griffiths et al., 1994). The latter "phagemid-display" system requires rescue with a helper phage, such as VCSM13 helper phage or M13KO7 helper phage, to generate particles displaying the scFv antibody fused to gene3. Phage particles derived in this way will display gene3 protein (g3p) from both the wild-type gene3 of the helper phage and the fusion gene3 from the resident phagemid. This is in contrast to display of g3p fusion encoded within the phage genome itself, where all g3p molecules are originally present as fusion. In this study, pCANTAB5E, a phagemid, and M13KO7, a helper phage was used to rescue the phages.

The digested pCANTAB5E vector is dephosphorylated by alkaline phosphatase to reduce the possibility of vector self-cyclation. The digested scFv gene can be purified either by the microspin column or by running an agarose gel, cutting the scFv out of the gel and then extracting it. The gel extraction method gives a much better yield than the microspin column, but the later method is easier and faster.

After the transformation to *E. coli* TG1 cells, at first only 3,000 clones are present. This is a relatively low number compared with some other libraries. The main reason could be the transformation method. Here we employed the heat shock method whose efficiency is much lower than that of electroporation. We took the same amount of ligation mix to transform the TG1 cells by electroporation, and got around 10,000 clones. We started from hybridoma cells, so in principle we could only generate one recombinant antibody. Although the heat shock method is a method that shows low efficiency, it is still good enough to obtain the positive clone. But in the case of other sources of material and other aims (for example, creating an universal library for different proteins), it would be preferable to use electroporation.

The phage rescue is done directly from transformed (or re-infected) TG1 cells without the step of culturing the TG1 cells on plates which is always a part of the phage production for the Griffin 1 library. Actually, the direct phage rescue is faster (saving one day), but it has the risk of losing candidate antibodies. The reason is that differently transformed (or re-infected) cells may grow at different rates. Some candidates may grow very slowly, have a very low number of bacteria and produce very low amount of phages which can never be enriched by

selection. For simple libraries such as our library cloned from hybridoma cells, which has not so many candidates, the direct phage rescue is useful. For a complex library which has many candidate antibodies (for example, the Griffin 1 library which has about 10^9 candidates), one additional day of culture on plates is recommended.

4.1.4. Selection of phages

With the selection method employing affinity column purification, the number of recovered phages is relatively low compared with that for the Griffin 1 library. This could be due to the stringency which is higher in the affinity column selection method than the immunotube selection method because the binding conditions such as incubation time, the flow rate for phages is not optimized. The advantage of a high stringency method is that phages can be rapidly enriched so that ELISA signal is high enough after only 2 rounds of selection (see Figure 17 in results). The disadvantage is of course the loss of antibody candidates. If we only want one antibody, the stringency selection method (for example, the selection employing the affinity column) is useful; if a set of antibodies against the same protein is desired, the lower stringency selection method is recommended (for example, the selection employing the immunotube), and in this case, the phages are enriched slowly (see Figure 22A in results). Other reasons could also speed up the rate of the phage enrichment (see Figure 22B in results), for example, the high affinity between a phage displayed antibody and the antigen, the high number of the phage antibodies against the antigen in the library. We don't know the reasons for the rapid enrichment in the selection using the phosphorylated K19, possibly the affinity between phage displayed antibodies and the phosphorylated K19 was high.

4.1.5. Screening of phages

Screening is always needed because unspecific phages can never be removed by selection. Even when the ELISA signal is very high (>1.0) after 4 rounds of selection, only 5 clones were obtained from 100 clones (see Table 1 in results). This means that unspecific phages contribute 95% of the whole phage population. The percentage of the specific binders in the whole phage population could vary a lot, a report (Griffiths et al., 1993) showed that specific binders could be up to 45% after three rounds of selection.

4.1.6. Expression and purification of the scFv antibody

Since the *E. coli* HB2151 bacteria are transfected quite poorly, the selection on a plate containing nalidixic acid ensures that the resulting clones are truly nalidixic acid-resistant HB2151 bacteria and not due to a carryover of infected TG1 cells.

The location of expressed the scFv antibody in the *E. coli* cells is important for purification. If the scFv antibody is secreted into the medium, its concentration in the supernatant is very low, not worthy of further handling. Inside cells, there is a valuable amount of the scFv antibody, but it is mostly inactive (see Table 3 in results). By contrast, the periplasmic space contains a substantial amount of active scFv antibody. Active antibody requires an intramolecular disulfide bridge (Harlow and Lane, 1988; Alberts et al., 1989). Inside HB2151 cells, there is a reducing environment, where it is difficult to form a disulfide bridge, therefore the scFv antibody inside the cells are not active. Outside cells (in the periplasma or the medium), the disulfide bridge can form and the scFv antibody is active.

The periplasmic extract is finally taken to purify the scFv antibody. The scFv antibody was rapidly and easily purified, and its molecular weight was about 30KD as expected (see Figure 18 in results). The disadvantage of this method is that the HiTrap anti-E tag affinity column is very expensive. Secondly, since the E-tag binds tightly to the column, it is hard to recover scFv antibodies completely, therefore if the same column is used to purify other scFv antibody, cross-contamination may occur.

4.1.7. Epitope of the scFv-Tau193-204 antibody

To identify the epitope of a phosphorylation-independent antibody, the Tau40-spot-membrane is a fast, convenient method. 193 peptides of 15 amino acids which comprise the Tau40 protein sequence are covalently bound to a Nylon membrane, each peptide is concentrated in a spot area, this is the so-called Tau40-spot-membane. There are 8 lines, each line contains 25 spots (the last line contains only 18 spots), each spot contains 100ng peptide. The neighboring two spots have 12-amino acids overlap, and 3-amino acid difference. Thus the peptides move in a step of three amino acids along the Tau40 sequence from N- to C-terminal, from one spot to the next (see 6.4 in the appendix). The Tau40-spot-membrane is very useful for searching

interaction sites between Tau and other proteins (including antibodies). In this study, the Tau40-spot-membrane was successfully used to identify the epitopes of antibodies including both the Tau-1 monoclonal antibody (Binder et al., 1985) and the different scFv recombinant antibodies.. It is physically very stable, can be regenerated and reused many times.

By using the Tau40-spot-membrane, the epitope of the scFv-Tau193-204 antibody was identified. It has successive Ser residues which may contribute to an interacting surface with the antibody (see 3.1.11 in results). The scFv-Tau193–204 antibody is a phosphorylation-independent antibody. By contrast, AT-8 recognizes the same region of Tau, but it is a phosphorylation-dependent antibody. It needs phosphorylated Ser202 and Thr205 residues for the antibody-antigen interaction (Mercken et al., 1992; Biernat et al., 1992; Goedert et al., 1993 and 1995). The main epitope AT-8 probably is ^{200}PGS(p)PGT(p)PG^{208}, the helix is interrupted by the two phosphates. In this case, the two phosphates play an important role for the interaction between the AT-8 and the phosphorylated Tau.

4.1.8. Amino acid sequence of the scFv-Tau193-204 antibody

The scFv-Tau193-204 antibody was sequenced. The amino acid sequence showed the expected linker fragment of $(Gly_4Ser)_3$. This is a commonly used linker (Huston et al., 1988); it is a flexible peptide which allows the free interaction between V_H and V_L fragments. Some other linkers are also used in different laboratories (Whitlow et al., 1993).

The sequence shows that there are two cysteines in $V_{H,}$ and also two cysteins in V_L (see Figure 20 in results) This confirms that the necessary intramolecular disulfide bridges (Harlow and Lane, 1988; Alberts et al., 1989) can be formed.

C7 and E3 are two positive clones which produce the scFv antibody against the Tau193-204 epitope (this is also the epitope of the Tau-1 monoclonal antibody). They showed similar amino acid sequences with minor differences, especially at the beginning of the scFv. It may seem strange that one can get different recombinant antibodies from one monoclonal hybridoma cell line. Since these differences are outside the complementarity determining regions (CDRs) which determine the antibody specificity, they do not represent true differences in antibody specificity. A simple explanation is the cloning procedure employed in

several PCR reactions. It is well known that PCR can generate mutations especially at the beginning of the products.

CDRs are the key regions for the interaction between the antibody and the antigen. There are special rules to identify the CDR by screening the amino acid sequence (Wu and Kabat, 1970; McCallum et al., 1996; Martin and Thornton, 1996):

CDR-L1:	Start:	Approx. residue 24
	Residue before:	Always a Cys
	Residue after:	Always a Trp. Typically W-Y-Q, but also, W-L-Q, W-F-Q,W-Y-L
	Length:	10 to 17 residues
CDR-L2:	Start:	16 residues after the end of L1
	Residues before:	Generally I-Y, but also, V-Y, I-K, I-F
	Length:	7 residues
CDR-L3:	Start:	33 residues after end of L2
	Residue before:	Always Cys
	Residues after:	Always F-G-X-G
	Length:	7 to 11 residues
CDR-H1:	Start:	Approx. residue 26 (always 4 after a Cys)
	Residues before:	Always Cys-X-X-X
	Residues after:	Always a W. Typically W-V, but also,W-I,W-A
	Length:	10 to 12 residues
CDR-H2:	Start:	15 residues after the end of CDR-H1
	Residues before:	Typically L-E-W-I-G, but many variations
	Residues after:	K/R-L/I/V/F/T/A-T/S/I/A
	Length:	16 to 19 residues
CDR-H3:	Start:	33 residues after the end of CDR-H2 (2 after a Cys).
	Residues before:	Always Cys-X-X (typically C-A-R)
	Residues after:	Always W-G-X-G
	Length:	3 to 25 residues

4.1.9. Analysis of Alzheimer Tau by the scFv-Tau193-204 antibody

Recombinant antibody of scFv-Tau193-204 showed similar western blotting results as Tau-1 monoclonal antibody when they were used to detect Tau protein purified from normal human brains and Alzheimer's disease (AD) patient brains (see Figure 21 in results). This means that scFv-Tau193-204 has a similar epitope as Tau-1. The pattern shows that the control–HTau is not phosphorylated, but the AD-HTau is phosphorylated at the site of 193-204 so that the

antibody did not recognize Alzheimer Tau. In principle, the scFv-Tau193-204 can be used to detect Tau in the cerebrospinal fluid in order to distinguish normal people from Alzheimer's disease patients. However, since the ELISA gives negative signals for Alzheimer Tau samples, it is not suitable for ELISA detection of AD-HTau in the cerebrospinal fluid (CSF). But it may be a useful antibody to capture Tau from samples in general for the ELISA.

4.2. The scFv antibodies against Tau from Griffin.1 library

4.2.1. The Griffin.1 library

Following the development of the phage display technique, the libraries which have bigger repertoires representing a whole spectrum of affinities were constructed (e.g., Griffiths et al., 1994; Vaughan et al., 1996). In this study, we used the Griffin.1 library (Griffiths et al., 1994) which was a generous gift from Prof. Winter. The Griffin.1 library contains exactly the same synthetic human V-genes as the Human Synthetic Fab (4-12) 2lox Library but is in a scFv format instead of Fab format (see Figure 4 in introduction). The vector pHEN2 is a phagemid so that the phage rescue requires the helper phage. The Griffin.1 library is similar to the Human Synthetic scFv Library or "Nissim Library" (Nissim et al., 1994).

4.2.2. Selection with immunotube

The phage number increases during successive rounds of selection. It increases 5-7 fold per round. After 4 rounds, the number saturates, which means that the capacity of the selection system is saturated. Normally, three rounds of selection are enough to get a high ELISA signal which reflects the amount of the positive phages. In some cases, four or even five rounds of selection yield some improvement, but more than six rounds of selection make little sense. The capacity of the selection system is determinated by a number of factors (McCafferty J., 1996; Bothmann and Plückthun, 1998) such as the display level of the antibodies (i.e., the amount of antibodies which are displayed on the surfaces of the phages) and the theoretical aspects between the antibodies and the antigen (i.e., the kinetic and equilibrium behavior of the binding interactions).

Only the successful selection strategy can lead to the specific anybody, an unfavorable strategy will bring us nothing (Bruin et al., 1999). At the moment, we are at the stage where the user can be inventive and devise novel selection schemes to enrich and select for the desired binding activity, many new strategies and methods are found (Hoogenboom, 1997; Hoogenboom et al., 1998), for example, the modified usual selection approaches (Sawyer et al., 1997; Wind et al., 1997), the streptavidin-biotinylated antigen selection system (Hawkins et al., 1992), the in situ selection (Tordsson et al., 1997); the cell-based selection (Watters et al., 1997), the subtractive selection (De Greeff et al., 2000; Shinohara et al., 2000), the in vivo selection (Johns et al., 2000).

4.2.3. Selection with Tau-spot-membrane

The Tau40-spot-membrane is a useful tool to select phages. But it does not work if the original Griffin.1 library is used; only the pre-selected and enriched library is useful for selection by Tau-spot-membrane. The explanation could be that the phage is too large compared with the scFv antibody exposed on its tip; the peptide on the spot-membrane could be too small so that the steric-hindrance blocks the binding between the scFv antibody and the peptide.

Another disadvantage is that Tau40-spot-membrane can not be used to selected phosphorylation-dependent scFv since all peptides immobilized on the membrane are dephosphorylated. In principle, it is possible to make a spot-membrane dotted with phosphorylated peptides to resolve this problem. But since phosphorylated peptide synthesis is quite complicated and expensive, and phosphorylation sites can be combined in different ways, it is nearly impossible to design such kind of a spot-membrane.

4.2.4. Screening of phages

If phages are obtained from immunotube selection, the probability of finding positive clones is about 10% (see Table 7 in results), it is comparable to the screening from the Hybri-Tau1 phage library which was selected by the affinity column (see Table 1 in results). If phages have been selected from the Tau40-spot-membrane or by the single peptide selection, the

probability of finding positive clones is about 50-90% (see Table 8 and 3.2.3 in results). This high value might be due to very specific peptides.

The Tau40-spot-membrane showed that there were many phosphorylation-independent scFv in the Tau40p library (see Figure 23 in results). This is due to the antigen, the hyper-phosphorylated Tau40, which is composed of both the phosphorylated sites and the dephosphorylated sites of Tau. But we did not obtain phosphorylation-independent scFv by screening 95 clones (see Table 7 in results). This means that phosphorylation-independent scFv are detectable by the sensitive Tau40-spot-membrane immunoblot, but their amount is too low (<1%) to be obtained by screening so that the phosphorylation-independent clones are the minor population in the Tau40p library. Screening is an easy way to get a single clone of the major phage population which recognize the phosphorylated sites, but a difficult way to obtain a single clone of the minor phage population which recognize the dephosphorylated sites. One solution is to do the further selection by the Tau40-spot-membrane and then screen the newly enriched library.

4.2.5. Transfection of HB2151 cells

The phages which showed positive ELISA signals against the antigen were used to transfect HB2151 cells in order to switch the antibody gene to the antibody protein expression system . One usually gets the transfected HB2151 clones, but many of them did not produce active soluble scFv proteins (see Table 9 in results), at least, the scFv antibody is not detectable by ELISA. One possible reason is that there are usually two copies of the scFv antibody on the tip of phages (Armstrong et al., 1996), and thus the functional affinity based on these two binding sites is high. By contrast, the soluble scFv antibody protein has only one binding site, its functional affinity is very low, it can be reduced by 10,000 times (Berzofsky and Berkower, 1984; Stryer, 1988). This would explain why the phages showed a positive ELISA signal, whereas the soluble scFv antibody showed no ELISA signal. This was also the reason why we constructed the tetravalent scFv antibody.

4.2.6. Expression and purification of the scFv antibody

The scFv from the Griffin 1 library has a His-tag and a myc-tag (see Figure 30 in results). The Myc-tag is used for detection, the His-tag is used for the purification of scFv. Ni-NTA-agarose chromatography is a simple and specific method. Although the eluate contained other components (see Figure 25 in results), the purity was sufficient. The part of the fraction that eluted from the column was quite pure (a single band on the Coomassie stained SDS gel), it can be taken for special experiments such as the Kd measurement. However, Ni-NTA-agarose is not as good as Hitrap Anti E-tag affinity chromatography which yielded a single band on the silver stained gel (see Figure 18 in results). In practice, considering the price and the stability, Ni-NTA-agarose chromatography is more practicable.

4.2.7. Epitope of the scFv antibody

The Tau40-spot-membrane is a fast and convenient method to identify antibody epitopes. But since the Tau40-spot-membrane containing phosphorylated Tau sequences is not available, in vitro phosphorylated Tau constructs were used as an alternative, although this traditional method is time-consuming.

4.2.8. Amino acid sequence of the scFv-TauS235p antibody

The amino acid sequence of scFv-TauS235p shows typical complementarity determining regions (CDRs) (see Figure 30 in results). There are three CDRs in V_H and three CDRs in V_L too. There are also 2 cysteines in V_H and V_L which are necessary to form intramolecular disulfate-bridges.

The six CDRs form the antigen binding domain. All CDRs are variable, particularly the CDR-H3 is the most variable one because its length is from 3 to 25 amino acids (see 4.1.7), namely, the CDR-H3 is the most important domain which contribute to the diversity of the antibodies (Figure 48). In this study, the length of CDR-H3 is 4 amino acids (see Figure 20 in results) and 8 amino acids (see Figure 30 in results), they are relative short. An extended CDR-H3 is not commonly present in V_H of human and mouse (Sanz, 1991; Wu et al., 1992), but it is frequently found in bovine V_H (Saini et al., 1997 and O'Brien et al., 1999). It would be interesting to determine if the longer CDR-H3 influences the affinity of the scFv antibody.

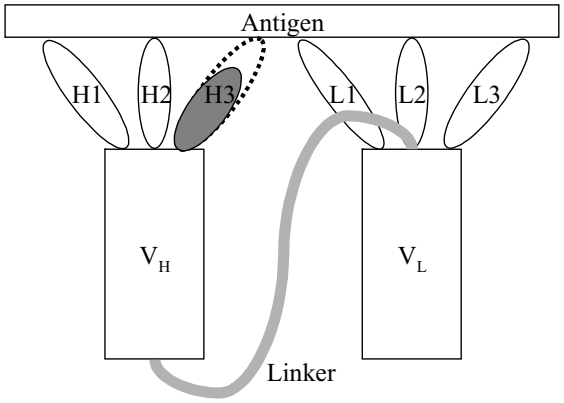

Figure 48. The CDRs of the scFv. The CDRs in the V_H are H1 to H3, likewise, the CDRs in the V_L are L1 to L3. CDR-H3 is more variable than others, its length could be too short to contribute to the binding to the antigen as the dark globule demonstrated; its length also can be as long as 25 amino acids which will be the longest and the most important one in CDRs as the dotlined globule demostrated.

4.2.9. The human Tau-specific scFv-Tau13-24

ScFvTau13-24 is a very interesting antibody because its epitope is a unique human Tau sequence which is not present in other types of Tau protein such as mouse Tau (Lee et al., 1988; Kampers et.al., 1999). It is similar to the human-Tau-specific HT7 monoclonal antibody whose epitope is ^{159}**PPGQK**^{164} (Mercken et al., 1992; Vanmechelen et al., 2000), which is also an unique sequence in human Tau (Kampers et al., 1999). Therefore it could be used to identify the human Tau in the case of human Tau transformed mouse cell lines or human Tau transgenic mice. Actually, we tested this scFv antibody by ELISA and Western blotting, it recognized human Tau specifically and did not crossreact with the mouse Tau.

4.2.10. Analysis of Tau in Alzheimer brain or in eucaryotic cells

The scFv-TauS235p antibody recognized the Alzheimer Tau but not the normal Tau, so in principle, it can be used to distinguish Alzheimer Tau from normal Tau, for instance, in the cerebrospinal fluid (CSF). But the test depends on whether Tau is phosphorylated in the CSF of AD-patient, thus it has not been shown stringently. It maybe a useful antibody, since at the moment, it is the only antibody available, which recognizes phosphorylated Ser235.

The scFv-TauS320p/S324p recognizes the phosphorylated Tau epitope in the third repeat of Tau (see Table 12). By Western blotting, we can distinguish the AD-brain-HTau from the control-brain-HTau.

Immunofluoresence (IF) is not as sensitive as Western blotting. Here we showed that scFv-TauS235p worked by IF to detect Tau in mitotic CHO cells. It shows that a recombinant antibody can be a useful tool for detection of antigens in cells.

4.3. Tetramerization of the scFv antibody

4.3.1. Construction of the vector for the tetravalent scFv antibody.

The expression-cassette has multi-cloning sites, it contains the myc-tag and the His-tag which are useful for the detection and the purification. It allows the insertion of the scFv antibody via SfiI and NotI restriction sites and the insertion of the hinge/p53 fragment via NotI and FseI restriction sites. It allows the insertion of V_H (via NcoI and ApaLI restriction sites) and V_L (via ApaLI and NotI restriction sites) separately which will lead to recombination of V_H from one scFv antibody and V_L from another scFv antibody (see Figure 49).

There are two ways to construct the tetravalent scFv. One way is first to insert scFv and then insert the hinge/p53 fragment; the other is first to insert the hinge/p53 fragment and then insert scFv (Figure 49). In this study, both methods worked.

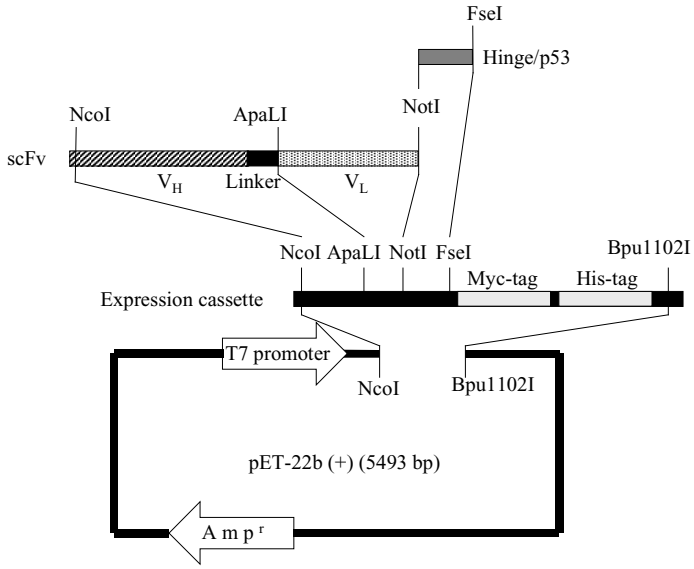

Figure 49. The vector construction for the tetravalent antibody. The starting source is the pET22b(+) vector. The first step was to insert the expression cassette into the pET22b(+) vector at the NcoI and Bpu1102I sites; the second step is to insert the scFv gene into the vector at the NcoI and NotI sites; the third step is to insert the hinge/p53 sequence behind the scFv gene at the NotI and FseI sites.

The hinge/p53 sequence is the oligomerization domain of human p53 (Jeffrey et al., 1995; Plückthun and Pack, 1997). It drive the scFv monomer to form an scFv tetramer: the monomer, which is fused to a beta strand and an alpha helix from the hinge/p53, associates with a second monomer across an antiparallel beta sheet and an antiparallel helix-helix

interface to form a dimer. Two of these dimers associate across a second and distinct parallel helix-helix interface to form the tetramer. The tetravalent scFv antibody has a spider-like shape, in which the binding sites point into the directions of a tetrahedron and can therefore easily adapt to distant antigens (see Figure 11 in introduction).

Another tetramerizing polypeptide which consist of the interaction between hydrophobic domains has been described by Harbury et al. (Harbury et al., 1993). Alternatively, the fusion of scFv fragments to streptavidin, a tetramer , has been investigated too (Dübel et al., 1995; Kipriyanov et al., 1996).

4.3.2. Expression and purification of the tetravalent antibody

Since expression is optimized, and the amount of expressed scFv antibody is very high, the soluble scFv antibody aggregates in the periplasma space (Field et al., 1990; Plückthun, 1992). Furthermore, some antibody fragments can not be expressed in periplasma, not even as insoluble fractions (Tsumoto et al., 1995). An alternative approach to obtaining the scFv antibody from *E. coli* is to produce proteins as cytoplasmic inclusion bodies and then refold the proteins in vitro (Buchner and Rudolph, 1991; Martineau et al., 1998; Tsumoto et al., 1998). In this study, urea was used to solublized scFv in order to purify the antibody by the Ni-NTA-agarose chromatography. After elution of the bound scFv, urea was removed by slow dialysis to allow the scFv antibody to refold (Sakurai et al., 1999). About 60-80% of the antibodies are active after this treatment.

Under the denaturing conditions of the SDS-PAGE, the modified scFv monomer, which was expressed from the tetravalent system, showed a higher molecular weight (around 36KD) than the previous monovalent scFv (around 30KD, see Figure 37 in results). The higher molecular weight of modified scFv monomer is due to the hinge/p53 fragment which is around 7KD (see Figure 36 in results).

4.3.3. Functional affinity of the tetravalent scFv antibody

The functional affinity of the scFv antibody improvement is very significant. In some cases, the monomer scFv shows no ELISA signal, but tetravalent scFv show a clear ELISA signal

against the antigen. The improvement was not accurately quantified, we estimate an improvement of 10−fold (see Table 14. in results), this estimation is similar as the data in other labs (Adams et al., 1998; Alt et al., 1999). The gain in functional affinity is dependent on correct alignment and orientation in the Fv modules of tetramer, otherwise gains in functional affinity are likely to be small (Hudson, 1999).

Two reasons are responsible for the improvement of functional affinity, elevating the affinity of the monomer or diminishing the off rate of the monomer (Mersmann et al., 1998). Therefore improved K_{on} or decreased K_{off}. will gain higher functional affinity for the tetravalent antibody. In principle, the decreased K_{off} is the main reason for the functional affinity improvement (Griffiths et al.,1993; Kipriyanov et al., 1996), but there are also examples published where tetramerization of scFv resulted in an increased K_{on} (Korrt et al., 1997).

4.4. The scFv antibody against MARK

4.4.1. Selection of the phages

During the selection by immunotube, phages were enriched and specific ELISA signals increased rapidly (see Figure 38 in results). Selected phages were not only against MARK1, but also MARK2 and MARK3. Since MARK isoforms have similar sequences, it is clear that even if we selected phages by MARK1, they could cross-react with other MARK isoforms. If MARK1 specific scFv are desired, there are in principle two ways: removing scFv-Pan-MARK antibody from a pre-selected library, or select phages by a MARK1 specific peptide. Since short peptides are not useful for selection due to the steric-hindrance, and long peptides still have the Pan-MARK domains, we selected MARK1 specific phages by the competition method in order to remove the phages against the common sequence of the MARK family.

Competition selection resulted in a different MARK1-spot-membrane pattern where phages against the common sequences of all MARK isoforms were reduced (see Figure 40 in results). By screening the MARK1-specific phage population, we obtained a clone that was specific for MARK1. This screening is not complicated and does not consume too much time and material. Although the competition could not remove all MARK unspecific phages completely, MARK1 specific phages were enriched enough.

4.4.2. Screening of phages

Since the phage library contained MARK1 specific and Pan-MARK phages, the screening strategy was more complicated. In the same screening, MARK1 and MARK2 coated plates were used for the same phage. In principle, we could get two kinds of phages: the phages that were specific for MARK1 but not MARK2; the phages that were against MARK1 and MARK2, so-called Pan-MARK phages. In practice, from 100 clones, 20% were positive, but none were MARK1-specific. It means that the phages against the Pan-MARK regions were the main population, phages which were against MARK1-specific domain were just a minor part of the phage population, they could be obtained after two rounds of competition selection and screening. This case is quite similar to the case of Tau40 phosphorylation-independent antibodies (see 4.2.4.).

4.4.3. Expression, purification and characterization of the scFv

The transfection of *E.coli* HB2151 cells sometimes leads to a loss of a positive ELISA signal. The same was true for scFv against MARK. For this reason we directly switched phages to the tetravalent scFv system, skipping the step of the transfection of HB2151.

The tetravalent scFv was purified under the denaturing conditions which involved urea, and was refolded by the slow dialysis.

The MARK1-spot-membrane showed similar good results as the Tau40-spot-membrane, and therefore the spot-membrane is a useful method to identify the antibody epitope. Some scFv antibodies from the Griffin/MARK1 phage library showed discontinuous epitopes, this could be explained that MARK is a globular folded protein compared with Tau which has nearly no structure, some sequences may get close to each other in the three dimensional structure although they are far away in the primary amino acid sequence. Even for Tau, as we have known, the Alz50 monoclonal antibody showed also the discontinuous epitope (Carmel et al., 1996).

4.4.4. Detection of MARK2 in the eucaryotic cells by the tetravalent scFv

The monomeric scFv does not have sufficiently high functional affinity, so it is not useful for Western blotting. The Tetravalent scFv were able to detect MARK by Western blotting. ScFv-MARK1-535-549 antibody was very specific to MARK1, it did not recognize other MARK isoforms. The scFv-PanMARK antibody did just recognize MARK isoforms but there were no cross-reaction in Western blotting with other enzymes such as PKA, cdk5 and so on.

MARK2 transfected into CHO cells has a HA-tag so that it can be detected by Anti-HA tag polyclonal antibodies. The tetravalent scFv antibody shows a similar pattern as Anti-HA tag polyclonal antibody against MARK2, which means that this scFv antibody also works in immunofluorescence (IF) although in IF it has a lower sensitivity as in Western blotting. But the tetravalent scFv antibody does not have high enough functional affinity to detect endogenous widespread MARK by Western blotting or IF.

4.5. Perspective of this study

In this study, I have established the method to generate recombinant antibody by phage display technique. I got five antibodies against Tau protein (including phosphorylated Tau protein) and two antibodies against MARK kinases, which were tested by ELISA, Western blotting and Immunofluorescence. Improvement of the system in the future is possible:

1. The higher affinity of the scFv antibodies could be achieved by DNA shuffling (Crameri et al., 1996; Ryn and Nam, 2000).
2. The selection approach could be improved such as using ribosome display techniques (Hanes and Plückthun, 1997; Hanes et al., 1998; Schaffitzel et al., 1999;).
3. The expression of scFv antibody still needs to be optimized by changing *E. coli* growth conditions (Horn et al., 1996; Kipriyanov et al., 1997; Mörbe J. L. and Riesenberg D., 1997; Kujau et al., 1998).
4. In order to avoid the urea extraction in the purification of tetravalent scFv, which leads to the loss of antibody activities, the cell-free translation system could be employed like Ryabova et. al. did (Ryabova et al., 1997).

5. In order to simplify the detection of the scFv antibody through the myc-tag or E-tag, a bispecific antibody fused with the marker enzymes such as ß-glactosidase and phosphatase should be developed (Kontermann et al., 1997; Lindner et al., 1997).

The recombinant antibodies generated were useful to analyze the Alzheimer Tau from Alzheimer's disease patient by Western blotting. They were also used to analyze, by immunofluorescence, Tau and MARK in CHO cells transfected with the proteins. Since the scFv antibody is also suitable for ELISA (Randolf et al., 1997), after the functional affinity of the antibody was improved by tetramerization, the possibility is opened to develop a more sensitive ELISA assay for Tau analysis in the cerebrospinal fluid (CSF), in particular, this ELISA should focus on the special forms of Tau such as the phosphorylated Tau at specific sites (Hoffmann et al., 1997), the truncated Tau fragments (Johnson et al., 1997) and special Alzheimer Tau conformations.

For a highly specific ELISA, we need to generate two types of scFv antibodies. One high affinity antibody to capture the Tau from CSF. A good candidate would be a scFv antibody recognizing the Tau sites which can not be phosphorylated (see Figure 23 in results). The other high affinity antibodies which are needed, are for detection of Alzheimer Tau, so they have to be specific for Alzheimer typical phosphorylation sites of Tau or for an Alzheimer typical conformation of Tau.

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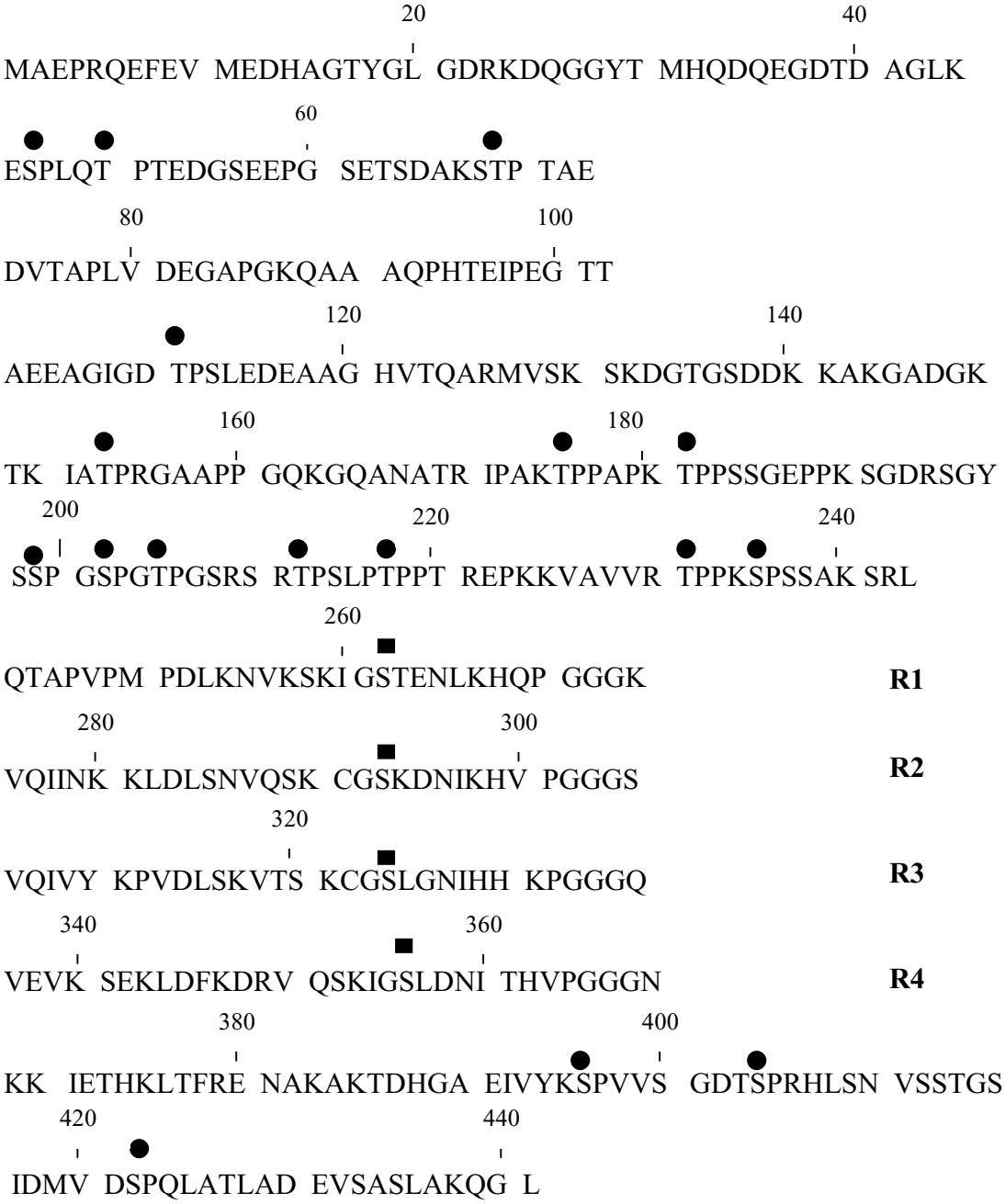
Appendices

I. Abbreviations

AD	Alzheimer's disease
AP	Amyloid plaque
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
bp	Base pairs
BSA	Bovine serum albumin
CCD	Charged couple device
CDR	Complementarity determining region
CSF	Cerebrospinal fluid
DMSO	Dimethylsulfoxide
ECL	Enhanced chemilluminscence
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
IF	Immunofluoresence
IPTG-b	Isopropyl-ß, D-thiogalacto-pyranoside
FAD	Familial Alzheimer's disease
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate (ITC)
HRP	Horseradish-peroxidase
KD	Kilo dalton
MAP	Microtubule associated protein
MARK	Microtubule affinity regulating kinase
ß-ME	ß–Mercaptoethanol
mRNA	Messenger RNA
NFT	Neuron fibrillary tangle
PEG	Polyethylene glycol
PHF	Paired helical filament
PS1	Presenilin 1
PS2	Presenilin 2
PVDF	Polyvinyldifluoride
scFv	Single chain fragment variant
RT-PCR	Reverse transcription-Polymerase chain reaction
rpm	Round per minute
PAGE	Polyacrylamide gel electrophoresis

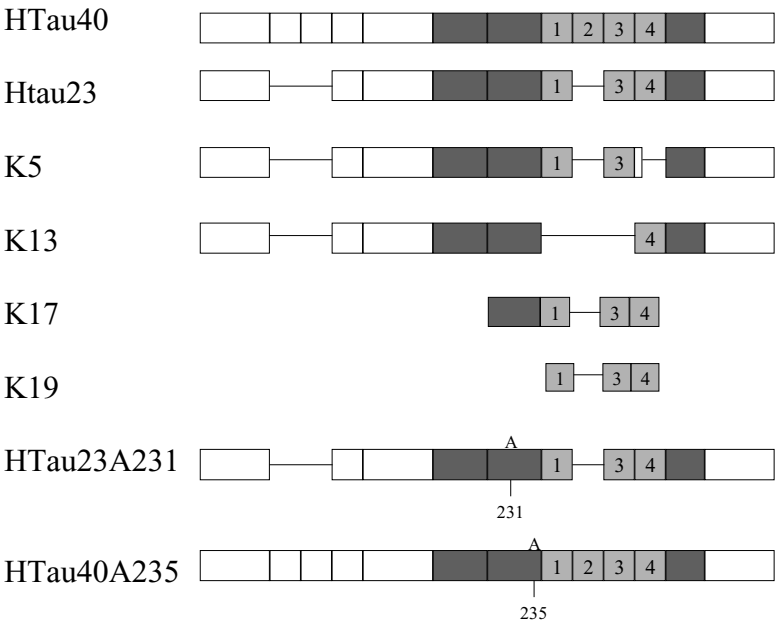
II. Amino acid sequence of Human Tau40



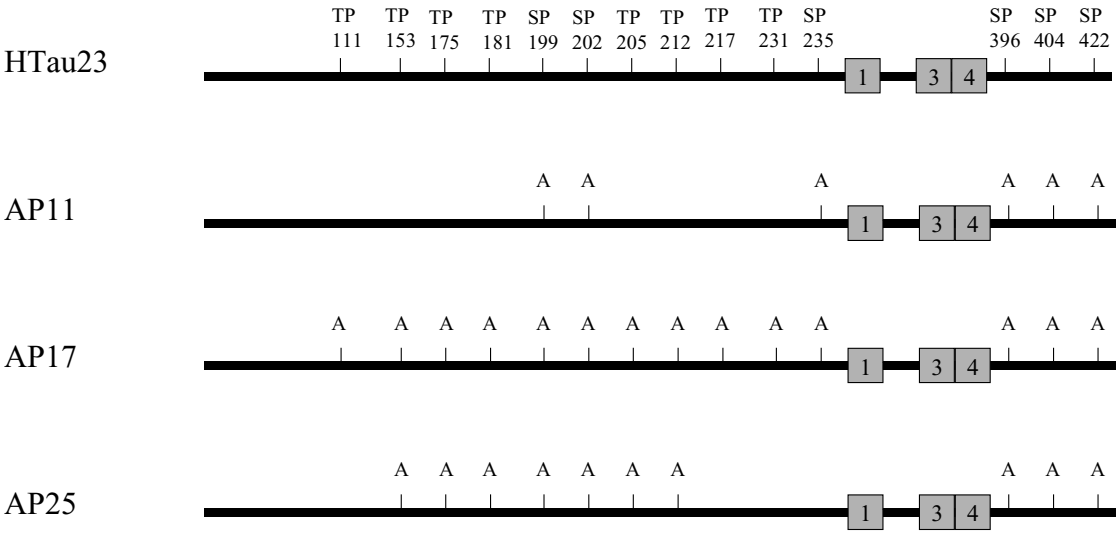
The R1 to R4 represent the repeat sequences. The dark points mark the Ser-Pro and Thr-Pro sites which could be phosphorylated by GSK, CDK and MAPK. The dark blocks mark the KXGS motives in the repeats which could be phosphorylated by MARK.

III. Constructs and mutants of Huamn Tau40

Constructs (K means the construct):



Mutants (AP means SP or TP sites are mutated to Alanine-Proline sites):



IV. Peptide sequences on Tau40-spot-membranes

In the following sequence, the sequence of the A1 spot (the first spot in the line A) is at the beginning of the N-terminal of Tau. The peptide sequence moves every three amino acids towards the C-terminal of Tau.

Lines	Spots	Sequences
A	1	1 MAEPRQEFEVMEDHA 15
	2	4 PRQEFEVMEDHAGTY 18
	3	7 EFEVMEDHAGTYGLG 21
	4	10 VMEDHAGTYGLGDRK 24
	5	13 DHAGTYGLGDRKDQG 27
	6	16 GTYGLGDRKDQGGYT 30
	7	19 GLGDRKDQGGYTMHQ 33
	8	22 DRKDQGGYTMHQDQE 36
	9	25 DQGGYTMHQDQEMHQ 39
	10	28 GYTMHQDQEMHQMHQ 42
	11	31 MHQDQEGDTDAGLKE 45
	12	34 DQEGDTDAGLKESPL 48
	13	37 GDTDAGLKESPLQTP 51
	14	40 DAGLKESPLQTPTED 54
	15	43 LKESPLQTPTEDGSE 57
	16	46 SPLQTPTEDGSEEPG 60
	17	49 QTPTEDGSEEPGSET 63
	18	52 TEDGSEEPGSETSDA 66
	19	55 GSEEPGSETSDAKST 69
	20	58 EPGSETSDAKSTPTA 72
	21	61 SETSDAKSTPTAEDV 75
	22	64 SDAKSTPTAEDVTAP 78
	23	67 KSTPTAEDVTAPLVD 81
	24	70 PTAEDVTAPLVDEGA 84
	25	73 EDVTAPLVDEGAPGK 87

Lines	Spots	Sequences
B	1	76 TAPLVDEGAPGKQAA 90
	2	79 LVDEGAPGKQAAAQP 93
	3	82 EGAPGKQAAAQPHTE 96
	4	85 PGKQAAAQPHTEIPE 99
	5	88 QAAAQPHTEIPEGTT 102
	6	91 AQPHTEIPEGTTAEE 105
	7	94 HTEIPEGTTAEEAGI 108
	8	97 IPEGTTAEEAGIGDT 111
	9	100 GTTAEEAGIGDTPSL 114
	10	103 AEEAGIGDTPSLEDE 117
	11	106 AGIGDTPSLEDEAAG 120
	12	109 GDTPSLEDEAAGHVT 123
	13	112 PSLEDEAAGHVTQAR 126
	14	115 EDEAAGHVTQARMVS 129
	15	118 AAGHVTQARMVSKSK 132
	16	121 HVTQARMVSKSKDGT 135
	17	124 QARMVSKSKDGTGSD 138
	18	127 MVSKSKDGTGSDDKK 141
	19	130 KSKDGTGSDDKKAKG 144
	20	133 DGTGSDDKKAKGADG 147
	21	136 GSDDKKAKGADGKTK 150
	22	139 DKKAKGADGKTKIAT 153
	23	142 AKGADGKTKIATPRG 156
	24	145 ADGKTKIATPRGAAP 159
	25	148 KTKIATPRGAAPPGQ 162

C	1	151	IATPRGAAPPGQKGQ	165
	2	154	PRGAAPPGQKGQANA	168
	3	157	AAPPGQKGQANATRI	171
	4	160	PGQKGQANATRIPAK	174
	5	163	KGQANATRIPAKTPP	177
	6	166	ANATRIPAKTPPAPK	180
	7	169	TRIPAKTPPAPKTPP	183
	8	172	PAKTPPAPKTPPSSG	186
	9	175	TPPAPKTPPSSGEPP	189
	10	178	APKTPPSSGEPPKSG	192
	11	181	TPPSSGEPPKSGDRS	195
	12	184	SSGEPPKSGDRSGYS	198
	13	187	EPPKSGDRSGYSSPG	201
	14	190	KSGDRSGYSSPGSPG	204
	15	193	DRSGYSSPGSPGTPG	207
	16	196	GYSSPGSPGTPGSRS	210
	17	199	SPGSPGTPGSRSRTP	213
	18	202	SPGTPGSRSRTPSLP	216
	19	205	TPGSRSRTPSLPTPP	219
	20	208	SRSRTPSLPTPPTRE	222
	21	211	RTPSLPTPPTREPKK	225
	22	214	SLPTPPTREPKKVAV	228
	23	217	TPPTREPKKVAVVRT	231
	24	220	TREPKKVAVVRTPPK	234
	25	223	PKKVAVVRTPPKSPS	237

D	1	225	VAVVRTPPKSPSSAK	240
	2	228	VRTPPKSPSSAKSRL	243
	3	231	PPKSPSSAKSRLQTA	246
	4	234	SPSSAKSRLQTAPVP	249
	5	237	SAKSRLQTAPVPMPD	252
	6	240	SRLQTAPVPMPDLKN	255
	7	243	QTAPVPMPDLKNVKS	258
	8	246	PVPMPDLKNVKSKIG	261
	9	249	MPDLKNVKSKIGSTE	264
	10	252	LKNVKSKIGSTENLK	267
	11	255	VKSKIGSTENLKHQP	270
	12	258	KIGSTENLKHQPGGG	273
	13	261	STENLKHQPGGGKVQ	276
	14	264	NLKHQPGGGKVQIIN	279
	15	267	HQPGGGKVQIINKKL	282
	16	270	GGGKVQIINKKLDLS	285
	17	273	KVQIINKKLDLSNVQ	288
	18	276	IINKKLDLSNVQSKC	291
	19	279	KKLDLSNVQSKCGSK	294
	20	282	DLSNVQSKCGSKDNI	297
	21	285	NVQSKCGSKDNIKHV	300
	22	288	SKCGSKDNIKHVPGG	303
	23	291	GSKDNIKHVPGGGSV	306
	24	294	DNIKHVPGGGSVQIV	309
	25	297	KHVPGGGSVQIVYKP	312

E	1	300 PGGGSVQIVYKPVDL 315
	2	303 GSVQIVYKPVDLSKV 318
	3	306 QIVYKPVDLSKVTSK 321
	4	309 YKPVDLSKVTSKCGS 324
	5	312 VDLSKVTSKCGSLGN 327
	6	315 SKVTSKCGSLGNIHH 330
	7	318 TSKCGSLGNIHHKPG 333
	8	321 CGSLGNIHHKPGGGQ 336
	9	324 LGNIHHKPGGGQVEV 339
	10	327 IHHKPGGGQVEVKSE 342
	11	330 KPGGGQVEVKSEKLD 345
	12	333 GGQVEVKSEKLDFKD 348
	13	336 VEVKSEKLDFKDRVQ 351
	14	339 KSEKLDFKDRVQSKI 354
	15	342 KLDFKDRVQSKIGSL 357
	16	345 FKDRVQSKIGSLDNI 360
	17	348 RVQSKIGSLDNITHV 363
	18	351 SKIGSLDNITHVPGG 366
	19	354 GSLDNITHVPGGGNK 369
	20	357 DNITHVPGGGNKKIE 372
	21	360 THVPGGGNKKIETHK 375
	22	363 PGGGNKKIETHKLTF 378
	23	366 GNKKIETHKLTFREN 381
	24	369 KIETHKLTFRENAKA 384
	25	372 THKLTFRENAKAKTD 387

F	1	375 LTFRENAKAKTDHGA 390
	2	378 RENAKAKTDHGAEIV 393
	3	381 AKAKTDHGAEIVYKS 396
	4	384 KTDHGAEIVYKSPVV 399
	5	387 HGAEIVYKSPVVSGD 402
	6	390 EIVYKSPVVSGDTSP 405
	7	393 YKSPVVSGDTSPRHL 408
	8	396 PVVSGDTSPRHLSNV 411
	9	399 SGDTSPRHLSNVSST 414
	10	402 TSPRHLSNVSSTGSI 417
	11	405 RHLSNVSSTGSIDMV 420
	12	408 SNVSSTGSIDMVDSP 423
	13	411 SSTGSIDMVDSPQLA 426
	14	414 GSIDMVDSPQLATLA 429
	15	417 DMVDSPQLATLADEV 432
	16	420 DSPQLATLADEVSAS 435
	17	423 QLATLADEVSASLAK 438
	18	426 TLADEVSASLAKQGL 441

V. Peptide sequences on MARK1-spot-membran

In the following sequence, the sequence of the A1 spot (the first spot in the line A) is at the beginning of the N-terminal of MARK1. The peptide sequence moves every three amino acids towards the C-terminal of MARK1.

Lines	Spots	Sequences
A	1	1 MSARTPLPTVNERDT 15
	2	4 RTPLPTVNERDTENH 18
	3	7 LPTVNERDTENHTSV 21
	4	10 VNERDTENHTSVDGY 24
	5	13 RDTENHTSVDGYTET 27
	6	16 ENHTSVDGYTETHIP 30
	7	19 TSVDGYTETHIPPTK 33
	8	22 DGYTETHIPPTKSSS 36
	9	25 TETHIPPTKSSSRQN 39
	10	28 HIPPTKSSSRQNIPR 42
	11	31 PTKSSSRQNIPRCRN 45
	12	34 SSSRQNIPRCRNSIT 48
	13	37 RQNIPRCRNSITSAT 51
	14	40 IPRCRNSITSATDEQ 54
	15	43 CRNSITSATDEQPHI 57
	16	46 SITSATDEQPHIGNY 60
	17	49 SATDEQPHIGNYRLQ 63
	18	52 DEQPHIGNYRLQKTI 66
	19	55 PHIGNYRLQKTIGKG 69
	20	58 GNYRLQKTIGKGNFA 72
	21	61 RLQKTIGKGNFAKVK 75
	22	64 KTIGKGNFAKVKLAR 78
	23	67 GKGNFAKVKLARHVL 81
	24	70 NFAKVKLARHVLTGR 84
	25	73 KVKLARHVLTGREVA 87

Lines	Spots	Sequences
B	1	76 LARHVLTGREVAVKI 90
	2	79 HVLTGREVAVKIIDK 93
	3	82 TGREVAVKIIDKTQL 96
	4	85 EVAVKIIDKTQLNPT 99
	5	88 VKIIDKTQLNPTSLQ 102
	6	91 IDKTQLNPTSLQKLF 105
	7	94 TQLNPTSLQKLFREV 108
	8	97 NPTSLQKLFREVRIM 111
	9	100 SLQKLFREVRIMKIL 114
	10	103 KLFREVRIMKILNHP 117
	11	106 REVRIMKILNHPNIV 120
	12	109 RIMKILNHPNIVKLF 123
	13	112 KILNHPNIVKLFEVI 126
	14	115 NHPNIVKLFEVIETE 129
	15	118 NIVKLFEVIETEKTL 132
	16	121 KLFEVIETEKTLYLV 135
	17	124 EVIETEKTLYLVMEY 138
	18	127 ETEKTLYLVMEYASG 141
	19	130 KTLYLVMEYASGGEV 144
	20	133 YLVMEYASGGEVFDY 147
	21	136 MEYASGGEVFDYLVA 150
	22	139 ASGGEVFDYLVAHGR 153
	23	142 GEVFDYLVAHGRMKE 156
	24	145 FDYLVAHGRMKEKEA 159
	25	148 LVAHGRMKEKEARAK 162

C	1	151	HGRMKEKEARAKFRQ	165
	2	154	MKEKEARAKFRQIVS	168
	3	157	KEARAKFRQIVSAVQ	171
	4	160	RAKFRQIVSAVQYCH	174
	5	163	FRQIVSAVQYCHQKC	177
	6	166	IVSAVQYCHQKCIVH	180
	7	169	AVQYCHQKCIVHRDL	183
	8	172	YCHQKCIVHRDLKAE	186
	9	175	QKCIVHRDLKAENLL	189
	10	178	IVHRDLKAENLLLDA	192
	11	181	RDLKAENLLLDADMN	195
	12	184	KAENLLLDADMNIKI	198
	13	187	NLLLDADMNIKIADF	201
	14	190	LDADMNIKIADFGFS	204
	15	193	DMNIKIADFGFSNEF	207
	16	196	IKIADFGFSNEFTVG	210
	17	199	ADFGFSNEFTVGNKL	213
	18	202	GFSNEFTVGNKLDTF	216
	19	205	NEFTVGNKLDTFCGS	219
	20	208	TVGNKLDTFCGSPPY	222
	21	211	NKLDTFCGSPPYAAP	225
	22	214	DTFCGSPPYAAPELF	228
	23	217	CGSPPYAAPELFQGK	231
	24	220	PPYAAPELFQGKKYD	234
	25	223	AAPELFQGKKYDGPE	237

D	1	226	ELFQGKKYDGPEVDV	240
	2	229	QGKKYDGPEVDVWSL	243
	3	232	KYDGPEVDVWSLGVI	246
	4	235	GPEVDVWSLGVILYT	249
	5	238	VDVWSLGVILYTLVS	252
	6	241	WSLGVILYTLVSGSL	255
	7	244	GVILYTLVSGSLPFD	258
	8	247	LYTLVSGSLPFDGQN	261
	9	250	LVSGSLPFDGQNLKE	264
	10	253	GSLPFDGQNLKELRE	267
	11	256	PFDGQNLKELRERVL	270
	12	259	GQNLKELRERVLRGK	273
	13	262	LKELRERVLRGKYRV	276
	14	265	LRERVLRGKYRVPFY	279
	15	268	RVLRGKYRVPFYMST	282
	16	271	RGKYRVPFYMSTDCE	285
	17	274	YRVPFYMSTDCENLL	288
	18	277	PFYMSTDCENLLKKL	291
	19	280	MSTDCENLLKKLLVL	294
	20	283	DCENLLKKLLVLNPI	297
	21	286	NLLKKLLVLNPIKRG	300
	22	289	KKLLVLNPIKRGSLE	303
	23	292	LVLNPIKRGSLEQIM	306
	24	295	NPIKRGSLEQIMKDR	309
	25	298	KRGSLEQIMKDRWMN	312

E	1	301	SLEQIMKDRWMNVGH	315
	2	304	QIMKDRWMNVGHEEE	318
	3	307	KDRWMNVGHEEEELK	321
	4	310	WMNVGHEEEELKPYS	324
	5	313	VGHEEEELKPYSEPE	327
	6	316	EEEELKPYSEPELDL	330
	7	319	ELKPYSEPELDLNDA	333
	8	322	PYSEPELDLNDAKRI	336
	9	325	EPELDLNDAKRIDIM	339
	10	328	LDLNDAKRIDIMVTM	342
	11	331	NDAKRIDIMVTMGFA	345
	12	334	KRIDIMVTMGFARDE	348
	13	337	DIMVTMGFARDEIND	351
	14	340	VTMGFARDEINDALV	354
	15	343	GFARDEINDALVSQK	357
	16	346	RDEINDALVSQKYDE	360
	17	349	INDALVSQKYDEVMA	363
	18	352	ALVSQKYDEVMATYI	366
	19	355	SQKYDEVMATYILLG	369
	20	358	YDEVMATYILLGRKP	372
	21	361	VMATYILLGRKPPEF	375
	22	364	TYILLGRKPPEFEGG	378
	23	367	LLGRKPPEFEGGESL	381
	24	370	RKPPEFEGGESLSSG	384
	25	373	PEFEGGESLSSGNLC	387

F	1	376	EGGESLSSGNLCQRS	390
	2	379	ESLSSGNLCQRSRPS	393
	3	382	SSGNLCQRSRPSSDL	396
	4	385	NLCQRSRPSSDLNNS	399
	5	388	QRSRPSSDLNNSTLQ	402
	6	391	RPSSDLNNSTLQSPA	405
	7	394	SDLNNSTLQSPAHLK	408
	8	397	NNSTLQSPAHLKVQR	411
	9	400	TLQSPAHLKVQRSIS	414
	10	403	SPAHLKVQRSISANQ	417
	11	406	HLKVQRSISANQKQR	420
	12	409	VQRSISANQKQRRFS	423
	13	412	SISANQKQRRFSDHA	426
	14	415	ANQKQRRFSDHAGPS	429
	15	418	KQRRFSDHAGPSIPP	432
	16	421	RFSDHAGPSIPPAVS	435
	17	424	DHAGPSIPPAVSYTK	438
	18	427	GPSIPPAVSYTKRPQ	441
	19	430	IPPAVSYTKRPQANS	444
	20	433	AVSYTKRPQANSVES	447
	21	436	YTKRPQANSVESEQK	450
	22	439	RPQANSVESEQKEEW	453
	23	442	ANSVESEQKEEWDKD	456
	24	445	VESEQKEEWDKDTAR	459
	25	448	EQKEEWDKDTARRLG	462

G	1	451 EEWDKDTARRLGSTT 465
	2	454 DKDTARRLGSTTVGS 468
	3	457 TARRLGSTTVGSKSE 471
	4	460 RLGSTTVGSKSEVTA 474
	5	463 STTVGSKSEVTASPL 477
	6	466 VGSKSEVTASPLVGP 480
	7	469 KSEVTASPLVGPDRK 483
	8	472 VTASPLVGPDRKKSS 486
	9	475 SPLVGPDRKKSSAGP 489
	10	478 VGPDRKKSSAGPSNN 492
	11	481 DRKKSSAGPSNNVYS 495
	12	484 KSSAGPSNNVYSGGS 498
	13	487 AGPSNNVYSGGSMTR 501
	14	490 SNNVYSGGSMTRRNT 504
	15	493 VYSGGSMTRRNTYVC 507
	16	496 GGSMTRRNTYVCERS 510
	17	499 MTRRNTYVCERSTDR 513
	18	502 RNTYVCERSTDRYAA 516
	19	505 YVCERSTDRYAALQN 519
	20	508 ERSTDRYAALQNGRD 522
	21	511 TDRYAALQNGRDSSL 525
	22	514 YAALQNGRDSSLTEM 528
	23	517 LQNGRDSSLTEMSAS 531
	24	520 GRDSSLTEMSASSMS 534
	25	523 SSLTEMSASSMSSTG 537

H	1	526 TEMSASSMSSTGSTV 540
	2	529 SASSMSSTGSTVASA 543
	3	532 SMSSTGSTVASAGPS 546
	4	535 STGSTVASAGPSARP 549
	5	538 STVASAGPSARPRHQ 552
	6	541 ASAGPSARPRHQKSM 555
	7	544 GPSARPRHQKSMSTS 558
	8	547 ARPRHQKSMSTSGHP 561
	9	550 RHQKSMSTSGHPIKV 564
	10	553 KSMSTSGHPIKVTLP 567
	11	556 STSGHPIKVTLPTIK 570
	12	559 GHPIKVTLPTIKDGS 573
	13	562 IKVTLPTIKDGSEAY 576
	14	565 TLPTIKDGSEAYRPG 579
	15	568 TIKDGSEAYRPGTAQ 582
	16	571 DGSEAYRPGTAQRVP 585
	17	574 EAYRPGTAQRVPAAS 588
	18	577 RPGTAQRVPAASPSA 591
	19	580 TAQRVPAASPSAHSI 594
	20	583 RVPAASPSAHSISAS 597
	21	586 AASPSAHSISASTPD 600
	22	589 PSAHSISASTPDRTR 603
	23	592 HSISASTPDRTRFPR 606
	24	595 SASTPDRTRFPRGSS 609
	25	598 TPDRTRFPRGSSSRS 612

I	1	601	RTRFPRGSSSRSTFH	615
	2	604	FPRGSSSRSTFHGEQ	618
	3	607	GSSSRSTFHGEQLRE	621
	4	610	SRSTFHGEQLRERRS	624
	5	613	TFHGEQLRERRSAAY	627
	6	616	GEQLRERRSAAYSGP	630
	7	619	LRERRSAAYSGPPAS	633
	8	622	RRSAAYSGPPASPSH	636
	9	625	AAYSGPPASPSHDTA	639
	10	628	SGPPASPSHDTAALA	642
	11	631	PASPSHDTAALAHAR	645
	12	634	PSHDTAALAHARRGT	648
	13	637	DTAALAHARRGTSTG	651
	14	640	ALAHARRGTSTGIIS	654
	15	643	HARRGTSTGIISKIT	657
	16	646	RGTSTGIISKITSKF	660
	17	649	STGIISKITSKFVRR	663
	18	652	IISKITSKFVRRDPS	666
	19	655	KITSKFVRRDPSEGE	669
	20	658	SKFVRRDPSEGEASG	672
	21	661	VRRDPSEGEASGRTD	675
	22	664	DPSEGEASGRTDTAR	678
	23	667	EGEASGRTDTARGSS	681
	24	670	ASGRTDTARGSSGEP	684
	25	673	RTDTARGSSGEPKDK	687

J	1	676	TARGSSGEPKDKEEG	690
	2	679	GSSGEPKDKEEGKEA	693
	3	682	GEPKDKEEGKEAKPR	696
	4	685	KDKEEGKEAKPRSLR	699
	5	688	EEGKEAKPRSLRFTW	702
	6	691	KEAKPRSLRFTWSMK	705
	7	694	KPRSLRFTWSMKTTS	708
	8	697	SLRFTWSMKTTSSMD	711
	9	700	FTWSMKTTSSMDPND	714
	10	703	SMKTTSSMDPNDMVR	717
	11	706	TTSSMDPNDMVREIR	720
	12	709	SMDPNDMVREIRKVL	723
	13	712	PNDMVREIRKVLDAN	726
	14	715	MVREIRKVLDANTCD	729
	15	718	EIRKVLDANTCDYEQ	732
	16	721	KVLDANTCDYEQRER	735
	17	724	DANTCDYEQRERFLL	738
	18	727	TCDYEQRERFLLFCV	741
	19	730	YEQRERFLLFCVHGD	744
	20	733	RERFLLFCVHGDARQ	747
	21	736	FLLFCVHGDARQDSL	750
	22	739	FCVHGDARQDSLVQW	753
	23	742	HGDARQDSLVQWEME	756
	24	745	ARQDSLVQWEMEVCK	759
	25	748	DSLVQWEMEVCKLPR	762

K	1	751	VQWEMEVCKLPRLSL	765
	2	754	EMEVCKLPRLSLNGV	768
	3	757	VCKLPRLSLNGVRFK	771
	4	760	LPRLSLNGVRFKRIS	774
	5	763	LSLNGVRFKRISGTS	777
	6	766	NGVRFKRISGTSIAF	780

K	7	769	RFKRISGTSIAFKNI	783
	8	772	RISGTSIAFKNIASK	786
	9	775	GTSIAFKNIASKIAN	789
	10	778	IAFKNIASKIANELK	792
	11	781	AFKNIASKIANELKL	795

VI. Nucleotide sequence of scFv-Tau193-204 (C7) clone

GGCCCAGCCGGCC ATGGCCTACGTGCAGCTGCAACAGTCTGGGGCAGAG
SfiI site → V_H

CTTGTGAGGTCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTCTGGCTT

CAATATTAAAGACTACTATATACAGTGGGTGAAGCAGAGGCCTGAACAG

GGCCTGGAGTGGATTGGATGGATTGATCCTGAGAATGGTGATAGTGATTC

TGTCCCGAAGTTCCAGGGCAAGGCCACTATGACTGTAGACACATCCTCCA

ACACAGCCTACCTGCAGCTCAGCAGCCTGACATCTGAGGACACTGCCGTC

TATTACTGTAATAGGGGCTGGGGCTACTGGGGCCAAGGCACCACGGTCA

CCGTCTCCTCA**GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGG**
Linker

TGGCGGATCGGACATCGAGCTCACTCAGTCTCCA CTCACTTTCTTGGT
→ V_L

TACCATTGGACAACCAGCCTCCATCTCTTGCAAGTCAAGTCAGAGCCTCT

TAAATAGTGATGGAAAGACATATTTGAGTTGGTTGTTACAGAGGCCAGG

CCAGTCTCCAAAGCGCCTAATCTATCTGGTCTCTAAACTGGACTCCGGAG

TCCCTGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTCACACTGAAA

ATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTATTGCTGGCAAG

GTACACATTTTCCTCAGACGTTCGGTGGAGGGACCAAGCTGGAAATGAA

ACGTGCGGCCGCAGGTGCGCCGGTGCCGTATCCGGATCCGCTGGAACCG
NotI site E-tag

CGT

VII. Nucleotide sequence of scFv-Tau193-204 (E3) clone

GGCCCAGCCGGCC ATGGCCCACGTGAAGCTGCAACAGTCTGGGGCAGAG
SfiI site → V_H

CTTGTGAGGTCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTCTGGCTT

CAATATTAAAGACTACTATATACAGTGGGTGAAGCAGAGGCCTGAACAG

GGCCTGGAGTGGATTGGATGGATTGATCCTGAGAATGGTGATAGTGATTC

TGTCCCGAAGTTCCAGGGCAAGGCCACTATGACTGTAGACACATCCTCCA

ACACAGCCTACCTGCAGCTCAGCAGCCTGACATCTGAGGACACTGCCGTC

TATTACTGTAATAGGGGCTGGGGCTACTGGGGCCAAGGCACCACGGTCA

CCGTCTCCTCA**GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGG**
Linker

TGGCGGATCGGACATCGAGCTCACTCAGTCTCCA CTCACTTTCTCGGT
→ V_L

TACCATTGGACAACCAGCCTCCATCTCTTGCAAGTCAAGTCAGAGCCTCT

TAAATAGTGATGGAAAGACATATTTGAGTTGGTTGTTACAGAGGCCAGG

CCAGTCTCCAAAGCGCCTAATCTATCTGGTCTCTAAACTGGACTCCGGAG

TCCCTGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTCACACTGAAA

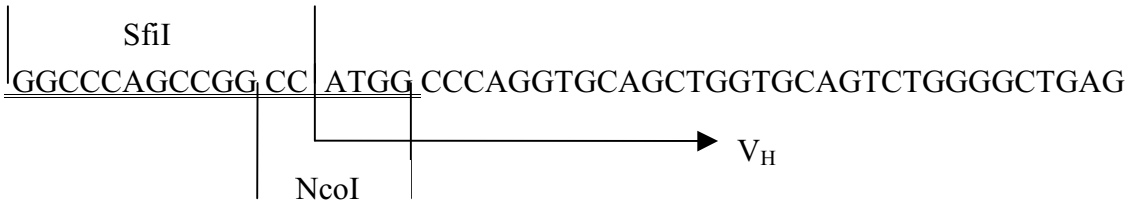
ATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTATTGCTGGCAAG

GTACACATTTTCCTCAGACGTTCGGTGGAGGGACCAATCTGGAACTGAAA

CGGGCGGCCGCAGGTGCGCCGGTGCCGTATCCGGATCCGCTGGAACCGC
NotI site E-tag

GT

VIII. Nucleotide sequence of scFv-TauS235p clone



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GCAGAGAAGTTCCAGGGCAGAGTCACCATAACCGCGGACACGTCTACAGA

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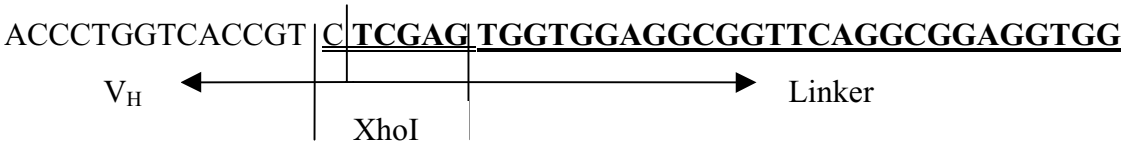

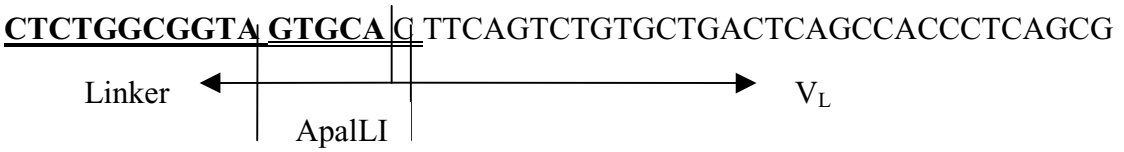

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CAACATCGGAAGTAATTATGTATACTGGTACCAGCAGCTCCCAGGAACGG

CCCCCAAACTCCTCATCTATAGGAATAATCAGCGGCCCTCAGGGGTCCCT

GACCGATTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCAGT

GGGCTCCGGTCCGAGGATGAGGCTGATTATTACTGTGCAGCATGGGATGA

CAGCCTGCCTTCTTTTGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAG

GTGCGGCCGCACATCATCATCATCATCACGGGGCCGCAGAACAAAAACTC

NotI 6x His-tag Myc-tag

ATCTCAGAAGAGGATCTGAAT

IX. List of scFv antibodies against Tau and MARK

ScFv antibody	Epitope	Characteristic
ScFv-Tau193-204	Tau, ^{193}DRSGYSSPGSPG^{204}	1: Phosphorylation-independent 2: Same epitope as Tau-1 monoclonal antibody
ScFv-Tau349-360	Tau, ^{349}RVQSKIGSLDNI^{360}	Phosphorylation-independent
ScFv-Tau13-24	Tau, ^{13}DHAGTYGLGDRK^{24}	1:phosphorylation-independent 2: Specific against the human Tau rather than the mouse Tau
ScFv-TauS235p	Tau, phosphorylated ^{235}Ser	1: Phosphorylation-dependent 2: Similar epitope as AT180 monoclonal antibody
ScFv-TauS320p/S324p	Tau, ^{316}SKVTS(p)KCGS(p)LGNI^{328}	Double phosphorylation-dependent
ScFv-PanMARK592-603	MARK, ^{592}HSISASTPDRTRFPR^{603}	1: Against all MARK isoforms 2: Not against other kinases
ScFv-MARK1-535-549	MARK, ^{535}STGSTVASAGPSARP^{549}	Specific against MARK1

X. Liste für Gefahrenmerkmale und Sicherheitsratschläge

Chemikalien	Gefahrensymbole	sicherheitsratschläge
Acrylamide	R: 45-46-24/25-48/23/24/25	S: 53-45
Ampicillin	R: 42/43	S: 23-36
Carbenicillin	R: 42/43	S: 36
DMSO	R: 36/37/38	S: 26-36-23
DTT	R: R: 36/37/38	S: 26-36
Doxcyclin	R: 20/21/22-36/37/38-63	S: 26-36-22
EDTA	R: 20/21/22	S: 36
Ethanol	R: 11	S: 7-16
Ethidium Bromide	R: 46-36/37/38	S: 45-26-22-36/37/39
Geneticin 418	R: 61-20/21/22	S: 45-36/37/39
Hydrogen Peroxide	R: 8-34	S: 17-45-26-36/37/39
Kanamycin	R: 61	S: 45-36/37/39
ß–Mercaptoethanol	R: 24-20/22-41-37/38	S: 53-45-26-36/37/39
Methanol	R: 11-23/25	S: 7-16-24-45
N,N‘-Methylene-bis-Acrylamide	R: 60-63-22-36/37/38	S: 53-45-36/37/39-22
Nalidixic acid	R: 45-46-20/21/22-36/37/38	S: 45-36/37/39-22-7
Penicillin	R: 45-46-23/24/25	S: 45-36/37739-22
Phenylenediamine	R: 20/21-25-43-50/53	S: 36/37-45-60-61
Puromycin	R: 20/22-36/37/38	S: 23-36/37/39
Silver Nitrite	R: 8-20/21/22-36/37/38	S: 17-26-36
Streptomycin	R: 61-20/21/22	S: 45-36/37/39-22

XI. Curriculum Vitae

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PERSONAL DATA

Birthday:	Feb. 07, 1969
Place of birth:	Shaanxi, P. R. China
Family status:	Married, no child
Nationality:	P. R. China

EDUCATION

1976-1987	Beijing Yu-ying school. Graduation of high school.
1987-1992	Department of Biochemistry, Beijing Normal University. Bachelor of Science. The thesis title was “Purification of calcineurin”.
1993-1996	Department of Biochemistry, Beijing Normal University. Master of Science. The thesis title was “Structure and function of PP2b (calcineurin)”.
1996-2000	Max-Planck-Arbeitsgruppen für strukturelle Molekularbiologie. Ph. D. student The thesis title was “Generation of scFv recombinant antibodies with the help of the phage display system against the microtubule associated protein Tau and the kinase MARK”.

XII. Statement (Erklärung)

I declare that I have carried out this thesis by myself and have not used external help except where explicitly indicated.

This thesis was not submitted to any other university.

I did not make any earlier attempt to submit this work as a doctoral thesis.

Hamburg, on

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfaßt, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt und die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Ferner versichere ich, daß ich diese Dissertation noch an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen.

Hiermit erkläre ich auch, daß ich keine anderen früheren Versuche gemacht habe, die Arbeit zur Promotion einzureichen.

Hamburg, den