

An Antibody Which Recognizes Phosphorylated Polypeptides

The present invention relates to an antibody which binds to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not
5 bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5 , wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu.

Alzheimer's Disease (AD) is the most common form of adult-onset dementia.
10 There is currently no reliable biochemical test or biomarker available for diagnosis of AD and conclusive diagnosis can only be made post-mortem.

Neuropathological examination of post-mortem brain shows large amounts of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) in characteristic regions of the brain. NFT deposition correlates better with disease severity
15 than plaque accumulation.

NFTs comprise paired helical filaments (PHFs) of the microtubule-associated protein tau (Delacourte, A, J., Neurol. Sci. 76 (1986) 173-180, Kosik, K., PNAS 83 (1986) 4044-4048, Kondo, J., Neuron 1 (1988) 82, Wood, J., PNAS 83 (1986) 4040-4043). The function of tau *in vivo* is to promote microtubule assembly and stability
20 within the axonal compartment of neurons (Lewis, S., Nature 342 (1989) 498-505).

Tau is a highly soluble protein, with no propensity whatsoever to aggregate into filaments. However, in AD, tau becomes hyperphosphorylated. This hyperphosphorylation initiates an aggregation processes which culminates in PHFs and ultimately NFTs. Phosphorylation is a normal feature of tau and is part of the control
25 mechanism which regulates the binding to microtubules (Lovestone, S., Biol. Psychiatry 45(1999) 995-1003).

There is a large number of potential phosphorylation sites within the molecule, these occur within two regions which flank the microtubule binding domain of tau. Phosphorylation at these sites is benign and does not lead to tau aggregation. In AD, a

phosphorylated amino acid) are found and it is this *hyperphosphorylation* or *abnormal* phosphorylation which most likely initiates the fibrillization process. An understanding of the mechanisms that lead to PHF-tau formation therefore requires knowledge of these phosphorylation sites

5 The abnormal phosphoepitopes in tau that occur in AD have been identified by isolation and characterization of PHFs from AD brain (Hasegawa, M., J. Biol. Chem. 267 (1992) 17047-17054, Cripps, D., J. Biol. Chem. 281 (2006) 10825-10838). One of these phosphoepitopes occurs at Ser422, towards the C-terminus of tau. Phosphorylation of Ser422 is tightly linked to NFT formation (Jicha, G., J. Neurochem 69 (1997) 2087-
10 2095). This group showed in cell culture experiments that phosphorylation of tau at Ser422 causes a decrease in tau solubility, implying a role in the initial steps of the aggregation process. Mutation of Ser422 to alanine abolished this effect (Ferrari, A., J. Biol. Chem. 278 (2003) 40162-40168).

 Phosphorylation of tau at Ser422 may be a sensitive marker for AD, as suggested
15 by an early study using a polyclonal antibody directed against this phosphoepitope (Bussiere, T., Acta. Neuropathol. 97 (1999) 221-230). However, a high affinity, highly selective monoclonal antibody has not been available for this phosphoepitope until now. Such an antibody must be capable of recognising tau that is phosphorylated at Ser422 against a high background of non-phosphorylated tau and of tau phosphorylated at other
20 pathogenic and non-pathogenic epitopes. The high degree of selectivity is crucial for allowing the clear distinction of pathological from non-pathological events in AD.

 A number of publications point to the importance of the single phosphoepitope, phospho-Ser422, in the development of tau pathology.

 In addition, mass spectrometric analysis of tau derived from NFTs or from normal
25 brain showed that this phosphoepitope is unique to PHF-tau (Hasegawa, M., J. Biol. Chem. 267 (1992) 17047-17054, Cripps, D., J. Biol. Chem. 281 (2006) 10825-10838).

 There are many phosphoepitopes in PHF-tau (paired helical filament-tau) and several monoclonal antibodies recognizing one or more of these phosphoepitopes are known e.g. TG3 (pThr231), PHF-1 (pSer396/pSer404), 12E8 (pS262 /pS356), AT8
30 (pSer202/pT205), AT100 (pSer212/pThr214), AT180 (pThr231) and AT270 (p181) (Seubert, P., J. Biol.Chem. 270 (1995) 18917-18922, Greenberg, S., J. Biol. Chem. 267 (1992) 564-569, Jicha, G., J.Neurochem. 69 (1997) 2087-2095, Mercken, M., Acta. Neuropathol. 84 (1992) 265-272).

 Some of these antibodies, for instance 12E8, AT180 and AT270, also recognize
35 tau from healthy brains and are not truly disease-state specific. All of these antibodies

(except 12E8) were raised using purified PHF-tau as an immunogen and consequently the antigenic epitope recognized by these antibodies is complex, comprising either double/multiple phosphorylations or a combination of conformational and phosphoepitope. These antibodies are therefore of limited use in analyzing the contribution of individual phosphoepitopes to the development of tau pathology.

Although many polyclonal antibodies have been raised against individual phosphoepitopes, many of these show some degree of cross-reactivity with normal tau. One monoclonal antibody, AP422, has previously been described which was raised against a tau-derived phosphopeptide containing phospho-Ser422. However, this antibody shows weak cross-reactivity with normal tau (Hasegawa, M., FEBS Letters 384 (1996) 25-30).

In order to assess the contribution of this phosphoepitope to tau pathology, and to develop immunoassays for measuring levels of this phosphotau variant in brain tissues derived from AD patients, a high affinity, highly selective monoclonal antibody showing no cross-reactivity with normal tau is required.

The disclosure provides an antibody which binds to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu. Furthermore, the disclosure provides an antibody which binds to tau comprising phospho-Ser422, and does not bind to tau comprising unphosphorylated Ser422. Furthermore, the disclosure provides a method for detecting tau comprising phospho-Ser422. Furthermore, the disclosure provides use of the antibody for detecting tau comprising phospho Ser-422. Furthermore, the disclosure provides a kit for detecting tau comprising phospho-Ser422. Furthermore, the disclosure provides a cell which produces the antibody.

According to a first aspect, there is provided a monoclonal antibody or a fragment thereof produced by the hybridoma DSM ACC2762 or DSM ACC2763, which binds to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-

Gln-Leu-Ala-Thr-Leu-Ala-A5, wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu.

According to a second aspect, there is provided a method for diagnostic of neurological disorder, which method comprises (a) contacting a sample with the antibody as defined above; and (b) detecting a complex formed between the antibody and tau or MAP2.

According to a third aspect, there is provided a method for detecting tau comprising phospho-Ser422, which method comprises (a) contacting a sample with the antibody as defined above; and (b) detecting a complex formed between the antibody and tau.

According to a fourth aspect, there is provided a method for detecting MAP2 comprising phosphoSer1808, which method comprises (a) contacting a sample with the antibody as defined above; and (b) detecting a complex formed between the antibody and MAP2.

According to a fifth aspect, there is provided an antibody as defined above for use in the vitro diagnosis of neurological disorders.

According to a sixth aspect, there is provided an antibody as defined above for use in detecting tau comprising phospho-Ser422.

According to a seventh aspect, there is provided an antibody as defined above for use in detecting MAP2 comprising phospho-Ser1808.

According to an eighth aspect, there is provided a kit for diagnosis of neurological disorders, which comprises the antibody as defined above.

According to a ninth aspect, there is provided a kit for detecting tau comprising phospho-Ser422, which comprises the antibody as defined above.

According to a tenth aspect, there is provided a kit for detecting MAP2 comprising phospho-Ser1808, which comprises the antibody as defined above.

According to an eleventh aspect, there is provided a hybridoma DSM ACC2762 or DSM ACC2763 which produces the antibody as defined above.

More specifically, the present invention provides the following (1) to (24).

- (1) An antibody or fragment thereof which binds to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu.

- (2) The antibody of (1), wherein A1 is Asp, A2 is Met, A3 is Val, A4 is Asp and A5 is Asp.
- (3) The antibody of (1), wherein A1 is Asn, A2 is Leu, A3 is Leu, A4 is Glu and A5 is Glu.
- 5 (4) The antibody of (1) or (2), wherein the antibody binds to tau comprising phospho-Ser422 and does not bind to tau comprising unphosphorylated Ser422.
- (5) The antibody of (1) to (4), wherein the antibody binds to MAP2 comprising phospho-Ser1808 and does not bind to MAP2 comprising unphosphorylated Ser1808.
- (6) The antibody of (2), wherein its Kd for the peptide consisting of Ser-Ile-Asp-Met-
10 Val-Asp-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp is lower than 100nM.
- (7) The antibody of (1) to (6), wherein the antibody is monoclonal antibody.
- (8) The antibody of (1) to (7), wherein the antibody is produced by the hybridoma DSM ACC2762 or DSM ACC2763.
- (9) A method for diagnostic of neurological disorder, which method comprises
- 15 (a) contacting a sample with the antibody of (1) to (8); and
- (b) detecting a complex formed between the antibody and tau or MAP2.
- (10) The method of (9), wherein neurological disorder is Alzheimer's disease.
- (11) A method for detecting tau comprising phospho-Ser422, which method comprises
- (a) contacting a sample with the antibody of (1) to (8); and
- 20 (b) detecting a complex formed between the antibody and tau.
- (12) A method for detecting MAP2 comprising phospho-Ser1808, which method comprises
- (a) contacting a sample with the antibody of (1) to (8); and
- (b) detecting a complex formed between the antibody and MAP2.
- 25 (13) The method of (11) or (12), wherein the complex is detected by western blotting, immunohistochemistry or ELISA.
- (14) Use of the antibody of (1) to (8) for diagnosis of neurological disorders.

(15) Use of (14), wherein neurological disorder is Alzheimer's disease.

(16) Use of the antibody of (1) to (8) for detecting tau comprising phospho-Ser422.

(17) Use of the antibody of (1) to (8) for detecting MAP2 comprising phospho-Ser1808.

5 (18) A kit for diagnosis of neurological disorders, which comprises the antibody of (1) to (8).

(19) A kit of (18), wherein neurological disorder is Alzheimer's disease.

(20) A kit for detecting tau comprising phospho-Ser422, which comprises the antibody of (1) to (8).

10 (21) A kit for detecting MAP2 comprising phospho-Ser1808, which comprises the antibody of (1) to (8).

(22) A cell which produces the antibody of (1) to (8).

(23) The cell of (22), wherein the cell is hybridoma.

(24) The cell of (23), wherein the hybridoma is DSM ACC2762 or DSM ACC2763.

15 The present invention provides an antibody which binds to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5 (SEQ ID NO:9), and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5 (SEQ ID NO:8), wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu. In the present invention,
20 "Ser(PO₃H₂)" means phosphorylated serine.

In one embodiment, the antibody of the present invention binds to an epitope consisting of Ser-Ile-Asp-Met-Val-Asp-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp (SEQ ID NO:6), and does not bind to an epitope consisting of Ser-Ile-Asp-Met-Val-Asp-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp (SEQ ID NO: 4).

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In the preferred embodiment, the present invention provides the antibody which binds to phosphorylated tau comprising phospho-Ser422, and does not bind to tau comprising unphosphorylated Ser422.

There are several splicing isoforms of tau, such as, Fetal-tau, Tau-A, Tau-B, Tau-C, Tau D, Tau-E, Tau-F (Swiss-Prot ; Entry name:TAU_HUMAN, Primary accession number:P10636). In the present invention, preferably, tau is a polypeptide having the sequence of SEQ ID NO:1. The term "tau" also contains splicing isoforms, variant,
5 derivatives, homologues or fragments of the polypeptide having the sequence of SEQ ID NO:1. In the case tau is splicing isoform, variant, derivative, homologue or fragment of the polypeptide having the sequence of SEQ ID NO:1, it is preferable that tau comprises the sequence of Ser-Ile-Asp-Met-Val-Asp-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp.

The term "phosphorylated tau" refers to tau in which at least one amino acid is
10 phosphorylated.

The term "phospho-Ser422" refers to a phosphorylated Serine at position 422 as defined by the position in the amino acid sequence of SEQ ID NO:1. The term "phospho-Ser422" also contains a phosphorylated Serine in splicing isoform, derivative, variant, homologue or fragment, which is corresponding to the phosphorylated Serine at position
15 422 of the polypeptide having the sequence of SEQ ID NO:1.

It is preferable that the antibody of the present invention has high binding affinity to tau comprising phospho-Ser422. The term "high binding affinity", as the term is used herein, means dissociation constant K_d of an antibody for tau comprising phospho-Ser422 is lower than 100nM. K_d of an antibody can be determined by methods known to
20 those skilled in the art. For example, K_d can be determined using surface plasmon resonance with Biacore under the condition described in the example 4. To use the peptide consisting of Ser-Ile-Asp-Met-Val-Asp-Ser(PO_3H_2)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp for determining K_d is preferable.

25 In one embodiment, the antibody of the present invention binds to an epitope consisting of Ser-Ile-Asn-Leu-Leu-Glu-Ser(PO_3H_2)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Glu (SEQ ID NO:7), and does not bind to an epitope consisting of Ser-Ile-Asn-Leu-Leu-Glu-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Glu (SEQ ID NO:5).

In a preferred embodiment, the antibody of the present invention binds to MAP2
30 (microtubule-associated protein 2) comprising phospho-Ser1808, and does not bind to MAP2 comprising unphosphorylated Ser1808.

There are a few isoforms of MAP2, such as isoform1, isoform2 or isoform3 (Swiss-Prot ; Entry name:MAP2_HUMAN, Primary accession number:P11137). In the present invention, preferably, MAP2 is a polypeptide having the sequence of SEQ ID

NO:2. The term "MAP2" also contains splicing isoform, variant, derivative, homologue or fragment of the polypeptide having the sequence of SEQ ID NO:2. In the case MAP2 is splicing isoform, variant, derivative, homologue or fragment of the polypeptide having the sequence of SEQ ID NO:2, it is preferable that MAP2 has the sequence of Ser-Ile-
5 Asn-Leu-Leu-Glu-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Glu.

The term "phosphorylated MAP2" refers to MAP2 in which at least one amino acid is phosphorylated.

The term "phospho-Ser1808" refers to a phosphorylated Serine at position 1808 as defined by the position in the amino acid sequence of SEQ ID NO:2. The term "phospho-
10 Ser1808" also contains a phosphorylated Serine in splicing isoform, derivative, variant, homologue or fragment, which is corresponding to the phosphorylated Serine at position 1808 of the polypeptide having the sequence of SEQ ID NO:2.

Whether an antibody binds to the epitope or not can be determined by well known
15 methods, such as western blot, enzyme immunoassay or surface plasma resonance. A polypeptide consisting of the sequence of the epitope, a whole protein comprising the sequence of the epitope or a fragment of the protein can be used for detecting the binding.

20 The antibody of the present invention may be polyclonal or monoclonal antibody. In a preferable embodiment, the antibody of the present invention is a monoclonal antibody.

The term "monoclonal antibody" refers to an antibody obtained from a group of substantially homogeneous antibodies, i.e., antibody group wherein the antibodies
25 constituting the group are homogeneous except for naturally occurring mutants that exist in a small amount.

In a preferred embodiment, the antibody of the present invention is the antibody produced by the hybridoma MAK<pTAU>M.2.5.2 or MAK<pTAU>M.2.20.4. The
30 hybridoma MAK<pTAU>M.2.5.2 was deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-38124 Braunschweig, Germany) on March 15, 2006 under No. DSM ACC2762. The hybridoma MAK<pTAU>M.2.20.4 was deposited at DSMZ (Deutsche Sammlung von

Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-38124 Braunschweig, Germany) on March 15, 2006 under No. DSM ACC2763.

The antibody produced by the hybridoma DSM ACC2762 (MAK<pTAU>M.2.5.2) or DSM ACC2763 (MAK<pTAU>M.2.20.4) has high affinity and high selectivity with
5 tau comprising phospho-Ser422, and does not have cross-reactivity with tau comprising unphosphorylated Ser422. Additionally, the antibody produced by the hybridoma binds to MAP2 comprising phospho-Ser1808, and does not bind to MAP2 comprising unphosphorylated Ser1808. These antibodies can be used for detecting tau comprising phospho-Ser422 or MAP2 comprising phospho-Ser1808. The antibody produced by the
10 hybridoma DSM ACC2762 or DSM ACC2763 is suitable for diagnosis of neurological diseases such as Alzheimer's disease. The antibody produced by the hybridoma DSM ACC2762 or DSM ACC2763 is also suitable for detecting tau comprising phospho-Ser422 or MAP2 comprising phospho-Ser1808.

15 The antibody of the present invention can be obtained as a polyclonal antibody or a monoclonal antibody, using known techniques. For example, the monoclonal antibody can be produced by the hybridoma method (Kohler G. and Milstein C., Nature 256 (1975) 495-497) or the recombinant method (U.S. patent No. 4,816,567). The monoclonal antibody can be also isolated from a phage antibody library (Clackson T.,
20 Nature 352(1991) 624-628).

Hybridoma producing the antibody can be prepared essentially using known techniques. For example, following methods can be used: An animal is immunized by a conventional immunization method using a sensitizing antigen to obtain an immune cell,
25 which is then fused to a known parent cell by a conventional cell fusion method. Fused cells are screened for monoclonal antibody-generating cells by a conventional screening method. More specifically, the monoclonal antibody can be obtained as follows.

First, a fragment which has the sequence of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5 (A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is
30 Asp or Glu and A5 is Asp or Glu), for use as a sensitized antigen is prepared. The fragment can be prepared by chemical synthesis or culturing host cell comprising a gene coding the fragment. Then, the fragment is phosphorylated by kinase, for example ERK2 kinase. A whole protein comprising the fragment may also be used as a sensitized antigen. In the present invention, preferable sensitized antigen is the phosphorylated

fragment of tau, which has the sequence of Ser-Ile-Asp-Met-Val-Asp-Ser(PO_3H_2)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp.

There is no limitation as to the type of mammalian species to be immunized with the sensitized antigen. However, a mammal is preferably selected based on its
5 compatibility with the parental cell to be used in cell fusion. Generally, rodents such as for example mice, rats, hamsters or rabbits, and monkeys can be used.

For immunization of animals with a sensitizing antigen, known methods may be employed. Animals can be immunized with a sensitized antigen by well known methods such as a routine method of injecting a sensitized antigen into a mammal
10 intraperitoneally or subcutaneously. Specifically, the sensitized antigen is diluted appropriately with phosphate-buffered saline (PBS), physiological saline and such, and then suspended. An adequate amount of a conventional adjuvant, for example, Freund's complete adjuvant, is mixed with the suspension, as necessary. An emulsion is then prepared for administering to a mammal several times over a 4- to 21-day interval. An
15 appropriate carrier may be used for the sensitized antigen in immunization.

A mammal is immunized as described above. After a titer increase of target antibody in the serum is confirmed, immunocytes are collected from the mammal and then subjected to cell fusion. Spleen cells are the preferred immunocytes.

Mammalian myeloma cells are used as the parental cells to be fused with the above
20 immunocytes. Preferable myeloma cells to be used include various known cell lines, for example, P3(P3x63Ag8.653) (Kearney JF, et al., J. Immunol. 123, 1548-1550 (1979)), P3x63Ag8U.1 (Yelton DE, et al., Current Topics in Microbiology and Immunology 81, 1-7 (1978)), NS-1 (Kohler, G. and Milstein, C. Eur. J. Immunol. 6, 511-519 (1976)), MPC-11 (Margulies, D. H. et al., Cell 8, 405-415 (1976)), SP2/0 (Shulman, M. et al.,
25 Nature 276, 269-270 (1978)), FO (deSt. Groth, S. F. et al., J. Immunol. Methods 35, 1-21 (1980)), S194 (Trowbridge, I. S., J. Exp. Med. 148, 313-323 (1978)), and R210 (Galfre, G. et al., Nature 277, 131-133 (1979)).

Cell fusions between the immunocytes and the myeloma cells as described above can be essentially carried out using methods known to a person skilled in the art, for
30 example, a method by Kohler and Milstein (Kohler, G. and Milstein, C., Methods Enzymol. 73: 3-46 (1981)).

More specifically, the above-described cell fusions may be carried out, for example, in a conventional culture medium in the presence of a cell fusion- promoting agent. The fusion-promoting agents include but are not limited to polyethylene glycol

(PEG) and Sendai virus (HVJ). If required, an auxiliary substance such as dimethyl sulfoxide may also be added to improve fusion efficiency.

The ratio of immunocytes to myeloma cells may be determined at one's own discretion, preferably, one myeloma cell for every one to ten immunocytes. Culture media to be used for the above cell fusions include, for example, media that are suitable for the growth of the above myeloma cell lines, such as RPMI 1640 media and MEM media, and other conventional culture media used for this type of cell culture. In addition, serum supplements such as fetal calf serum (FCS) may also be used in combination.

Cell fusion is carried out as follows. As described above, predetermined amounts of immunocytes and myeloma cells are mixed well in the culture medium. PEG solution (for example, mean molecular weight of about 1,000- 6,000) pre-heated to 37°C is added to the cell suspension typically at a concentration of 30% to 60% (w/v), and mixed to produce fused cells (hybridomas). Then, an appropriate culture medium is successively added to the mixture, and the sample is centrifuged to remove supernatant. This treatment is repeated several times to remove the unwanted cell fusion-promoting agent and others that are unfavorable to hybridoma growth.

Screening of the resulting hybridomas can be carried out by culturing them in a conventional selective medium, for example, hypoxanthine, aminopterin, and thymidine (HAT) medium. Culturing in the above-described HAT medium is continued for a period long enough (typically, for several days to several weeks) to kill cells (non-fused cells) other than the desired hybridomas. Then, hybridomas are screened for single-cell clones capable of producing the target antibody by conventional limiting dilution methods.

In addition to the method for preparing the above-described hybridomas by immunizing non-human animals with antigens, human antibodies can also be obtained by sensitizing human lymphocytes with a sensitized antigen in vitro; and fusing the sensitized lymphocytes with human myeloma cells capable of dividing permanently (see, for example, Japanese Patent Application No. (JP-B) Hei 1-59878).

Alternatively, it is possible to obtain human antibodies from immortalized cells producing the antibodies. In this method, the cells producing the antibodies are prepared by administering a sensitized antigen to transgenic animals comprising a repertoire of the entire human antibody genes (see for example WO 94/25585, WO 93/12227, WO 92/03918, and WO 94/02602).

The monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium, and stored in liquid nitrogen over long periods of time.

Monoclonal antibodies can be prepared from the above-described hybridomas by, for example, a routine procedure of culturing the hybridomas and obtaining antibodies
5 from the culture supernatants.

Once monoclonal antibody is obtained from a hybridoma, recombinant antibody can also be prepared by conventional methods.

The antibody of the present invention may be a labelled antibody. For the labelling
10 of the antibody, known labels such as radioactive substance (for example, ^{32}P , ^{125}I , ^3H , ^{131}I , ^{14}C), fluorochrome (for example fluorescein, rhodamine, umbeliferone), enzyme (for example luciferases, oxidase, alkaline phosphatase, β -galactosidase, β -glucosidase, lysozyme, glucoamylase), coenzyme (for example biotin) or chemiluminescence substance can be used. The labeling of the antibody can be done by well known methods,
15 such as for example glutaraldehyde method, maleimide method, pyridyl disulfide method or periodic acid method.

The antibody of the present invention may also be fragment of the antibody as long as the fragment can bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO_3H_2)-
20 Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5 (A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu). The fragment of antibody may be, but not limited, Fab, Fab', Fv, F(ab')₂, scFv, sc(Fv)₂ or diabody.

25 Preferably, the antibody of the present invention forms an immunological complex with tau or fragment thereof comprising a phosphorylated Ser at position 422 as defined by the position of the amino acid in SEQ ID NO:1. More preferably, said tau or fragment thereof is isolated from autopsy-derived brain tissue of AD patients or patients having any other neurological disorders with NFT present. Preferably, the antibody of the
30 present invention does *not* form such an immunological complex with autopsy-derived brain material from patients having died or suffering from other diseases without the occurrence of NFTs in the brain.

The antibody of the present invention can be used for diagnosis of a neurological disorder such as Alzheimer's disease by detecting phosphorylated polypeptide. The antibody of the present invention can be also used for detection of phosphorylated polypeptide comprising the sequence of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-
5 Leu-Ala-Thr-Leu-Ala-A5, wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu, such as phosphorylated tau comprising phospho-Ser422 or phosphorylated MAP2 comprising phosho-Ser1808.

Moreover, the present invention provides a cell which produces the antibody of the
10 present invention. The cell of the present invention may be a hybridoma or a recombinant cell.

The hybridoma which produces the antibody of the present invention can be obtained by conventional methods described above. In a preferable embodiment, hybridoma of the present invention is hybridoma DSM ACC2762 or DSM ACC2763.
15 The hybridoma DSM ACC2762 was deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-38124 Braunschweig, Germany) on March 15, 2006 under No. DSM ACC2762. The hybridoma DSM ACC2763 was deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-38124 Braunschweig, Germany) on
20 March 15, 2006 under No. DSM ACC2763.

A recombinant cell of the invention can be obtained by well known methods. Specifically, such recombinant cell can be obtained as follows.

An mRNA encoding the variable (V) region of the antibody which binds to an
25 epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu, is isolated from hybridomas producing such an antibody.

30 For mRNA isolation, total RNAs are first prepared by conventional methods such as guanidine ultracentrifugation methods (Chirgwin, J. M. et al., Biochemistry 18, 5294-5299 (1979)), or acid guanidinium thiocyanate-phenol- chloroform (AGPC) methods (Chomczynski, P. et al., Anal. Biochem. 162, 156-159 (1987)), and then the target mRNA is prepared using an mRNA Purification Kit (Pharmacia) and such. Alternatively,

the mRNA can be directly prepared using the QuickPrep mRNA Purification Kit (Pharmacia).

A cDNA of the antibody V region is synthesized from the resulting mRNA using reverse transcriptase. cDNA synthesis is carried out using the AMV Reverse
5 Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.), or such. Alternatively, cDNA can be synthesized and amplified by the 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA 85, 8998-9002 (1988); Belyavsky, A. et al., Nucleic Acids Res. 17, 2919-2932 (1989)) using the 5'- Ampli FINDER RACE Kit (Clontech) and PCR.

10 Target DNA fragments are purified from the obtained PCR products and then ligated with vector DNAs to prepare recombinant vectors. The vectors may be introduced into *E. coli* and such, and colonies are selected for preparing the recombinant vector of interest. The target DNA nucleotide sequence is then confirmed by conventional methods such as the dideoxynucleotide chain termination method.

15 Once a DNA encoding the V region of target antibody is obtained, the DNA is inserted into an expression vector which comprises a DNA encoding the constant region (C region) of a desired antibody.

The method for obtaining the cells which produce the antibody typically comprises the steps of: inserting the antibody gene into an expression vector, so that the
20 gene is expressed under the regulation of expression regulatory regions, such as enhancer and promotor; and transforming host cells with the resulting vectors to express the antibody. Polynucleotide encoding H chain and L chain, respectively, can be inserted into separate expression vectors and co- transfected into a host cell. Alternatively, polynucleotides encoding both H chain and L chain can be inserted into a single
25 expression vector and transfected into a host cell (see for example WO 94/11523). The host cells are not limited as long as the cells can produce the antibody. For example, the cells may be CHO, COS, NIH3T3, myeloma, BHK, Hela, Vero, amphibian cells, insect cells, plants cells or bacterial cells such as *E. coli*.

30 Furthermore, the present invention provides a method for diagnosis of neurological disorder. The method for diagnosis of neurological disorder comprises the following steps:(a) contacting a sample with the antibody of the present invention, and (b) detecting the complex formed between the antibody and tau or Map2.

The present invention further provides a method for detecting tau comprising phospho-Ser422. The method for detecting tau comprising phospho-Ser422 comprises (a) contacting a sample with the antibody of the present invention; and (b) detecting a complex formed between the antibody and tau.

5

The present invention further provides a method for detecting MAP2 comprising phospho-Ser1808. The method for detecting MAP2 comprising phospho-Ser1808 comprises (a) contacting a sample with the antibody of the present invention; and (b) detecting a complex formed between the antibody and MAP2.

10

The term "neurological disorder", as used herein, refers to disease which are related to tau comprising phospho-Ser422 or MAP2 comprising phospho-Ser1808, such as diseases caused by tau comprising phospho-Ser422 or MAP2 comprising phospho-Ser1808, or diseases of which tau comprising phospho-Ser422 or MAP2 comprising phospho-Ser1808 is detected from a patient. The term "neurological disorder" includes, but is not limited to, Alzheimer's disease, Down's Syndrome, Pick's Disease, progressive supranuclear palsy and corticobasal degeneration.

Although a sample used in the methods of the present invention are not limited as long as there is a possibility the sample comprises tau or MAP2, a biological sample obtained from human is preferred. A biological sample includes, but is not limited to, cerebrospinal fluid, serum, blood, tissue (for example, brain tissue) and cell (for example, brain cell).

A contact of a sample with the antibody of the present invention can be achieved under conditions which allow the formation of a complex formed between the antibody and tau or a complex formed between the antibody and MAP2. Such conditions are well known to a person skilled in the art.

The detection of a complex formed between the antibody and tau or a complex formed between the antibody and MAP2 can be carried out by conventional manner, such as western blot, enzyme immunoassay such as enzyme-linked immunosorbent assay(ELISA), radioimmunoassay, fluorescent immunoassay, luminescent immunoassay, immunoprecipitation, immunostaining, immunodiffusion and surface plasma resonance biosensor (such as BIAcore).

The method of the present invention may be carried out *in vitro* or *in vivo*, however, *in vitro* method is preferable.

5 The present invention also provides use of the antibody of the present invention for diagnosis of neurological disorders, such as Alzheimer's disease. The present invention also provides use of the antibody of the present invention for detecting a phosphorylated polypeptide such as tau comprising phospho-Ser422 or MAP2 comprising phospho-Ser1808.

10 The antibody of the present invention may be used *in vivo* or *in vitro*. In the preferred embodiment, the antibody of the present invention is used *in vitro*.

The present invention provides a kit comprising the antibody which binds to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-
15 Leu-Ala-Thr-Leu-Ala-A5, wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu. This kit can be used, for example, for diagnosis of neurological disorders, detecting tau comprising phospho-Ser422 or detecting MAP2 comprising phospho-Ser1808.

20 The kit may comprise a carrier, such as insoluble polysaccharide(for example, agarose or cellulose), synthetic resin(for example, polystyrene resin, silicon resin, polyacrylamide resin, polycarbonate resin or nylon resin) or insoluble support(for example, glass). The antibody may be immobilized to the carrier.

The kit may comprise a reagent, such as reaction liquid, blocking solution, or other reagent used in immunoassay.

25

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures.

30

Figures

Figure 1 shows specificity of the monoclonal antibodies. Specificity of the monoclonal antibodies was tested by western blot analysis of various tau species with 441aa. A: Pan α -tau (control), antibodies 2.5.2 and 2.20.4; B: monoclonal antibody

5 AP422

Lane1 : tau comprising unphosphorylated Ser at position 422

Lane2 : phosphorylated tau comprising phosphorylated Ser at position 422

Lane3 : tau comprising Ser→Ala mutation at position 422

Lane4 : phosphorylated tau comprising Ser→Ala mutation at position 422

10

Figure 2 shows the result of western blot analysis of soluble- and insoluble extracts from Braak-staged AD brain tissue. Several tau bands are visible due to the fact that the human brain contains multiple splicing isoforms of tau. Note that neither Braak stage II/control brain, nor the soluble fraction, contain tau phosphorylated at Ser422. M = lane

15 containing marker proteins (Novex); I= insoluble fraction; S= soluble fraction; II, IV, VI= Disease severity (Braak stage)

Figure 3 shows the result of Immunohistochemical analysis of AD brain tissue (Braak stage VI). NFT = neurofibrillary tangle; NT = neuropil thread; DN = dystrophic neuritis (surrounding amyloid plaque). Antibody used was 2.5.2. Control brain showed

20 no staining.

Figure 4 shows the result of western blot analysis of okadaic acid-treated LAN-5 cells (OA) using antibody 2.5.2. The antibody reacted with three proteins which were all

25 phosphoproteins, as indicated by the lack of reactivity with vehicle/DMSO extract or with extract from cells treated with the pan-kinase inhibitor K252a. The individual molecular weights were consistent with the largest protein being MAP 2a/b (phosphorylated at S1808) and the two smaller proteins being two different isoforms of tau (phosphorylated at S422). Lane 1 = vehicle; lane 2 = OA, lane 3 = OA + K252a.

30

Figure 5 shows the result of ELISA for determination of tau/pSer422 levels in okadaic acid-treated LAN-5 cells. The amount of tau/pSer422 was calculated on the basis of 1×10^6 cells.

EXAMPLES

The following examples serve merely to illustrate certain aspects of the present invention and should not be viewed as limiting the present invention in scope.

5 Example 1: Generation of monoclonal antibodies

A. Immunization of mice

Mice were immunized with a phosphopeptide comprising the following amino acid sequence

Cys-Ser-Ile-Asp-Met-Val-Asp-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp

- 10 which corresponds to amino acids 415-430 of the longest isoform of human tau (peptide synthesized by NeoMPS, Strasbourg). The naturally occurring Asp 415 was replaced by Cys to allow directed coupling via the thiol to KLH.

- 15 10-12-week old female Balb/c (Jackson Laboratory, stock#001026) and NMRI mice (Jackson Laboratory, stock#003076) were injected intraperitoneally with 100 µg of phosphopeptide in Complete Freund's Adjuvant. The mice received three further interperitoneal injections of 100 µg peptide in Freund's Incomplete Adjuvant at monthly intervals. Final immunizations were made at 3, 2 and 1 day before spleen removal by intravenous injection of 50 µg peptide in PBS.

B. Fusion and Cloning

- 20 Fusion of the spleen cells from immunized mice was carried out according to Galfré and Milstein, Methods in Enzymology, 73 (1981) 3-46. Thus 1×10^8 spleen cells from each immunized mouse were mixed with 2×10^7 myeloma cells (P3X63-Ag8-653, ATCC CRL1580) and centrifuged at $300 \times g$ for 10 min at RT. The cells were then washed once with RPMI-1640 medium (without FCS) and centrifuged once more at $300 \times g$ for 10 min in a conical tube. The cells were loosened by tapping the tube and warmed to 37°C in a pre-heated waterbath. Within the space of 1 minute, 1 ml of PEG was added (polyethylenglycol with molecular weight 1500, Roche Diagnostics). Next, 5 ml of RPMI-1640 medium (without FCS) was added dropwise with gentle shaking and finally the volume was adjusted to 50 ml by addition of RPMI-1640 medium containing
- 25 10% FCS. The cell suspension was then centrifuged at $300 \times g$ for 10 min. The cell pellet was resuspended in RPMI-1640 medium containing 10% FCS and seeded into
- 30 hypoxanthine-azaserine selection medium (100 mM hypoxanthine [Sigma], 1 µg/ml

azaserine [Sigma] in RPMI-1640 + 10% FCS). The medium was supplemented with 50U/ml interleukin-6 (Roche Applied Science).

After 10 days the primary cultures were tested for specific antibody production (see Example 2). Primary cultures which showed a positive reaction with phosphopeptide and
5 no reaction with unphosphorylated peptide were cloned by fluorescent-activated cell sorting in 96-well cell culture plates. The medium used here was RPMI-1640 medium supplemented with 10%FCS and 25 U/ml interleukin-6.

Two of the hybridoma cell lines/clones obtained in this way were deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,
10 Mascheroder Weg 1b, D-38124 Braunschweig).

Table 1 shows the details of the hybridoma.

Clone	Deposited at DSMZ on 15.03.2006 under the following number	IgG Subclass
2.5.2	DSM ACC2762	Ig G2a; κ -light chain
2.20.4	DSM ACC2763	Ig G2a; κ -light chain

Table 1

15

C. Antibody purification from cell culture supernatant

Hybridoma cells thus obtained were seeded at a density of 1×10^5 cells/ml in RPMI-1640 medium supplemented with 10% FCS and 25U/ml interleukin-6 and grown to a cell density of about 3×10^5 /ml. The cells were then diluted to 1×10^6 /ml in a final
20 volume of 250 ml and allowed to grow to maximal cell density. The cells were then removed by centrifugation. The hybridoma culture supernatants typically contained ca. 40-50 μ g/ml antibody.

Each antibody was purified as follows. Cell-free hybridoma culture supernatant (250-300 ml) was loaded on a 25 ml MEP HyperCel column (Pall Biosciences) which
25 was equilibrated with 50 mM TrisCl pH 8.0. After washing with equilibration buffer the antibody was eluted with 30 mM sodium citrate / 100 mM NaCl pH 4.1. Antibody containing fractions were pooled and then dialysed (Spectra/Por 6-8000) overnight at 4°C against 5 l of SourceQ buffer A. The dialysed MEP pool was loaded on a 10 ml Source 15Q column (GE Healthcare) which was equilibrated with 10 mM TrisCl pH 8.0

(buffer A). After washing with buffer A the antibody was eluted with a gradient from 0 – 25 % buffer B in 10 column volumes. Buffer B contained 10 mM TrisCl /1 M NaCl pH 8.0. The antibody eluted at about 200 mM NaCl. The purity of the individual fractions was checked by SDS-PAGE and the purest fractions pooled. The yield of antibody thus purified was ca. 10 mg/250 ml hybridoma culture supernatant.

Example 2: Screening for anti-Tau/pSer422 specific antibodies

A. Determination of specificity of antibodies for phosphopeptide (tau 416-430/pSer422)

In order to measure the specificity of the antibodies in the cell culture supernatants, streptavidin-coated microtiter plates (MicroCoat, Bernried, DE) were coated with 0.1 µg/ml biotinylated phosphopeptide (tau 416-430/pSer422) in PBS, 0.5% Byco C (100 µl/well, 1 h incubation at R.T. with shaking). The plates were then washed 3 times with wash buffer (0.9 % NaCl / 0.05% Tween 20). Next, 100 µl of antibody-containing culture supernatant was added in each well and the plates incubated for 1 h at room temperature (R.T.) with shaking. The plates were then washed 3 times with wash buffer. In order to detect bound antibody, 100 µl/well of a polyclonal anti-mouse antibody /peroxidase conjugate (Dianova) for 1 h at R.T.. The plates were subsequently washed again. Finally, 100 µl/well of ABTS solution (Roche Diagnostics) was added and the plates incubated for 20 min at R.T. The plates were then read at 405 nm in an X Read Plus microplate reader (Tecan) .

B. Measurement of antibody cross-reactivity with non-phosphorylated peptide (tau 416-430)

The same procedure as described above was used, except that the microtitre plates were coated with non-phosphorylated peptide (tau 416-430).

C. Measurement of binding to free phosphopeptide (tau 416-430/pSer422)

100 µl/ well of each cell culture supernatant was pipetted into a microtitreplate that had previously been coated with anti-mouse Fcy antibody (MicroCoat, Bernried, DE). The plate was then incubated for 1 h at R.T. with shaking and subsequently washed 3 times with wash buffer. Next, 100 µl/well of 50 nM biotinylated phosphopeptide (tau 416-430/pSer422) was added and the plate incubated for 1 h at R.T. with shaking. The plate was washed three times and then incubated with 100 µl/well of 50U/ml streptavidin/peroxidase conjugate (Dianova) for 1h at R.T. The plate was washed once

again and then developed with 100/μl well ABTS solution (Roche Applied Science) for 20 min at R.T. The plate was then read at 405 nm in a X Read Plus microplate reader.

D. Measurement of antibody binding to free non-phosphorylated peptide (tau 416-430)

- 5 The same procedure as described above was used, except that the microtitre plates were coated with non-phosphorylated peptide (tau 416-430).

Using the methods described above, both of the deposited antibodies showed good binding to immobilized and free phosphopeptide (tau 416-430/pSer422). No cross-reactivity was observed with non-phosphorylated peptide (tau 416-430).

10

Example 3: Determination of antibody specificity towards full-length phosphorylated tau protein and phosphorylated mutant tau protein (S422A) by western blot

- Antibodies were tested for their ability to recognise each of four different tau species, namely tau, tauS422A (Ser→Ala mutation at position 422), p-tau (phosphorylated tau) and p-tauS422A (phosphorylated tau comprising Ser→Ala mutation at position 422). Tau and tauS422A were expressed in *E. coli* and purified according to standard method. Both proteins were then phosphorylated by ERK2 kinase, which was shown to introduce a phosphogroup at S422 as well as a number of other sites in tau.
- 20 SDS-PAGE gel was loaded with 150 ng of each of the four tau species; subsequent to electrophoresis the proteins were transferred to nitrocellulose by standard western blotting protocol. Blots were incubated overnight at 4°C with individual hybridoma cell culture supernatants that had been diluted two-fold with StartingBlock (Perbio). After a standard washing procedure, the blots were incubated in 2 ng/ml anti-mouse antibody
- 25 /horseradish peroxidase conjugate (Perbio) for 1 hr at RT. The blots were then washed and developed with LumiLight ECL substrate (Roche Applied Science). As shown in Figure 1A, antibodies 2.5.2 and 2.20.4 (table 1) showed no cross-reactivity with non-phosphorylated tau nor with tau phosphorylated at Ser (or Thr) residues other than Ser422. The antibodies are thus highly selective for the target phosphoepitope. Figure 1B
- 30 shows the specificity of the monoclonal antibody AP422. The monoclonal antibody AP422 was obtained from Prof. M. Hasegawa (University of Tokyo, Japan) and diluted 1:2000 with 1% milk in TBSt buffer. The blot was incubated overnight at 4°C. The following day it was washed and incubated for 1 hr at room temperature with anti-mouse/horseradish peroxidase (Pierce) diluted 1:5000 with 1% milk in TBSt buffer.
- 35 Detection was with the Roche ECL system. The antibody AP422 showed cross-reactivity with unphosphorylated tau.

Example 4: Determination of k_{on} , k_{off} , K_A and K_d

The rate constants k_{on} and k_{off} and the resulting dissociation constant K_d were determined using surface plasma resonance (BIAcore 2000 from BIAcore AB).

5 NHS/EDC-activated Sensorchips (CM5, BIAcore AB) were coated with rabbit-anti mouse IgG at a concentration of 15 $\mu\text{g/ml}$ in 10mM sodium acetate-acetic acid pH5.0 for 5 min at a flow rate of 20 $\mu\text{l/min}$. Cell culture supernatants were diluted to a final antibody concentration of 50 nM in 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% Polysorbate and injected at a flow rate of 10 $\mu\text{l/min}$ over a period of 2
10 min. Phosphopeptide or non-phosphorylated peptide (0-1000 nM) in 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% Polysorbate was then injected at a flow rate of 100 $\mu\text{l/min}$ for 2 min. This was followed by dissociation for 5 min in 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% Polysorbate.

k_{on} and k_{off} were then calculated from the sensogram after double referencing using
15 BIAcore evaluation software (version 4.1, BIAcore AB). Models were fitted globally across the data sets using the 1:1 (Langmuir) binding interaction. The association constant K_a was calculated from k_{on} / k_{off}

The values obtained are summarized below. Both antibodies have K_d values in the low nanomolar range and thus can be considered to bind pSer422 with high affinity.
20 Neither of the antibodies showed any interaction with the non-phosphorylated peptide, indicating that they are highly selective for the phosphopeptide and confirming the western blot results in example 3.

Table 2 shows K_{on} , K_{off} , K_a and K_d of the antibody 2.5.2 and the antibody 2.20.4.

25

Tau 416-430/pSer422				
Clone	k_{on}	k_{off}	K_a	K_d
	1 / Ms	1 / s	1 / M	nM
2.5.2	8×10^5	8×10^{-3}	9×10^7	11
2.20.4	7×10^5	1×10^{-2}	7×10^7	14

Table 2

Example 5: Western blots of AD brain extracts

Microtome sections were prepared from Braak-staged AD brains and control brains. Ca. 50 mg wet weight tissue from each brain was dispensed into Eppendorf tubes. Each tissue was then homogenized in 10 volumes of ice-cold RAB-HS buffer using a hand-held glass-homogenizer. Homogenates were then centrifuged at 50000xg for 40 min at 4°C. The resulting pellets were resuspended in Tris-sucrose-SDS by homogenization and centrifuged at 50000xg for 40 min at RT. The resulting supernatants were retained for analysis. SDS-PAGE gel was loaded with 4.5 µl each supernatant. Western blotting was carried out as described above, except that the first antibody (2.5.2, purified as described above) was diluted to 1 µg/ml in StartingBlock. Results are shown in Figure 2. The antibody specifically detected tau isoforms phosphorylated at Ser422 in the RAB-insoluble/SDS-soluble brain extracts. This is to be expected since hyperphosphorylated tau usually occurs in this fraction of the extract. Normal tau occurs in the RAB-soluble fraction and clearly there is no cross-reaction of the antibody with this fraction in any of the samples. The intensity of staining correlates with disease severity and no staining is evident with the control brain extract.

Example 6: Immunohistochemical analysis of AD brain sections

Cryostat sections of unfixed brain tissue from cortical human brain regions, obtained postmortem from a patient that was positively diagnosed for Alzheimer's disease, Braak stage VI. Sections were labeled by indirect immunofluorescence with anti-p-tauS422A antibody (Wheatley S. and Wang Y., Methods Cell Bio 57 (1998) 313-332). A successive two-step incubation was used to detect bound anti-p-tauS422A antibodies, which are revealed by affinity-purified goat anti-mouse (GAM) IgG (H+L) conjugated to Alexa 488 (Molecular Probes). A counterstaining against Aβ peptide was done to reveal amyloid-β plaques.

In detail, Sections were cut at -18°C using a cryostat (Leica, CM 3050 S) at a nominal thickness of 10 µm. After adhesion to pre-cooled glass slides (Super Frost Plus, Menzel, Germany), sections were hydrated in PBS and treated with 100% acetone pre-cooled at -20°C for 2 min. Washing with PBS was done twice for two minutes. Blocking of unspecific binding sites was done by incubation in PBS with 1% bovine serum albumin (BSA) and 1% ovalbumin (OVA) and 1% normal goat serum for 20 min. Anti-p-tauS422A antibodies were used at a concentration of 10 µg/ml in PBS with 1% BSA, 1% OVA and 1% normal goat serum for 1 hour. After washing with PBS and 1% BSA, slides were incubated with affinity-purified goat anti-mouse (GAM) IgG (H+L) conjugated to Alexa 488 (Molecular Probes) at 15 µg/ml in PBS with 1% BSA for 1

hour. Slides were washed 3 x 5 minutes with PBS and 1 % BSA and counterstained with a monoclonal mouse antibody against A β (BAP-2, Dr. M. Brockhaus, F.Hoffmann La Roche, EP130424) at 5 μ g/ml in PBS with 1% BSA for 1 hour. Slides were washed 3 x 5 minutes with PBS rinsed with water and immersed in 0.3% sudan black in 70% aqueous ethanol for 5 minutes to reduce autofluorescence of lipofuscin. After one rinse with 70% ethanol and 2 rinses with water slides were washed with 2 x 3 minutes PBS and embedded with fluorescence mounting medium (S3023 Dako). Controls included unrelated mouse IgG1 antibodies (Sigma) and the secondary antibody alone, which all gave negative results.

Images were recorded with a Zeiss Axioplan2 using a 10x/0.3 objective. Images of both recorded fluorescence channels were merged with Photoshop.

Results are shown in Figure 3. The antibody stained typical structures associated with the pathology of Alzheimer's Disease, the most prominent of these being neurofibrillary tangles. Numerous neuropil threads were also evident, as were dystrophic neurites surrounding plaques (visualised by counter staining with an amyloid-specific antibody).

Example 7: Western blots for detecting pSer422 in tau and MAP2 in neuroblastoma cell extracts

The peptide immunogen used to generate the monoclonal antibodies is based on the amino acid sequence immediately surrounding S422 in tau, namely S416-IDMVDSPQLATLA-D430. This sequence occurs in tau towards its C-terminus. A homology search of protein sequence databases revealed that the highly homologous amino acid sequence S1802-INLLESPQLATLA-E1816 occurs towards the C-terminus of the microtubule-associated protein MAP2. LAN-5 cells treated with okadaic acid and analyzed by western blot using the antibodies described above do indeed contain a high molecular weight protein which cross-reacts with antibodies 2.5.2 and 2.20.4. The molecular weight of this protein is consistent with it being MAP2.

Cells (1×10^7 /well in serum-free medium) were treated with DMSO or 10 μ M K252a (Alexis) for 1 hr at 37°C; the medium was then supplemented with DMSO or 2 μ M okadaic acid for a further 1 hr at 37°C. Medium was removed and the cells extracted into 100 μ l of Cytobuster (Novagen) and 25 μ l used for western blot analysis. Western blots were performed as described in example 5. The result is shown in Fig. 4. The antibody 2.5.2 showed cross-reactivity with phosphorylated MAP2.

Example 8: ELISA assay for detecting tau/pSer422 in okadaic acid-treated neuroblastoma cells

LAN-5 neuroblastoma cells express tau endogenously. Phosphorylation of
5 endogenous tau at Ser422 occurs when the cells are treated with okadaic acid.

LAN-5 neuroblastoma cells are cultured in medium at 37°C and plated into 96-well microtitre plates at a cell density of 2.5×10^5 cells/well in 100µl of serum-free medium. After 24 h in culture the cells are then treated with 2.5 µM okadaic acid for 2h. Subsequently, 10 µl of a solution comprising 1mg/ml digitonin, 10 mM EDTA is added
10 and the cells shaken for 30 min at 4°C. This extract is then used directly in the ELISA assay described below.

Antibody 2.5.2 was biotinylated using biotin-NHS at a ratio of 20:1 in phosphate buffered saline, pH7.2. Antibody 5A6 (Developmental Studies Hybridoma Bank, University of Iowa) was labelled with BV-TAG™ at a ratio of 8:1 according to the
15 protocol supplied by BioVeris (BioVeris Corporation, Gaithersburg, Maryland). In a typical assay, 25 µl streptavidin-coated magnetic beads (Dyna/Invitrogen Corporation) at a dilution of 1:50 was preincubated for 30 min with 25 µl of 1 µg/ml biotin-antibody at RT. To this mixture was then added 50 µl of cell extract and 25 µl BV-tagged antibody 2.5.2 (prepared as described above). The mixture was then incubated for 3h at 4°C with
20 shaking. All samples were prepared in 96-well microtitre plates. Subsequently 125 ul buffer was added to each sample and plate measured in BioVeris M384 analyser. Tau/pSer422 standard curve was prepared by diluting ERK2- or P38-phosphorylated tau in cell extraction buffer; calculation of tau/pSer422 level in cells assumes 1 mole phospho-Ser422 phosphate/mole tau for the phospho-tau standard. Figure 5 shows a
25 standard curve and typical values obtained (on the basis of 1×10^6 cells) before and after okadaic acid treatment.

Claims

1. A monoclonal antibody or a fragment thereof produced by the hybridoma DSM ACC2762 or DSM ACC2763, which binds to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu.
2. The antibody of claim 1, wherein A1 is Asp, A2 is Met, A3 is Val, A4 is Asp and A5 is Asp.
3. The antibody of claim 1, wherein A1 is Asn, A2 is Leu, A3 is Leu, A4 is Glu and A5 is Glu.
4. The antibody of claim 1 or 2, wherein the antibody binds to tau comprising phospho-Ser422 and does not bind to tau comprising unphosphorylated Ser422.
5. The antibody according to any one of claims 1 to 4, wherein the antibody binds to MAP2 comprising phospho-Ser1808 and does not bind to MAP2 comprising unphosphorylated Ser1808.
6. The antibody of claim 2, wherein its K_d for the peptide consisting of Ser-Ile-Asp-Met-Val-Asp-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp is lower than 100nM.
7. A method for diagnostic of neurological disorder, which method comprises
 - (a) contacting a sample with the antibody of any one of claims 1 to 6; and
 - (b) detecting a complex formed between the antibody and tau or MAP2.
8. The method of claim 7, wherein neurological disorder is Alzheimer's disease.
9. A method for detecting tau comprising phospho-Ser422, which method comprises
 - (a) contacting a sample with the antibody of any one of claims 1 to 6; and
 - (b) detecting a complex formed between the antibody and tau.
10. A method for detecting MAP2 comprising phospho-Ser1808, which method comprises

- (a) contacting a sample with the antibody of any one of claims 1 to 6; and
 - (b) detecting a complex formed between the antibody and MAP2.
11. The method of claim 9 or 10, wherein the complex is detected by western blotting, immunohistochemistry or ELISA.
 - 5 12. An antibody according to any one of claims 1 to 6 for use in the in vitro diagnosis of neurological disorders.
 13. The antibody of claim 12, wherein the neurological disorder is Alzheimer's disease.
 14. An antibody according to any one of claims 1 to 6 for use in detecting tau comprising phospho-Ser422.
 - 10 15. An antibody according to any one of claims 1 to 6 for use in detecting MAP2 comprising phospho-Ser1808.
 16. A kit for diagnosis of neurological disorders, which comprises the antibody according to any one of claims 1 to 6.
 17. A kit of claim 16, wherein neurological disorder is Alzheimer's disease.
 - 15 18. A kit for detecting tau comprising phospho-Ser422, which comprises the antibody according to any one of claims 1 to 6.
 19. A kit for detecting MAP2 comprising phospho-Ser1808, which comprises the antibody according to any one of claims 1 to 6.
 - 20 20. A hybridoma DSM ACC2762 or DSM ACC2763 which produces the antibody according to any one of claims 1 to 6.

An Antibody Which Recognizes Phosphorylated Polypeptides

Abstract

The present invention relates to an antibody which recognizes an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser(PO₃H₂)-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5, and does not
5 bind to an epitope consisting of Ser-Ile-A1-A2-A3- A4-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-A5 , wherein A1 is Asp or Asn, A2 is Met or Leu, A3 is Val or Leu, A4 is Asp or Glu and A5 is Asp or Glu, a hybridoma producing the antibody, a kit comprising the antibody, and a method for diagnosis of a neurological disorder.

10 Figure 1

SEQUENCE LISTING

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<130> 23732
<150> EP06116550.2
<151> 2006-07-04
<160> 9
<170> PatentIn version 3.3
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<211> 441
<212> PRT
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20 25 30
Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Glu Ser Pro Leu
35 40 45
Gln Thr Pro Thr Glu Asp Gly Ser Glu Glu Pro Gly Ser Glu Thr Ser
50 55 60
Asp Ala Lys Ser Thr Pro Thr Ala Glu Asp Val Thr Ala Pro Leu Val
65 70 75 80
Asp Glu Gly Ala Pro Gly Lys Gln Ala Ala Ala Gln Pro His Thr Glu
85 90 95
Ile Pro Glu Gly Thr Thr Ala Glu Glu Ala Gly Ile Gly Asp Thr Pro
100 105 110
Ser Leu Glu Asp Glu Ala Ala Gly His Val Thr Gln Ala Arg Met Val
115 120 125
Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp Asp Lys Lys Ala Lys Gly
130 135 140
Ala Asp Gly Lys Thr Lys Ile Ala Thr Pro Arg Gly Ala Ala Pro Pro
145 150 155 160
Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg Ile Pro Ala Lys Thr Pro
165 170 175
Pro Ala Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro Lys Ser Gly
180 185 190

Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser Pro Gly Thr Pro Gly Ser
195 200 205

Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu Pro Lys
210 215 220

Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys
225 230 235 240

Ser Arg Leu Gln Thr Ala Pro Val Pro Met Pro Asp Leu Lys Asn Val
245 250 255

Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu Lys His Gln Pro Gly Gly
260 265 270

Gly Lys Val Gln Ile Ile Asn Lys Lys Leu Asp Leu Ser Asn Val Gln
275 280 285

Ser Lys Cys Gly Ser Lys Asp Asn Ile Lys His Val Pro Gly Gly Gly
290 295 300

Ser Val Gln Ile Val Tyr Lys Pro Val Asp Leu Ser Lys Val Thr Ser
305 310 315 320

Lys Cys Gly Ser Leu Gly Asn Ile His His Lys Pro Gly Gly Gly Gln
325 330 335

Val Glu Val Lys Ser Glu Lys Leu Asp Phe Lys Asp Arg Val Gln Ser
340 345 350

Lys Ile Gly Ser Leu Asp Asn Ile Thr His Val Pro Gly Gly Gly Asn
355 360 365

Lys Lys Ile Glu Thr His Lys Leu Thr Phe Arg Glu Asn Ala Lys Ala
370 375 380

Lys Thr Asp His Gly Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser
385 390 395 400

Gly Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser
405 410 415

Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Val
420 425 430

Ser Ala Ser Leu Ala Lys Gln Gly Leu
435 440

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Met Ala Asp Glu Arg Lys Asp Glu Gly Lys Ala Pro His Trp Thr Ser
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Ala Pro Leu Thr Glu Ala Ser Ala His Ser His Pro Pro Glu Ile Lys
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Asp Gln Gly Gly Ala Gly Glu Gly Leu Val Arg Ser Ala Asn Gly Phe
35 40 45

Pro Tyr Arg Glu Asp Glu Glu Gly Ala Phe Gly Glu His Gly Ser Gln
50 55 60

Gly Thr Tyr Ser Asn Thr Lys Glu Asn Gly Ile Asn Gly Glu Leu Thr
65 70 75 80

Ser Ala Asp Arg Glu Thr Ala Glu Glu Val Ser Ala Arg Ile Val Gln
85 90 95

Val Val Thr Ala Glu Ala Val Ala Val Leu Lys Gly Glu Gln Glu Lys
100 105 110

Glu Ala Gln His Lys Asp Gln Thr Ala Ala Leu Pro Leu Ala Ala Glu
115 120 125

Glu Thr Ala Asn Leu Pro Pro Ser Pro Pro Pro Ser Pro Ala Ser Glu
130 135 140

Gln Thr Val Thr Val Glu Glu Asp Leu Leu Thr Ala Ser Lys Met Glu
145 150 155 160

Phe His Asp Gln Gln Glu Leu Thr Pro Ser Thr Ala Glu Pro Ser Asp
165 170 175

Gln Lys Glu Lys Glu Ser Glu Lys Gln Ser Lys Pro Gly Glu Asp Leu
180 185 190

Lys His Ala Ala Leu Val Ser Gln Pro Glu Thr Thr Lys Thr Tyr Pro
195 200 205

Asp Lys Lys Asp Met Gln Gly Thr Glu Glu Glu Lys Ala Pro Leu Ala
210 215 220

Leu Phe Gly His Thr Leu Val Ala Ser Leu Glu Asp Met Lys Gln Lys
225 230 235 240

Thr Glu Pro Ser Leu Val Val Pro Gly Ile Asp Leu Pro Lys Glu Pro
245 250 255

Pro Thr Pro Lys Glu Gln Lys Asp Trp Phe Ile Glu Met Pro Thr Glu
260 265 270

Ala Lys Lys Asp Glu Trp Gly Leu Val Ala Pro Ile Ser Pro Gly Pro

275	280	285
Leu Thr Pro Met Arg Glu Lys Asp Val Phe Asp Asp Ile Pro Lys Trp 290 295 300		
Glu Gly Lys Gln Phe Asp Ser Pro Met Pro Ser Pro Phe Gln Gly Gly 305 310 315 320		
Ser Phe Thr Leu Pro Leu Asp Val Met Lys Asn Glu Ile Val Thr Glu 325 330 335		
Thr Ser Pro Phe Ala Pro Ala Phe Leu Gln Pro Asp Asp Lys Lys Ser 340 345 350		
Leu Gln Gln Thr Ser Gly Pro Ala Thr Ala Lys Asp Ser Phe Lys Ile 355 360 365		
Glu Glu Pro His Glu Ala Lys Pro Asp Lys Met Ala Glu Ala Pro Pro 370 375 380		
Ser Glu Ala Met Thr Leu Pro Lys Asp Ala His Ile Pro Val Val Glu 385 390 395 400		
Glu His Val Met Gly Lys Val Leu Glu Glu Glu Lys Glu Ala Ile Asn 405 410 415		
Gln Glu Thr Val Gln Gln Arg Asp Thr Phe Thr Pro Ser Gly Gln Glu 420 425 430		
Pro Ile Leu Thr Glu Lys Glu Thr Glu Leu Lys Leu Glu Glu Lys Thr 435 440 445		
Thr Ile Ser Asp Lys Glu Ala Val Pro Lys Glu Ser Lys Pro Pro Lys 450 455 460		
Pro Ala Asp Glu Glu Ile Gly Ile Ile Gln Thr Ser Thr Glu His Thr 465 470 475 480		
Phe Ser Glu Gln Lys Asp Gln Glu Pro Thr Thr Asp Met Leu Lys Gln 485 490 495		
Asp Ser Phe Pro Val Ser Leu Glu Gln Ala Val Thr Asp Ser Ala Met 500 505 510		
Thr Ser Lys Thr Leu Glu Lys Ala Met Thr Glu Pro Ser Ala Leu Ile 515 520 525		
Glu Lys Ser Ser Ile Gln Glu Leu Phe Glu Met Arg Val Asp Asp Lys 530 535 540		
Asp Lys Ile Glu Gly Val Gly Ala Ala Thr Ser Ala Glu Leu Asp Met 545 550 555 560		

Pro Phe Tyr Glu Asp Lys Ser Gly Met Ser Lys Tyr Phe Glu Thr Ser
565 570 575

Ala Leu Lys Glu Glu Ala Thr Lys Ser Ile Glu Pro Gly Ser Asp Tyr
580 585 590

Tyr Glu Leu Ser Asp Thr Arg Glu Ser Val His Glu Ser Ile Asp Thr
595 600 605

Met Ser Pro Met His Lys Asn Gly Asp Lys Glu Phe Gln Thr Gly Lys
610 615 620

Glu Ser Gln Pro Ser Pro Pro Ala Gln Glu Ala Gly Tyr Ser Thr Leu
625 630 635 640

Ala Gln Ser Tyr Pro Ser Asp Leu Pro Glu Glu Pro Ser Ser Pro Gln
645 650 655

Glu Arg Met Phe Thr Ile Asp Pro Lys Val Tyr Gly Glu Lys Arg Asp
660 665 670

Leu His Ser Lys Asn Lys Asp Asp Leu Thr Leu Ser Arg Ser Leu Gly
675 680 685

Leu Gly Gly Arg Ser Ala Ile Glu Gln Arg Ser Met Ser Ile Asn Leu
690 695 700

Pro Met Ser Cys Leu Asp Ser Ile Ala Leu Gly Phe Asn Phe Gly Arg
705 710 715 720

Gly His Asp Leu Ser Pro Leu Ala Ser Asp Ile Leu Thr Asn Thr Ser
725 730 735

Gly Ser Met Asp Glu Gly Asp Asp Tyr Leu Pro Ala Thr Thr Pro Ala
740 745 750

Leu Glu Lys Ala Pro Cys Phe Pro Val Glu Ser Lys Glu Glu Glu Gln
755 760 765

Ile Glu Lys Val Lys Ala Thr Gly Glu Glu Ser Thr Gln Ala Glu Ile
770 775 780

Ser Cys Glu Ser Pro Phe Leu Ala Lys Asp Phe Tyr Lys Asn Gly Thr
785 790 795 800

Val Met Ala Pro Asp Leu Pro Glu Met Leu Asp Leu Ala Gly Thr Arg
805 810 815

Ser Arg Leu Ala Ser Val Ser Ala Asp Ala Glu Val Ala Arg Arg Lys
820 825 830

Ser Val Pro Ser Glu Thr Val Val Glu Asp Ser Arg Thr Gly Leu Pro

835	840	845
Pro Val Thr Asp Glu Asn His Val Ile Val Lys Thr Asp Ser Gln Leu 850 855 860		
Glu Asp Leu Gly Tyr Cys Val Phe Asn Lys Tyr Thr Val Pro Leu Pro 865 870 875 880		
Ser Pro Val Gln Asp Ser Glu Asn Leu Ser Gly Glu Ser Gly Thr Phe 885 890 895		
Tyr Glu Gly Thr Asp Asp Lys Val Arg Arg Asp Leu Ala Thr Asp Leu 900 905 910		
Ser Leu Ile Glu Val Lys Leu Ala Ala Ala Gly Arg Val Lys Asp Glu 915 920 925		
Phe Ser Val Asp Lys Glu Ala Ser Ala His Ile Ser Gly Asp Lys Ser 930 935 940		
Gly Leu Ser Lys Glu Phe Asp Gln Glu Lys Lys Ala Asn Asp Arg Leu 945 950 955 960		
Asp Thr Val Leu Glu Lys Ser Glu Glu His Ala Asp Ser Lys Glu His 965 970 975		
Ala Lys Lys Thr Glu Glu Ala Gly Asp Glu Ile Glu Thr Phe Gly Leu 980 985 990		
Gly Val Thr Tyr Glu Gln Ala Leu Ala Lys Asp Leu Ser Ile Pro Thr 995 1000 1005		
Asp Ala Ser Ser Glu Lys Ala Glu Lys Gly Leu Ser Ser Val Pro 1010 1015 1020		
Glu Ile Ala Glu Val Glu Pro Ser Lys Lys Val Glu Gln Gly Leu 1025 1030 1035		
Asp Phe Ala Val Gln Gly Gln Leu Asp Val Lys Ile Ser Asp Phe 1040 1045 1050		
Gly Gln Met Ala Ser Gly Leu Asn Ile Asp Asp Arg Arg Ala Thr 1055 1060 1065		
Glu Leu Lys Leu Glu Ala Thr Gln Asp Met Thr Pro Ser Ser Lys 1070 1075 1080		
Ala Pro Gln Glu Ala Asp Ala Phe Met Gly Val Glu Ser Gly His 1085 1090 1095		
Met Lys Glu Gly Thr Lys Val Ser Glu Thr Glu Val Lys Gln Lys 1100 1105 1110		

Val	Ala	Lys	Pro	Asp	Leu	Val	His	Gln	Glu	Ala	Val	Asp	Lys	Glu
1115						1120					1125			
Glu	Ser	Tyr	Glu	Ser	Ser	Gly	Glu	His	Glu	Ser	Leu	Thr	Met	Glu
1130						1135					1140			
Ser	Leu	Lys	Ala	Asp	Glu	Gly	Lys	Lys	Glu	Thr	Ser	Pro	Glu	Ser
1145						1150					1155			
Ser	Leu	Ile	Gln	Asp	Glu	Ile	Ala	Val	Lys	Leu	Ser	Val	Glu	Ile
1160						1165					1170			
Pro	Cys	Pro	Pro	Ala	Val	Ser	Glu	Ala	Asp	Leu	Ala	Thr	Asp	Glu
1175						1180					1185			
Arg	Ala	Asp	Val	Gln	Met	Glu	Phe	Ile	Gln	Gly	Pro	Lys	Glu	Glu
1190						1195					1200			
Ser	Lys	Glu	Thr	Pro	Asp	Ile	Ser	Ile	Thr	Pro	Ser	Asp	Val	Ala
1205						1210					1215			
Glu	Pro	Leu	His	Glu	Thr	Ile	Val	Ser	Glu	Pro	Ala	Glu	Ile	Gln
1220						1225					1230			
Ser	Glu	Glu	Glu	Glu	Ile	Glu	Ala	Gln	Gly	Glu	Tyr	Asp	Lys	Leu
1235						1240					1245			
Leu	Phe	Arg	Ser	Asp	Thr	Leu	Gln	Ile	Thr	Asp	Leu	Gly	Val	Ser
1250						1255					1260			
Gly	Ala	Arg	Glu	Glu	Phe	Val	Glu	Thr	Cys	Pro	Ser	Glu	His	Lys
1265						1270					1275			
Gly	Val	Ile	Glu	Ser	Val	Val	Thr	Ile	Glu	Asp	Asp	Phe	Ile	Thr
1280						1285					1290			
Val	Val	Gln	Thr	Thr	Thr	Asp	Glu	Gly	Glu	Ser	Gly	Ser	His	Ser
1295						1300					1305			
Val	Arg	Phe	Ala	Ala	Leu	Glu	Gln	Pro	Glu	Val	Glu	Arg	Arg	Pro
1310						1315					1320			
Ser	Pro	His	Asp	Glu	Glu	Glu	Phe	Glu	Val	Glu	Glu	Ala	Ala	Glu
1325						1330					1335			
Ala	Gln	Ala	Glu	Pro	Lys	Asp	Gly	Ser	Pro	Glu	Ala	Pro	Ala	Ser
1340						1345					1350			
Pro	Glu	Arg	Glu	Glu	Val	Ala	Leu	Ser	Glu	Tyr	Lys	Thr	Glu	Thr
1355						1360					1365			
Tyr	Asp	Asp	Tyr	Lys	Asp	Glu	Thr	Thr	Ile	Asp	Asp	Ser	Ile	Met

1370		1375		1380
Asp Ala 1385	Asp Ser Leu Trp	Val 1390	Asp Thr Gln Asp	Asp 1395
Ile Met 1400	Thr Glu Gln Leu	Glu 1405	Thr Ile Pro Lys	Glu 1410
Glu Lys 1415	Glu Ala Arg Arg	Ser 1420	Ser Leu Glu Lys	His 1425
Lys Pro 1430	Phe Lys Thr Gly	Arg 1435	Gly Arg Ile Ser	Thr 1440
Lys Val 1445	Ala Lys Lys Glu	Pro 1450	Ser Thr Val Ser	Arg 1455
Arg Arg 1460	Lys Lys Ala Val	Tyr 1465	Lys Lys Ala Glu	Leu 1470
Thr Glu 1475	Val Gln Ala His	Ser 1480	Pro Ser Arg Lys	Phe 1485
Pro Ala 1490	Ile Lys Tyr Thr	Arg 1495	Pro Thr His Leu	Ser 1500
Arg Lys 1505	Thr Thr Ala Ala	Gly 1510	Gly Glu Ser Ala	Leu 1515
Val Phe 1520	Lys Gln Ala Lys	Asp 1525	Lys Val Ser Asp	Gly 1530
Ser Pro 1535	Glu Lys Arg Ser	Ser 1540	Leu Pro Arg Pro	Ser 1545
Pro Pro 1550	Arg Arg Gly Val	Ser 1555	Gly Asp Arg Asp	Glu 1560
Ser Leu 1565	Asn Ser Ser Ile	Ser 1570	Ser Ser Ala Arg	Arg 1575
Ser Glu 1580	Pro Ile Arg Arg	Ala 1585	Gly Lys Ser Gly	Thr 1590
Thr Thr 1595	Pro Gly Ser Thr	Ala 1600	Ile Thr Pro Gly	Thr 1605
Tyr Ser 1610	Ser Arg Thr Pro	Gly 1615	Thr Pro Gly Thr	Pro 1620
Arg Thr 1625	Pro His Thr Pro	Gly 1630	Thr Pro Lys Ser	Ala 1635

Pro Ser Glu Lys Lys Val Ala Ile Ile Arg Thr Pro Pro Lys Ser
1640 1645 1650

Pro Ala Thr Pro Lys Gln Leu Arg Leu Ile Asn Gln Pro Leu Pro
1655 1660 1665

Asp Leu Lys Asn Val Lys Ser Lys Ile Gly Ser Thr Asp Asn Ile
1670 1675 1680

Lys Tyr Gln Pro Lys Gly Gly Gln Val Gln Ile Val Thr Lys Lys
1685 1690 1695

Ile Asp Leu Ser His Val Thr Ser Lys Cys Gly Ser Leu Lys Asn
1700 1705 1710

Ile Arg His Arg Pro Gly Gly Gly Arg Val Lys Ile Glu Ser Val
1715 1720 1725

Lys Leu Asp Phe Lys Glu Lys Val Gln Ala Lys Val Gly Ser Leu
1730 1735 1740

Asp Asn Ala His His Val Pro Gly Gly Gly Asn Val Lys Ile Asp
1745 1750 1755

Ser Gln Lys Leu Asn Phe Arg Glu His Ala Lys Ala Arg Val Asp
1760 1765 1770

His Gly Ala Glu Ile Ile Thr Gln Ser Pro Gly Arg Ser Ser Val
1775 1780 1785

Ala Ser Pro Arg Arg Leu Ser Asn Val Ser Ser Ser Gly Ser Ile
1790 1795 1800

Asn Leu Leu Glu Ser Pro Gln Leu Ala Thr Leu Ala Glu Asp Val
1805 1810 1815

Thr Ala Ala Leu Ala Lys Gln Gly Leu
1820 1825

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Cys Ser Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp

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<211> 15
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Ser Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp
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<210> 5
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Ser Ile Asn Leu Leu Glu Ser Pro Gln Leu Ala Thr Leu Ala Glu
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Ser Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp
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<210> 7
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<223> PHOSPHORYLATION
<400> 7

Ser Ile Asn Leu Leu Glu Ser Pro Gln Leu Ala Thr Leu Ala Glu
1 5 10 15

<210> 8
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<223> Xaa =Asp or Asn

<220>
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<222> (4)..(4)
<223> Xaa =Met or Leu

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<223> Xaa =Val or Leu

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<223> Xaa =Asp or Glu

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<400> 8

Ser Ile Xaa Xaa Xaa Xaa Ser Pro Gln Leu Ala Thr Leu Ala Xaa
1 5 10 15

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<223> Xaa =Asp or Glu

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Ser	Ile	Xaa	Xaa	Xaa	Xaa	Ser	Pro	Gln	Leu	Ala	Thr	Leu	Ala	Xaa
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