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(54) Title: METHOD FOR SCREENING OF MODULATORS OF CALCINEURIN ACTIVITY (57) Abstract A method for screening of modulators of calcineurin is provided, which uses the interaction between calcineurin and superoxide dismutase. Modulators of calcineurin are potential candidates for drugs, e.g. for immunosuppressive drugs. The forming of a complex comprising calcineurin and superoxide dismutase is monitorable in the presence of potential activators or inhibitors of calcineurin. Complex formation is performed within the cell by the use of appropriate expression vectors or in vitro using isolated proteins. Preferably, complex formation is monitored by fluorescence detection, especially by laser fluctuation correlation spectroscopy.		

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5 Method for screening of modulators of calcineurin activity
Specification:

The invention relates to a method for screening of modula-
10 tors of calcineurin.

Calcineurin (E.C. 3.1.3.16) is a serine/threonine phospho-
protein phosphatase and is composed of a catalytic (calcineu-
rin A) and regulatory (calcineurin B) subunit (about 60 and
15 about 18 kDa, respectively). In mammals, three distinct genes
(A-alpha, A-beta, A-gamma) for the catalytic subunit have
been characterized, each of which can undergo alternative
splicing to yield additional variants. Although mRNA for all
three genes appears to be expressed in most tissues, two
20 isoforms (A-alpha and A-beta) are most predominant in brain.

Calcineurin has been cloned from various organisms including human (Guerini et al., 1989), (Guerini and Klee, 1989), (Kincaid et al., 1991), (Kuno et al., 1989), (Ito et al., 1989), (Muramatsu and Kincaid, 1993). The crystal structure has shown that calcineurin A contains a binuclear metal center with unknown enzymatic function (Griffith et al., 1995). Recombinant expression of rat calcineurin A subunit in bacteria or SF9-cells were not effective and yielded only poor enzymatic activities since calcineurin A is not stable in the absence of calcineurin B (Perrino et al., 1992), (Perrino et al., 1995), (Haddy and Rusnack, 1994). Coexpression of calcineurin A and calcium binding subunit calcineurin B yielded a more stable and active enzyme (Mondragon et al.,

1997). Calcineurin has been implicated in various neuronal signaling pathways (Klee et al., 1988), (Yakel, 1997) but the neuronal function is only poorly understood (Guerini, 1997).

5 Calcineurin is the only protein phosphatase known to be under the control of Ca^{2+} and calmodulin. Binding of Ca^{2+} and calmodulin is necessary for enzymatic activity. Calmodulin is bound by the catalytic subunit whereas the regulatory subunit possesses four Ca^{2+} binding sites.

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Calcineurin is discussed in the context of immunosuppression. It has been shown that calcineurin acts via the transcription factor NFAT (nuclear factor of activated T cells) on the T cell response. The functions of NFAT proteins are directly
15 controlled by calcineurin in a calcium- and calmodulin-dependent manner. Activation of NFAT by calcineurin is mediated by the cytosolic binding protein FKBP.

Substances which are able to block the calcineurin signal
20 pathway are suitable agents in order to block the T cell activation and thereby suppressing the immune response. Suppression of immune response has important clinical relevance, for example in transplantation surgery for preventing rejection episodes. Therefore, calcineurin as pharmacological
25 target is of great importance and several attempts were made to develop agents which block the calcineurin signal pathway. Examples of such immunosuppressive drugs are FK506 (Fujisawa) and cyclosporine (Novartis) (Liu et al, 1991). These antibiotics inhibit calcineurin phosphatase activity in the
30 presence of immunophilin receptor proteins (FKBP, cyclophilin) and thereby suppress immune response by preventing the activation of the T cell transcription factor NFAT (Liu et al., 1992), (Nelson et al., 1993). FK506 (tacrolimus) binds to the binding protein FKBP and thereby prevents calcineurin

from binding to FKBP. Accordingly the signal pathway is interrupted. No activation of the transcription factor NFAT is achieved and the T cell activation is disturbed.

5 Nevertheless, there are several severe disadvantages and side-effect of said drugs. In clinical trials with liver and renal transplant recipients it has been shown that FK506-based therapy was associated with increased toxicities in comparison to conventional therapy. Furthermore FK506 has
10 negative effects on the bone mineral physiology.

Besides the role of the calcineurin signal pathway in immune response it has been shown that calcineurin is involved in apoptosis induction by glutamate excitotoxicity in neuronal
15 cells (Ankarcrona et al., 1996). Low enzymatic levels of calcineurin have been associated with Alzheimers disease (Ladner et al., 1996), (Kayyali et al., 1997). Calcineurin inhibitors (FK506, Cyclosporin) prevented epileptogenesis in model organisms (Moriwaki et al., 1996). In the heart or in
20 the brain calcineurin also plays a key role in the stress response after hypoxia or ischemia (Butcher et al., 1997), (Hashimoto et al., 1998), (Molkentin et al., 1998).

In summary, calcineurin is a crucial target to develop new
25 substances suitable as drugs, especially as immunosuppressive drugs. Former screening systems using purified calcineurin and conventional assays like radioactive or HPLC assays (Klee, 1991), (Enz et al., 1994) did not lead to appropriate new substances. Therefore, the invention has the object to
30 provide a new screening system for modulators of calcineurin taking advantage of new insights into the signal pathway of calcineurin. By the use of this new screening system it is possible to develop new pharmaceuticals with respect to the field of transplantation surgery, cardiac infarction and

apoplexy, chronic or acute neurodegeneration and inflammatory diseases, for example. This object is solved by a method according to claim 1. Preferred embodiments of the inventive method are depicted in the dependent claims 2 to 17. A kit,
5 vectors, cells and a peptide suitable for performing the inventive method are claimed in claim 18 to 23. The wording of all claims is hereby made to the content of the specification by reference.

- 10 The inventive method is based on results showing that a physiological interaction between calcineurin and superoxide dismutase takes place which provides a suitable target for developing of a new screening system.
- 15 For a long time it was not understood why recombinant or even purified calcineurin exhibited only 1 to 2 % of the specific activity estimated in crude brain extracts until it was detected that the binuclear metal center of the enzyme contains a redoxsensitive Fe^{2+} (Yu et al., 1997). After
20 calcium activation or during purification procedure the Fe^{2+} is oxidized by oxygen species and turns the enzyme inactive (Stemmer et al., 1995), (Wang et al., 1996).

Recently it has been shown that copper/zinc superoxide
25 dismutase (CuZnSOD, EC 1.15.1.1) protects calcineurin against oxidative inactivation (Wang et al., 1996). The phosphatase activity of calcineurin is strongly dependent on the presence of calcium and calmodulin. The addition of Ca^{2+} in the presence of calmodulin leads to a drastic increase in activi-
30 ty. But during several minutes this activity is lost. By the addition of copper/zinc superoxide dismutase the activity can be maintained.

Superoxide dismutase (SOD) dismutates the hyperoxide anion (superoxide) into hydroperoxide and molecular oxygen. There are two forms of this enzyme: the mitochondrial form containing manganese and the cytosolic form containing copper and zinc. In general superoxide dismutase is considered to be a catcher of radicals and is discussed in the field of detoxification of reactive oxygen species. Therefore, the role of superoxide dismutase in the protection of the activity of calcineurin found by Wang et al. was considered to be the result of general redox function of superoxide dismutase. Now, surprising results of the inventor lead to the knowledge that a physiological interaction between calcineurin and superoxide dismutase takes place. Several mutants of copper/zinc superoxide dismutase lacking the enzymatic function showed the protective effect on the activity of calcineurin. That means that the effect of CuZnSOD is not due to the function of superoxide dismutase in redox regulation. These results teach that superoxide dismutase interacts physiologically with calcineurin and that CuZnSOD is one component of the calcineurin pathway which is important for the physiological functions of calcineurin.

These results are used to develop a new screening system for modulators of calcineurin in order to find inhibitors or activators of the calcineurin signal pathway. The inventive method is based on the complex formation between calcineurin and superoxide dismutase in the presence of potential modulators of this physiological interaction. If a potential modulator disturbs the complex formation, this substance is a good candidate for inhibiting the calcineurin signal pathway and could possibly be used as immunosuppressive drug, for example. On the other hand it could be favourable to identify a substance which promotes complex formation and thereby stimulates the calcineurin signal pathway, e.g. the T cell

response in result. Such a substance could be used in order to strengthen immune response. By the term "modulator" is meant any substance which influences the complex formation relating to the inventive method. Additionally is meant any substance which influences the interaction between calcineurin and its substrates, e.g. the peptide RII. Furthermore is meant any substance which influences the superoxide dismutase and/or calcineurin on the transcriptional, the translational and/or the posttranslational level.

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Calcineurin as used in the inventive method is build up by the regulatory subunit A and the catalytic subunit B. The presence of both subunits is essential for physiological activity of calcineurin. Nevertheless, it is possible to perform the inventive method using only one of the subunits. There are several isoforms of calcineurin consisting of subunit calcineurin B and one out of the group comprising subunit calcineurin A-alpha, A-beta and A-gamma. Each isoform represents a special cell and tissue specific distribution. Therefore, the choice of isoform could be crucial for cell and tissue specificity of the substance to be screened. With respect to clinical application of the substances to be screened preferably human forms of the proteins are used.

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Furthermore it is preferred to perform the inventive method in the presence of calmodulin and calcium, because the activity of calcineurin is dependent on these factors. Preferably the cytosolic form of superoxide dismutase containing copper and zinc is used for complex formation, because interaction between the mitochondrial form of superoxide dismutase containing e.g. manganese normally does not occur under physiological conditions. The complex formation is performed in the presence of at least one potential modulator of calcineurin or the calcineurin signal pathway, respec-

tively. The complex comprising calcineurin A, calcineurin B, superoxide dismutase and preferably calmodulin is the target for potential modulators which could stabilize or disturb the complex.

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Advantageously, the complex formation is monitored during the whole process. It is possible to add the modulator before or after the complex formation has been performed. Preferably the modulator is added before complex formation because the
10 effect of a weak modulator will possibly not be monitorable when complex formation has already finished.

In principle, there are two possibilities to monitor the complex formation. Firstly, the complex formation is directly
15 monitored by the use of labeled components in the complex, preferably by fluorescence detection. Secondly, the complex formation is monitored by the activity of the complex, especially the enzymatic activity of calcineurin. This second method can be performed in addition to the firstly mentioned
20 method or as an alternative. Clearly, the inventive method is not restricted by the method for detecting the influence of the modulator on complex formation.

In a preferred embodiment of the invention, a mixture of
25 substances comprising at least one potential modulator is analyzed by the inventive method. By isolating the complex together with the possibly interacting modulator it is possible to separate the modulator out of the mixture and to identify it by common methods.

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In one preferred embodiment of the invention the calcineurin and/or superoxide dismutase are labeled. Especially preferred is the use of fluorescent labels. Preferably, the labeled proteins are fusion proteins comprising a fluorescent prote-

in, e.g. enhanced green fluorescent protein (EGFP). These fusion proteins are provided by genetic engineering methods. It is also possible to label said proteins by other methods known to experts in the art, e.g. by the use of radioactive isotopes which are incorporated into the proteins.

Advantageously the components of the complex, i.e. calcineurin and superoxide dismutase are expressed in the cell, especially in an eukaryotic cell, as fluorescent fusion proteins. By the use of laser fluctuation correlation spectroscopy the complex formation of labeled proteins is monitored directly within the cell. This embodiment of the invention is described in greater detail in the example. The invention comprises several vectors useful for the expression of calcineurin and/or superoxide dismutase in eukaryotic cells. These vectors encode the proteins, especially CuZnSOD and the different subunits of calcineurin, as fusion proteins in connection with the fluorescent protein EGFP (enhanced green fluorescent protein). EGFP is only one example of possible labels useful in respect of the inventive method. Furthermore, the invention comprises cells, especially eukaryotic cells, stably transfected with the above-mentioned vectors thereby expressing superoxide dismutase and/or calcineurin. Preferably, these proteins are coexpressed, i.e. expressed within the same cell.

In an especially preferred embodiment, the genetic information of fusion proteins is integrated in the cell by homologous recombination. That means that the gene encoding the recombinant protein, especially the fluorescent fusion protein, is incorporated in the genome of the cell in the place of the naturally occurring gene. This leads to a cell essentially lacking the natural protein. By the use of such cells it is possible to identify modulators by the inventive method which

influence the transcriptional, translational or posttranslational level of calcineurin and/or superoxide dismutase expression.

5 In another embodiment of the inventive method the components of the complex are isolated and preferably purified before complex formation is performed in vitro. Advantageously, the proteins are provided with a tag in order to facilitate purification, e.g. a histidin (his) tag consisting of several
10 histidines in sequence which permits affinity purification by known procedures. Corresponding vectors encoding the tagged proteins are comprised by the invention. These vectors are especially useful as prokaryotic expression vectors. Furthermore, the invention comprises cells bearing said vectors.

15 Advantageously, following purification of the his-tagged proteins the tag is excised by appropriate enzymatic digestion, e.g. by the use of cathepsin-C or carboxypeptidase-A. Especially preferred is the purification of calcineurin by
20 ferro-nitrilotriacetat-metal (Fe-NTA) affinity chromatography and the purification of superoxide dismutase by copper/-zinc-nitrilotriacetat-metal (CuZn-NTA) affinity chromatography. Nevertheless, other purification procedures known to experts in the art are possible. Naturally occurring protein
25 could also be used in the inventive manner.

Besides the use in purification of calcineurin and/or superoxide dismutase Ni (nickel)-NTA, Fe-NTA and/or Cu/Zn-NTA is used to immobilize the his-tagged calcineurin and/or super-
30 oxide dismutase in order to isolate naturally occurring ligands of these proteins using this inventive matrix. By the term "ligand" is meant any low- or highmolecular endogenous, exogenous or synthetic substance which interacts with said proteins. This could be a peptide, protein, carbohydrate,

lipid, nucleic acid or a synthetic polymer, for example. These so-identified ligands are potential candidates for modulators of the calcineurin signal pathway.

- 5 When performing complex formation in vitro it could be preferred to add calmodulin and/or calcium to the reaction because these factors are necessary for enzymatic activity of calcineurin.
- 10 In another preferred embodiment of the invention the complex formation is monitored indirectly by analyzing the enzymatic activity of calcineurin. As outlined above the phosphatase activity of calcineurin is strictly dependent on the interaction with superoxide dismutase. Therefore, it is possible to
- 15 monitor the complex formation indirectly by the measurement of phosphatase activity according to standard procedures. This is especially preferred if the laboratory equipment to perform fluorescent measurements as described above is not available. Furthermore, enzymatic analysis could be used in
- 20 addition to fluorescence detection like laser fluctuation correlation spectroscopy, e.g. as control.

Preferably the enzymatic activity is analyzed by the use of a labeled substrate of calcineurin. The substrate is preferably

25 labeled by fluorescence. One especially preferred substrate is the peptide RII characterized by the sequence:

30 Asp - Leu - Asp - Val - Pro - Ile - Pro - Gly - Arg -
Phe - Asp - Arg - Arg - Val - Ser - Val - Ala - Ala -
Glu.

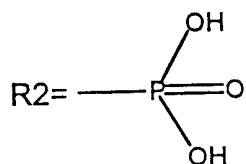
In a preferred embodiment this peptide carries a fluorescent label at serine in position 15. This amino acid is

labeled with fluoresceine by incubating the peptide with fluoresceine-phosphoamidit, thereby providing a labeled substrate (RII-Fluophos). RII interacts with the active center of calcineurin, but it is not converted by the phosphatase. Hereby it is possible to label calcineurin in the active state. Furthermore, it is possible to phosphorylate RII-Fluophos at the fluoresceine moiety as depicted below at tyrosine residues. Due to the phosphorylation RII-Fluophos loses its fluorescence and thereby provides a phosphatase substrate which becomes fluorescent subsequent to dephosphorylation.

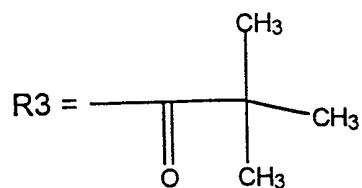
15

R1 = H

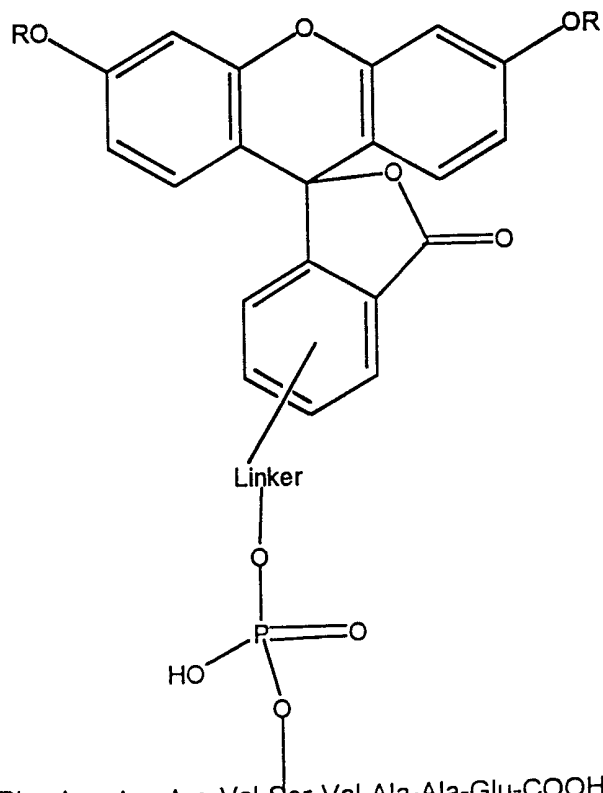
20



25



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-NH₂-Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu-COOH-

This peptide could be provided synthetically or it is expressed by cells, especially eucaryotic cells, which have been transfected with appropriate vectors encoding said peptide or other peptides useful as phosphatase substrates. In one
5 embodiment of the invention the fluorescent peptide is used as peptide label in fluorescence microscopy. This provides another method in order to analyze the active state of the calcineurin/superoxide complex.

- 10 The inventive method as outlined above is suitable for developing a high-throughput bioassay to identify inhibitors and/or activators of the calcineurin signal pathway. Details are described in the example.
- 15 The invention comprises the use of inhibitors of activators of the calcineurin signal pathway for the treatment of acute and/or chronic neurological and cardiovascular diseases like Alzheimer, Parkinson, epilepsy, ischemia and heart-failure. Furthermore the use as immunosuppressive drugs, e.g. in the
20 field of transplantation surgery and inflammatory diseases is included.

Finally the invention comprises a kit for screening of modulators of calcineurin. The kit provides calcineurin and
25 superoxide dismutase enabling complex formation for the screening for modulators of calcineurin as described above. In a first embodiment of the kit the components of the complex are provided as proteins. This kit is suitable for performing the inventive method in vitro. In a second embodi-
30 ment of the kit the proteins are provided in the form of vectors. These vectors have to be transformed/transfected into cells leading to the expressed proteins. These vectors are prokaryotic or eukaryotic expression vectors, respectively, and could be used to produce the proteins for the in

vitro assay or for the assay using complete cells as described above. In a third embodiment of the inventive kit cells transformed/transfected with the said vectors are provided saving the step of transforming/transfecting for the user.

5 For details of the inventive kit reference is made to the above description.

The new approach to identify new substance classes of calcineurin/CuZnSOD inhibitors comprises inter alia:

10

- coexpression of CuZnSOD/calcineurin A and calcineurin B to generate a oxidative stable enzyme which is suitable for drug screening,

15

- efficient purification of CuZnSOD on CuZn-nitrilotriacetat-metal affinity chromatography to retain enzymatic activity,

- efficient purification of calcineurin on Fe-nitrilotriacetat-metal affinity chromatography to retain enzymatic activity and prohibit Fe^{2+} oxidation,

20

- identifying that mutations in the CuZnSOD associated with a neurological disorder (amyotrophic lateral sclerosis) are also critical for calcineurin-CuZnSOD interaction,

- use of fluorescent labeled recombinant CuZnSOD and calcineurin to screen for CuZnSOD/calcineurin activators or

25

- inhibitors,

- use of fluorescent labeled RII-peptide and calcineurin to screen for calcineurin activators or inhibitors,

- identification of calcineurin/CuZnSOD inhibitors or activators by using the recombinant enzymes as affinity ligands to

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- purify new drugs from natural sources,

- inclusion of all isoforms, all known and two newly identified splicevariants into the screening procedure, which allows the identification of less toxic and tissue specific

drugs which are more suitable for the therapeutical treatment of different clinical indications.

The described features of the invention and further features
5 result in greater detail from the examples in combination with the subclaims. The features could be realized in combination with each other or alone.

Example

10

1. Cloning of CuZnSOD transcript from human brain poly-A-RNA - Cloning of human CuZnSOD was performed by reverse transcription PCR using human brain poly-A-RNA as template (Clontech, Palo Alto, CA, USA). The oligonucleotides SODs1
15 5'-ttc cgt tgc agt cct cgg aac-3', SODas1 5'-taa ggg gcc tca gac tac atc-3', SOD-PQE60s2 5'-caa gcc atg gcg acg aag gcc gtg tgc gtg ctg-3', SOD-PQE60as2 5'-gaa gat ctt tgg gcg atc cca att aca cca c-3', SOD-PQE30-s2 5'- cgc gga tcc gcg acg aag gcc gtg tgc gtg -3' and SOD-PQE30-as2 5'-ggg ttc gaa tta
20 ttg ggc gat ccc aat tac-3' were supplied by Interactiva (Ulm, Germany). Reverse transcription was performed with the SODas1 primer and 100 ng of poly-A-RNA according to the manufacturer's protocol (Expand reverse transcriptase, Boehringer Mannheim, Germany). The human CuZnSOD cDNA was amplified by
25 nested PCR. The first PCR was performed in 20 μ l, using 0,5 μ l reverse transcription product, 10 μ M SODs1 and SODas1 primers, 300 μ M dNTPs, 2 μ l of the manufacturer's 10 x PCR buffer and 2.5 U Taq-polymerase with 30 cycles of 1 min 95 $^{\circ}$ C, 1 min 45 $^{\circ}$ C, 1 min 72 $^{\circ}$ C followed by a second PCR (50 μ l)
30 with 5 μ l of the purified first PCR product, 10 μ M SOD-PQE60s2 and SOD-PQE60as2 primers, 300 μ M dNTPs, 5 μ l of the manufacturer's PCR buffer and 2,5 U Taq-polymerase with 30 cycles of 1 min 95 $^{\circ}$ C, 1 min 60 $^{\circ}$ C, 1 min 72 $^{\circ}$ C (Taq-polymerase, Pharmacia Biotech, Uppsala, Sweden). For the subcloning

into pQE30 expression vector the primers SOD-pQE30-s2 and SOD-pQE30-as2 were used instead of SOD-pQE60s2/SOD-pQE60as2.

2. Subcloning of human CuZnSOD into pQE60 expression vector

5 **(C-terminal fusion protein)** - The SOD-pQE60 PCR product was purified by gel extraction prior to NcoI/BglIII restriction (New England Biolabs). In order to generate a C-terminal histidin tag fusion protein the CuZnSOD transcript was ligated into the NcoI/BglIII treated prokaryotic expression
10 vector pQE60 (QIAexpress expression kit type IV and type ATG, Qiagen, Hilden, Germany). For selection, amplification and sequencing of the CuZnSOD vector construct (CuZnSOD-pQE60), 10 µl ligation product was transformed into E.coli M15[pREP4] cells (QIAexpress expression kit type ATG, Qiagen, Hilden,
15 Germany). Correct reading frames and exclusion of mismatches were confirmed by radioactive and automated sequencing on both strands (T7-sequencing kit, Pharmacia Biotech, Uppsala, Sweden; ABI 377 sequencer, Applied Biosystems, USA).

3. Subcloning of human CuZnSOD into pQE30 expression vector

(N-terminal fusion protein) - The SOD-pQE30 PCR product was purified by gel extraction prior to direct ligation into the pCR2.1 vector according to the manufacturer's protocols (TA-Cloning Kit, Invitrogen, De Schelp, Netherlands). After
25 amplification and plasmid purification the pCR2.1-CuZnSOD vector construct was restricted with BamHI to yield a CuZnSOD transcript extended at the 3'-end with the sequence 5'-GAATTCAGCACACTGGCGGCCGTTACTAGTGGATCC-3' which originates from PCR2.1 vector and includes additional EcoRI/BstX-I/
30 SpeI/BamHI restriction sites. In order to generate a N-terminal histidin tag fusion protein the extended transcript was ligated into the BamHI/HindIII treated prokaryotic expression vector pQE30 (QIAexpress expression kit type IV, Qiagen, Hilden, Germany), blunted by incubation with Klenow-DNA-poly-

merase and circularized by a second treatment with T4-DNA-Li-
gase (Boehringer Mannheim, Germany). For selection, amplifi-
cation and sequencing of the CuZnSOD vector construct (CuZn-
SOD-pQE30), 10 μ l ligation product was transformed into
5 E.coli M15[pREP4] cells (QIAexpress expression kit type IV
and type ATG, Qiagen, Hilden, Germany). Correct reading
frames and exclusion of mismatches were confirmed by radioac-
tive and automated sequencing on both strands (T7-sequencing
kit, Pharmacia Biotech, Uppsala, Sweden; ABI 377 sequencer,
10 Applied Biosystems, USA).

**4. Site directed mutagenesis (point mutations associated
with the neurological disorder Amyotrophic Lateral Sclerosis
and important for calcineurin/CuZnSOD protein interaction) -**

15 Amino acid substitutions were introduced according to the
manufacturer's protocol, using the primers SOD-PQE60-A4V
(5'-caa gcc atg gcg acg aag gtc gtg-3'), SOD-A4V (5'-tcc gcg
acg aag gtc gtg tgc gtg ctg-3'), SOD-G37R (5'-gg aag catt aaa
aga ctg act gaa ggc-3'), SOD-D90A (5'-aat gtg act gct gcc aaa
20 gat ggt gtg-3'), SOD-G93A (5'-gct gac aaa gat gct gtg gcc gat
gtg-3'), SOD-AflIII (5'-acg cag gaa aga aca tgt gag caa
aag-3'), SOD-BglII (5'-acg cag gaa aga aga tct gag caa
aag-3') and the expression vector CuZnSOD constructs CuZn-
SOD-pQE30 and CuZnSOD-pQE60, respectively (Chameleon site
25 directed mutagenesis kit, Stratagene, San Diego, CA, USA).
Incorporation of the site-directed mutations was confirmed by
DNA sequencing of the expression vector. Site directed
mutagenesis yielded eight additional vector sequences corres-
ponding to eight protein sequences with clinical relevant
30 amino acid substitutions:

Vector-construct	amino acid subst. (pos. in protein)	nucleic acid subst. (pos. in sequ. prot.)
CuZnSOD-pQE60	WT = wild-type	= SEQ ID NO 15

CuZnSOD-pQE60-(A4V)	Ala-4	-> Val-4	c-128	-> t-128
CuZnSOD-pQE60-(G37R)	Gly-37	-> Arg-37	g-226	-> a-226
CuZnSOD-pQE60-(D90A)	Asp-90	-> Ala-90	a-386	-> c-386
CuZnSOD-pQE60-(G93A)	Gly-93	-> Ala-93	g-395	-> c-395

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CuZnSOD-pQE30	WT = wild-type = SEQ ID NO 13			
CuZnSOD-pQE30-(A4V)	Ala-4	-> Val-4	c-161	-> t-161
CuZnSOD-pQE30-(G37R)	Gly-37	-> Arg-37	g-259	-> a-259
CuZnSOD-pQE30-(D90A)	Asp-90	-> Ala-90	a-419	-> c-419
10 CuZnSOD-pQE30-(G93A)	Gly-93	-> Ala-93	g-428	-> c-428

5. Recombinant expression and purification of wild-type and mutated CuZnSOD

The CuZnSOD-pQE60 or CuZnSOD-pQE30 vector transformed E.coli M15[pREP4] cells were plated on LB /

15 ampicillin (100 µg/ml) / kanamycin (25 µg/ml) agar. Expression cultures were grown in 250 ml LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) until the OD₆₀₀ was 0.6.

Constitutive leakage expression of human CuZnSOD was fully prevented by the repressor plasmid pREP4-lacI. Production of

20 the human CuZnSOD fusion proteins was induced by addition of IPTG (1 mM). After two hours the bacterial cells were harvested by centrifugation (4000 g, 20 min), resuspended in 8 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) and homogenized by three freeze thaw cycles and sonication

25 on ice (Bandelin sonoplus GM70, 300 W, 3 x 10 sec). The lysate was centrifuged (10.000 g, 20 min) and incubated with 750 µl CuZn-NTA (nitrilotriacetat)-agarose for batch affinity binding for 1 h at 4 °C (Qiagen expressionist kit, Qiagen, Hilden, Germany). CuZn-NTA-agarose was prepared from Ni-

30 NTA-agarose (Qiagen expressionist kit, Qiagen, Hilden, Germany) by subsequent washes in:

- 1) 2 volumes of bidestilled water
- 2) 3 volumes of regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)

- 3) 5 volumes bidestilled water
- 4) 3 volumes 2% SDS
- 5) 1 volume 25% ethanol
- 6) 1 volume 50% ethanol
- 5 7) 1 volume 75% ethanol
- 8) 5 volumes 100% ethanol
- 9) 1 volume 75% ethanol
- 10) 1 volume 50% ethanol
- 11) 1 volume 25% ethanol
- 10 12) 1 volume bidestilled water
- 13) 5 volumes 100 mM Na-EDTA pH 8.0
- 14) 5 volumes bidestilled water
- 15) 2 volumes 100 mM CuSO_4 / 100 mM ZnSO_4 / 1 mM reduced glutathion / 1 mM dithiothreitol
- 15 16) 2 volumes bidestilled water
- 17) 2 volumes regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)
- 18) 2 volumes buffer buffer A2 (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl, 200 μM CuSO_4 / 200 μM ZnSO_4 / 1 mM
- 20 reduced glutathion / 1 mM dithiothreitol

The batch was applied to a 30 ml chromatography column, washed with 15 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) and subsequently with 8 ml buffer B

25 (20 mM Tris-HCl pH 7.9, 60 mM imidazole, 500 mM NaCl). C-terminal or N-terminal histidin tagged CuZnSOD was eluted three times with 1,2 ml buffer C (10 mM Tris-HCl, 500 mM imidazole, 250 mM NaCl). Purity and correct expression products were checked by immunoblotting or N-terminal protein

30 sequencing after separation of 20 μl eluate in SDS-PAGE (discontinuous 12,5 % SDS-PAGE). To examine the protein levels in bacterial culture all CuZnSOD variants were induced synchronously at $\text{OD}_{600} = 0.6$ with 1 mM IPTG. After 1 h, 2 h, 3 h, 4 h and 20 h, aliquots (1 ml) of E.coli cultures were

taken, centrifuged and homogenized in buffer A as described. The pellet was resuspended in 1 ml H₂O. Subsequently, 20 µl of the supernatant (soluble fractions) or 20 µl of the sonicated pellet suspension (insoluble fractions) were mixed
5 with 7 µl of denaturing sample buffer (10 % SDS, 10 % mercaptoethanol, 20 % glycerol, 130 mM Tris-HCl pH 6.8, 0.03 % bromphenol blue). The samples were heated for 2 minutes at 80 °C and analyzed by 12 % SDS-PAGE. After coomassie staining, the electropherograms were digitized with a CCD camera
10 (Gel Doc 1000, BioRAD) and analyzed by densitometry using NIH-Image software (1.61).

6. Processing of CuZnSOD - In order to remove the nonphysiological histidin tag and to yield CuZnSOD useful for
15 clinical applications the N-terminal histidin tagged CuZnSOD was proteolytically processed with cathepsin-C or the C-terminal variant was processed with carboxypeptidase-A according to the manufacturer's protocols (Boehringer-Mannheim, Mannheim, Germany). Treatment with cathepsin-C yielded a processed
20 CuZnSOD starting with the amino acids NH₂-GSAT KAVCVLKGDGP (indicated in sequence protocol CuZnSOD-pQE30 SEQ ID NO 13). C-terminal fusion protein was yielded the C-terminal amino acid sequence VIGIAQR-COOH (indicated in sequence protocol CuZnSOD-pQE30 SEQ ID NO 13). Verification was done
25 by peptide sequencing.

7. Reactivation of CuZnSOD - In order to yield physiologically relevant active homodimeric CuZnSOD, the CuZn-NTA eluate was ultrafiltered through a 5 kD membrane (Omegacell,
30 Filtron, Northborough, MA, USA). For buffer exchange the samples were washed three times in reconstitution buffer (50 mM sodium citrate pH 5.5, 1 mM DTT). The protein solutions were incubated at 8 °C for 7 days (250 µg/ml protein). After distinct time intervals aliquots of the refolding mixture

were either analyzed by native gel electrophoresis (2.6 μ g CuZnSOD) and activity staining or assayed in a spectrophotometer (0.5 - 1 μ g CuZnSOD, superoxide dismutase assay kit, Calbiochem, San Diego, CA, USA). For visualization of protein bands native gels were stained with coomassie blue. For the production of larger CuZnSOD amounts M15-E.coli cells were subsequently grown in 15 ml, 200 ml, 2500 ml and 20 L flasks. Refolded CuZnSOD proteins were dialyzed against 100 volumes of buffer D (10 mM Tris-HCl 0.1 % Saccharose) and lyophilized.

8. SOD assay and activity staining - Enzymatic activity of the CuZnSOD proteins were either analyzed by 10 % native gel electrophoresis and activity staining with nitrotetrazolium blue dye or by a quantitative spectrophotometrically assay according to published protocols (Beauchamp and Fridovich, 1971; Nebot et al., 1993). Protein yields were determined by the Bradford method (Protein assay kit, BioRAD, Hercules, CA, USA). The concentration of purified CuZnSOD was determined spectrophotometrically using the extinction coefficient $\epsilon_{265} = 1.84 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

9. Subcloning of human CuZnSOD into pEGFP eukaryotic expression vector and generation of stable transfected PC12 cells (C-terminal fusion protein with enhanced green fluorescent protein as a fluorescent marker/label) - Using 10 μ M of the primers SOD-pEGFP-s 5'-ccg cgg gcc cgc cat ggc gac gaa ggc cgt gtg cgt gc-3' and SOD-pEGFP-as 5'-gct cac cat ggt ggt ttg ggc gat ccc aat tac acc ac-3', 10 ng CuZnSOD-pQE60 vector, 300 μ M dNTPs, 5 μ l of the manufacturer's PCR buffer and 2,5 U Taq-polymerase with 25 cycles of 1 min 95 °C, 1 min 60 °C, 1 min 72°C (50 μ l total volume, Taq-polymerase, Pharmacia Biotech, Uppsala, Sweden) a PCR product was generated which was cleaved by ApaI/NcoI digestion. The purified

PCR product was ligated into ApaI/NcoI treated pEGFP-N3 vector (Clontech Laboratories, Palo Alto, CA, USA). After amplification in XL2-Blue cells (25 µg/ ml kanamycin) and plasmid purification the CuZnSOD-pEGFP vector construct was
5 transfected into PC12 rat adrenal pheochromocytoma cells using the CalPhosTM Transfection Kit according to the manufacturer's protocols (Clontech Laboratories, Palo Alto, CA, USA). Stable transfected CuZnSOD-pEGFP clones were selected by fluorescence microscopy (excitation 488 nm/ emission 520
10 nm, MRC 1024 confocal microscope, BioRAD Laboratories, Hercules, CA, USA).

10. Cloning of the regulatory subunit human calcineurin-B -

Cloning of human calcineurin-B was performed by reverse
15 transcription PCR using human brain poly-A-RNA as template (Clontech, Palo Alto, CA, USA). The oligonucleotides CNBa-s1 5'-ccg ccg acc cgc cga gca-3', CNBa-as1 5'-ggc act ctc tga taa gag-3', CNBa-s3 5'-gga att ccc cgg gga aag agg aga aat
20 taa cta tgg gaa atg agg caa gtt atc-3', CNBa-as2 5'-ttc cgg gcc caa gct tct aat taa tca cac atc tac cac cat c-3' were supplied by Interactiva (Ulm, Germany). Reverse transcription was performed with the CNBa-as1 primer and 100 ng of poly-A-RNA according to the manufacturer's protocol (Expand reverse transcriptase, Boehringer Mannheim, Germany). The
25 human calcineurin-B cDNA was amplified by nested PCR. The first PCR was performed in 20 µl, using 0.5 µl reverse transcription product, 10 µM CNBa-s and CNBa-as1 primers, 300 µM dNTPs, 2 µl of the manufacturer's 10 x PCR buffer and 2.5 U Pfu-polymerase with 20 cycles of 1 min 95 °C, 1 min 55 °C, 2
30 min 72 °C followed by a second PCR (50 µl) with 5 µl of the purified first PCR product, 10 µM CNBa-s3 and CNBa-as2 primers, 300 µM dNTPs, 5 µl of the manufacturer's PCR buffer and 2.5 U Pfu-polymerase with 20 cycles of 1 min 95 °C, 1 min

55 °C, 1 min 72°C (Pfu-polymerase, Stratagene, San Diego, CA, USA).

11. Cloning of the catalytic subunit human calcineu-

5 **rin-A-Alpha and splicevariants** - Cloning of human calcineu-
rin-A-alpha was performed by reverse transcription PCR using
human brain poly-A-RNA as template (Clontech, Palo Alto, CA,
USA). The oligonucleotides CNAa-s1 5'-gcg tcg ctg tcc tcc ggc
agc-3', CNAa-as1 5'-gtg aac agg aag tgg tca ctg-3', CNAa-s2
10 5'-cat gcc atg gatc cat gtc cga gcc caa ggc-3', CNAa-as4
5'-tcc ccc cgg ggta ccc tag tta atc act gaa tat tgc tgc tat
tac-3' were supplied by Interactiva (Ulm, Germany). Reverse
transcription was performed with the CNAa-as1 primer and 100
ng of poly-A-RNA according to the manufacturer's protocol
15 (Expand reverse transcriptase, Boehringer Mannheim, Germany).
The human calcineurin-A-Alpha cDNA was amplified by nested
PCR. The first PCR was performed in 25 µl, using 0,5 µl
reverse transcription product, 10 µM CNAa-s1 and CNAa-as1
primers, 200 µM dNTPs, 2.5 µl of the manufacturer's 10 x PCR
20 buffer and 1.25 U Pfu-polymerase with 30 cycles of 40 seconds
at 95 °C, 40 seconds at 55 °C, 3 min 72 °C followed by a
second PCR (25 µl) with 2.5 µl of the purified first PCR
product, 10 µM CNAa-s2 and CNAa-as2 primers, 200 µM dNTPs,
2.5 µl of the manufacturer's PCR buffer and 2.5 U Pfu-poly-
25 merase with 25 cycles of 40 seconds at 95 °C, 40 seconds at
55 °C, 3 min 72 °C (Pfu-polymerase, Stratagene, San Diego,
CA, USA).

Hereby a new splicevariant was identified, which is impor-
30 tant for calcium regulation and proteolytic regulation of
calcineurin-A. The splicevariant lacks the whole catalytic
phosphatase domain and part of calcineurin-binding-site
(Elimination of nucleic bases 208-1317 in sequence protocol

CNAa1-pQE30 SEQ ID NO 17). The corresponding vector is named CNAa3-pQE30:

Location/Qualifiers

- 5 151..606 /note="splicevariant: Calcineurin A alpha 1 lacking
phosphatase domain, newly generated N-terminus exhibits
protease activity"
115..150 /note="His-Tag"
649..1161 /note="Calcineurin B;Calcineurin B alpha Ca²⁺
10 binding"

12. Cloning of the catalytic subunit human calcineurin-A-Beta and splicevariants - PCR was performed as described under 11.

- with the exception that the primers CNAb-s1 5'-gag cct agc
15 cga gcc ccg gg-3' and CNAb-as1 5'-ctg gga agt agt ggg tca
ctg-3' were used for the first PCR and the primers and
CNAb-s2 5'- cat gcc atg gat cca tgg ccg ccc cgg agc c-3' and
CNAb-as4 5'- tcc ccc cgg ggt acc cta gtt aat cac tgg gca gta
tgg ttg cca g-3' were used for second PCR.

20

13. Cloning of the catalytic subunit human calcineurin-A-Gamma and splicevariants - PCR was performed as described under

11. with the exception that the primers CNAg-s1 5'-gga gcc
tgg agg agg ccg ag-3' and CNAg-as1 5'-cgg cag gac tct aag tca
25 tga-3' were used for the first PCR and the primers and
CNAg-s2 5'-cat gcc atg gat cca tgt ccg gga ggc gct tc-3' and
CNAg-as4 5'-tcc ccc cgg ggt acc cta gtt aat cat gaa tgg gct
ttc ttc cct t-3' were used for second PCR.

- 30 Hereby a new splicevariant was identified, which is important
for calcium regulation and proteolytic regulation of calci-
neurin-A. The splicevariant with human exon is not yet
available in gene database (substitution of nucleic bases
1474-1503 in sequence protocol CNAg2-pQE30 SEQ ID NO 32) with

5'-ACA GTA GAA GCG GTA GAG GCC CGG GAA GCC-3' (corresponding peptide: NH₂-TVEAVEAREA-COOH). The corresponding vector is named CNAg3-pQE30.

5 Location/Qualifiers

115..150 /note="His-Tag"

151..1689 /note="Calcineurin-A-Gamma-2"

1474..1503 /note="human brain calcineurin-A-gamma alternative exon = interaction domain with cytoskelett, death-do-

10 main homolog, stomatin homolog"

1690..1731 /note="RBS&MCS2"

1732..2244 /note="Calcineurin-B"

14. Subcloning of calcineurin-B and calcineurin-A variants

15 **into pQE30** - For the recombinant expression in procaryotic cells calcineurin-B was subcloned with either calcineurin-A-alpha1, calcineurin-A-alpha2, calcineurin-A-beta1, calcineurin-A-beta2, calcineurin-A-gamma1 or calcineurin-A-gamma2. The purified calcineurin-A-alpha, calcineurin-B-alpha or calcineurin-A-gamma PCR products (described in 11. - 13.) were restricted with BamHI/XmaI. The purified calcineurin-B product (described in 10.) was restricted with XmaI/HindIII and ligated together with the respective calcineurin-A-fragment into the BamHI/HindIII treated vector
20 pQE30 to yield the final procaryotic expression vector constructs CNAa1-pQE30, CNAa2-pQE30, CNAa3-pQE30, CNAb1-pQE30, CNAb2-pQE30, CNAg1-pQE30, CNAg2-pQE30 and CNAg3-pQE30.

15. Recombinant coexpression and purification of calcineu-

30 **rin-B/calcineurin-A heterodimers with CuZnSOD** - CNAa1-pQE30, CNAa2-pQE30, CNAa3-pQE30, CNAb1-pQE30, CNAb2-pQE30, CNAg1-pQE30, CNAg2-pQE30 or CNAg3-pQE30 were transformed into E.coli M15[pREP4][CuZnSOD-pQE30] to yield cells able to coexpress calcineurin-A, calcineurin-B and CuZnSOD. cells

were plated on LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) agar. Expression cultures were grown in 250 ml LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) until the OD₆₀₀ was 0.6. Constitutive leakage expression was prevented by the repressor plasmid pREP4-lacI. Production of the human calcineurin-A/calcineurin-B histidin tagged heterodimers was induced by addition of IPTG (1 mM). After four hours the bacterial cells were harvested by centrifugation (4000 g, 20 min), resuspended in 8 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) and homogenized by three freeze thaw cycles and sonication on ice (Bandelin sonoplus GM70, 300 W, 3 x 10 sec). The lysate was centrifuged (10,000 g, 20 min) and incubated with 750 µl Fe-NTA-agarose for batch affinity binding for 1 h at 4 °C (Qiagen expressionist kit, Qiagen, Hilden, Germany). Fe-NTA-agarose was prepared from Ni-NTA-agarose (Qiagen expressionist kit, Qiagen, Hilden, Germany) by subsequent washes in:

- 1) 2 volumes of bidestilled water
- 2) 3 volumes of regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)
- 3) 5 volumes bidestilled water
- 4) 3 volumes 2% SDS
- 5) 1 volume 25% ethanol
- 6) 1 volume 50% ethanol
- 7) 1 volume 75% ethanol
- 8) 5 volumes 100% ethanol
- 9) 1 volume 75% ethanol
- 10) 1 volume 50% ethanol
- 11) 1 volume 25% ethanol
- 12) 1 volume bidestilled water
- 13) 5 volumes 100 mM Na-EDTA pH 8.0
- 14) 5 volumes bidestilled water
- 15) 2 volumes 100 mM FeSO₄ / 1 mM reduced glutathion / 1 mM dithiothreitol/ 100 mM ascorbic acid

- 16) 2 volumes bidestilled water
17) 2 volumes regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)
18) 2 volumes buffer buffer A3 (20 mM Tris-HCl pH 7.9, 5 mM
5 imidazole, 500 mM NaCl, 200 μ M FeSO₄ / 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid

The batch was applied to a 30 ml chromatography column, washed with 15 ml buffer A4 (20 mM Tris-HCl pH 7.9, 5 mM
10 imidazole, 500 mM NaCl/ 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid) and subsequently with 8 ml buffer B (20 mM Tris-HCl pH 7.9, 60 mM imidazole, 500 mM NaCl/ 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid). N-terminal histidin tagged calcineurin-A/cal-
15 cineurin-B heterodimer was eluted three times with 1.2 ml buffer C (10 mM Tris-HCl, 500 mM imidazole, 250 mM NaCl/1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid, buffer was degased and subsequently saturated with
20 nitrogen). To prevent oxidation of calcineurin, the eluate was stored at -80 °C in nitrogen containing and oxygen free vials. Purity and correct expression products were checked by immunoblotting or N-terminal protein sequencing after separation of 20 μ l eluate in SDS-PAGE (discontinuous 12,5 %
SDS-PAGE).

25

- 16. Subcloning of human calcineurin-A-Alpha into pEGFP eukaryotic expression vector and generation of stable transfected PC12 cells (C-terminal fusion protein with enhanced green fluorescent protein as a fluorescent marker) -** The
30 vector CNAa2-pQE30 was digested with BamHI/XmaI to generate a sticky end CNAa2 fragment. The purified fragment was ligated into Bgl-II/XmaI treated pEGFP-C1 vector (Clontech Laboratories, Palo Alto, CA, USA). After amplification in XL2-Blue cells (25 μ g/ ml kanamycin) and plasmid purification the

CNAa-pEGFP vector construct was transfected into PC12 rat adrenal pheochromocytoma cells using the CalPhosTM Transfection Kit according to the manufacturer's protocols (Clontech Laboratories, Palo Alto, CA, USA). Stable transfected
5 CNAa-pEGFP clones were selected by fluorescence microscopy during a three month propagation procedure (excitation 488 nm/emission 520 nm, MRC 1024 confocal microscope, BioRAD Laboratories, Hercules, CA, USA).

10 **17. Subcloning of calcineurin-A-Beta into pEGFP** - The same procedure as described in 16. was applied except that the CNAa2-pQE30 vector was substituted by CNAb2-pQE30 to generate CNAb-pEGFP.

15 **18. Subcloning of calcineurin-A-Gamma into pEGFP** - The same procedure as described in 16. was applied except that the CNAa2-pQE30 vector was substituted by CNAg2-pQE30 to generate CNAg-pEGFP.

20 **19. Western blotting and protein sequencing** - Transfer of purified proteins from 12% SDS-PAGE to PVDF membranes (Boehringer-Mannheim, Mannheim, Germany) was performed according to standard protocols using transfer buffer (48 mM Tris, 39mM Glycine, 20% methanol, 1% SDS, pH 9.2) and following blotting
25 conditions: 75 min at 25 V/110 mA. Blocking, washing and detection (HRP detection system) were performed according to the manufacturer's protocols (ECL kit, Amersham, Buckinghamshire, UK). An anti-human CuZnSOD antibody (1:5,000 dilution, rabbit polyclonal anti-human SOD1 antibody; BIOMOL, Hamburg,
30 Germany) was used as primary antibody and an anti-rabbit IgG antibody (1:10,000 dilution) labeled with HRP was used as secondary antibody. For the detection of calcineurin-A (alpha, beta, gamma isoforms) a polyclonal calcineurin-A antibody was used as 1:5000 dilution (Sigma Aldrich, Deisen-

hofen, Germany). For N-terminal protein sequencing the PVDF membrane was soaked in 100% methanol. Proteins which seemed to be blocked by N-terminal posttranslational modifications were treated with acylamino-acid-peptidase according to the manufacturer's protocol (Boehringer-Mannheim, Mannheim, Germany). Coomassie brilliant blue stained bands were cut out. Automated Edman degradation of peptides was performed on an Applied Biosystems protein sequencer (476A).

10 **20. Calcineurin phosphatase assay** - 100 ng - 4 μ g recombinant calcineurin (calcineurin-A/B heterodimer), 100 ng - 1 μ g purified bovine brain calcineurin (Sigma Aldrich, Deisenhofen, Germany) or 100 μ g homogenized tissue or cell extracts were used for classical calcineurin phosphatase assays. 100
15 μ g cells or tissue were homogenized exactly as described (Stemmer et al., 1995). Partly purified and redox sensitive calcineurin was prepared by centrifugation at 14,000 rpm at 4°C for 10 min (Eppendorf Centrifuge 5417R) and the resulting supernatant was separated on a 1.5 x 10 cm Sephadex-G50
20 gel filtration column as described (Stemmer et al., 1995), (Gold et al., 1997). Phosphotyrosine phosphatase assay was performed in microplates (100 μ l total assay volume) either using 30 μ M fluoresceinmonophosphate or 20 mM para-nitrophenylphosphate (Sigma Aldrich, Deisenhofen, Germany); 10 μ l
25 recombinant, purified or partly purified and assay buffer (25 mM Tris/HCl, pH 7; 2 mM CaCl_2 ; 0.1 μ M calmodulin; 25 μ M FK506). After starting the enzymatic reaction with para-nitrophenylphosphate or fluoresceinmonophosphate the absorbance at 405 nm (para-nitrophenylphosphate) or fluorescence (λ
30 excitation = 485 nm; λ emission = 520 nm) was monitored over 20 min at 30°C using a UV/VIS/fluorescence microplate photometer (Biolumin 960 kinetic fluorescence/absorbance photometer, Molecular Dynamics). Phosphoserine phosphatase assay was performed as described (Hubbard and Klee, 1991), (Wang et

al., 1996). In short: 40 μ l recombinant or partly purified calcineurin was mixed with test buffer (40 mM Tris/HCl pH 8; 0.1 M KCl; 0.4 mg/ml BSA; 0.67 mM DTT; 0.67 μ M calmodulin; 1 μ M FKBP binding protein; 0.5 μ M ocadaic acid for inhibition of phosphatase A1 and A2) and enzymatic reaction and calcium induced redox-inactivation of calcineurin started by addition of 20 μ l substrate buffer (7.7 μ M radioactive phosphorylated RII-peptid, 2.0 mM CaCl_2). The assay was performed in duplicates and the addition of 1 μ M FK506 or cyclosporine was used to verify calcineurin activity for each reading point. The protective effect of CuZnSOD against redox inactivation of calcineurin was determined by addition of 3 μ g recombinant human wild-type or mutated CuZnSOD (constant CuZnSOD protein) or addition of 1.67 units of recombinant human wild-type or mutated CuZnSOD (constant CuZnSOD activity). The reaction mixture was incubated for 2 min at 30°C and stopped with 100 mM potassium phosphate / 5% TCA. The reaction mixture was passed through a 0.5 ml ion-exchange column (Dowex; AG 50W-X8, BioRad) and the unbound phosphate eluted with 0.5 ml water. The quantity of released phosphate was determined by a scintillation counting.

An enzymatic protein phosphatase assay was established using the nonphysiological substrate fluoresceinmonophosphate (FMP). Assuming a Michaelis-Menten kinetic for FMP and using the Lineweaver-Burk method for analysis of kinetic data a K_M of 40 μ M and a $V_{\max}$ of 400 μ mol / min was determined. The assay was applicable to calcineurin and magnesium dependent proteinphosphatase 2C (data not shown, (Grothe et al., 1998)). The enzymatic activity is linear in the range of 12.5 pM to 75 pM calcineurin. FMP is more sensitive than para-nitrophenylphosphate (pNPP). Neither FMP nor pNPP are useful to measure calcineurin activity in crude preparations by inhibition with the immunosuppressive drugs FK506 or cyclosporine

(cell homogenate, partly purified calcineurin). Both substrates also failed to measure calcium induced redox-inactivation of calcineurin or CuZnSOD mediated protection of this inactivation. The inhibition assay also failed when calcium was substituted against other divalent cations (Ni^{2+} , Mg^{2+}). Only the physiological relevant substrate could be used in an immunosuppressive drug inhibitory assay (RII-peptide phosphopeptide). In the classic radioactive assay 95% inhibition with 1 μM FK506 or cyclosporine was determined. It is concluded that inhibition of calcineurin activity by immunosuppressive drugs needs larger molecular weight substrates than pNPP and FMP. Furthermore it is concluded that redox-sensitivity is linked to phosphoserine phosphatase activity and therefore not detectable with phosphotyrosine analoges like pNPP or FMP. The recombinant human wild-type CuZnSOD and purified human erythrocyte CuZnSOD (Sigma Aldrich, Deisenhofen, Germany) were effective to protect 50-100% of calcineurin after calcium induced redox inactivation. Mutated CuZnSOD proteins, associated with the severe neurological disorder amyotrophic lateral sclerosis, were less effective to protect calcineurin against redox inactivation.

Protective effect of CuZnSOD of calcium induced inactivation of calcineurin

25

Percentage of FK506 inhibitable RII-phosphopeptide activity after 20 min compared with the activity at 0 min

30 human CuZnSOD	constant protein	constant activity
	(3 μg)	(1.67 U)
erythrocyte wild-type (8330 U/mg)	57 +/-10 %	57 +/-10 %
recombinant wild-type	70 +/-33 %	58 +/-22 %

	(6380 U / mg)		
	recomb. mutation D90A	42 +/-17 %	32 +/-15 %
	(4590 U/ mg)		
	recomb. mutation G93A	16 +/-16 %	21 +/-22 %
5	(2130 U/ mg)		
	recomb. mutation A4V	22 +/-27 %	8 +/-3 %
	(1820 U/ mg)		
	control (no CuZnSOD)	9 +/-7 %	9 +/-7 %
	(0 U / mg)		

10

The protective effect does not depend on CuZnSOD activity since higher protein amounts of mutated CuZnSOD corresponding to a higher enzymatic activity were even less effective in protection of calcineurin.

15

Therefore it is concluded that amino acid substitutions, associated with familial amyotrophic lateral sclerosis, are important for the protein interaction of calcineurin and CuZnSOD and therefore are involved in the CuZnSOD mediated protection of calcium induced redox inactivation of calcineurin. Since this protective effect is disturbed in amyotrophic lateral sclerosis and protection of calcineurin by CuZnSOD it may also be important in other neurological and cardiovascular diseases (Alzheimer, Parkinson, epilepsy, ischemia, heart-failure).

25

An high-throughput bioassay was developed to detect and isolate artificial or endogenous drugs enhancing (activators) CuZnSOD-calcineurin interaction and therefore protecting calcineurin against redox-inactivation or drugs reducing (inhibitors) CuZnSOD-calcineurin interaction and therefore inhibit calcineurin activity. Inhibitors are useful to substitute toxic immunosuppressive drugs like FK506 or cyclosporine. Activators and inhibitors may be useful for the

30

therapeutical treatment of amyotrophic lateral sclerosis, Parkinson, Alzheimer, epilepsy, ischemia and cardiovascular diseases.

- 5 **21. High Throughput BioAssay using recombinant calcineurin-A, recombinant calcineurin-B, calmodulin and recombinant CuZnSOD (analytical assay to identify activators or inhibitors of CuZnSOD/calcineurin interaction)** - Laser fluctuation correlation spectroscopy (FCS) is a useful tool to quantify ligand-
 10 ligand interactions. The fluorescence $F(t)$ of a optical well defined volume element which is excited by a confocal laser is monitored as a function of time. The temporal autocorrelation of the fluorescence fluctuation $\delta F(t)$ yields the time scale of this dynamics and the average number of independent
 15 fluorophores in the probe volume. If the fluorescence fluctuation arise from diffusive motion and from fluorescence sensitive reaction, fluorescent fluctuation correlation function signal is approximated by the formula:

$$G_{DR}(\tau) = G_{Diff}(\tau) * [1 + A * \exp(-k_R * \tau)]$$

20

τ = fluorescence correlation time

k_R = apparent binding constant of the fluorescent labeled ligand

A = equilibrium coefficient dependent constant

25

- If one measurement is performed with a solution only containing the fluorescent labeled ligand and a second measurement is performed with a solution containing the fluorescent labeled ligand and an interacting molecule the correlation
 30 function G_{DR} can be separately analyzed and yields binding parameters of the interacting molecules. Upon binding of the ligand to the interacting molecule the hydrodynamic radius increases and therefore the diffusion coefficient decreases resulting in a longer correlation time.

A fluorescence labeled recombinant CuZnSOD as a fluorescent label was used to monitor the binding dynamics to calcineurin. CuZnSOD was labeled with Oregon-Green-514 dye according to the manufacturer's protocols (FluoReporter Protein labeling Kit, Molecular Probes, Leiden, Netherlands). The amount of fluorescent dye labels per CuZnSOD dimer was quantified by determining the ratio of the absorbance at 265 nm (CuZnSOD protein) / 514 nm (Oregon-dye). The diffusion constant and correlation time of the labeled CuZnSOD (100 nM) was measured on a bovine serum albumin treated glass plate with an confocal laser microscope attached to an autocorrelator (λ excitation = 488 nm, λ emission = 511 nm) in 10 μ l assay buffer containing 50 mM sodiumphosphate pH 7.1, 150 mM NaCl, 0.67 mM DTT, 0.67 μ M calmodulin, 0.67 mM CaCl_2 , 1 mM MgCl_2 . The beam from a modelocked Ti:Sa or cw argon ionlaser was collimated to fill the back aperture of a immersion microscope objective (Zeiss C-Apochromat 63x1.2w), producing a small diffractionlimited spot. The emitting fluorescent light was collected by the same objective separated from the excitation light by a beamsplitter/filter combination and imaged first to a variable pinhole and than to the detector (Avalanche Photodiode EG&G SPCM AQ161 or PMT Hamamatsu R5600-03). The labeled CuZnSOD exhibited an autocorrelation time corresponding to a hydrodynamic radius of 41,000 Dalton which is comparable to the expected molecular weight of the homodimer (34,600 Dalton). Next 0.2 μ l calcineurin-A/B heterodimer (5 μ M) was added to a labeled CuZnSOD mixture and the fluorescence correlation signal was determined. The hydrodynamic radius increases from 41 kDa to 90 kDa indicating that approximatly one calcineurin heterodimer interacts with one CuZnSOD dimer (expected: 114 kDa). Using mutated D90A CuZnSOD yielded an apparent molecular weight of 180.000 kDa indicating the formation of calcineurin/CuZnSOD aggrega-

tes. The apparent binding constant between human wild-type CuZnSOD and calcineurin was estimated as $k_D = 2 \times 10^{-6} \text{ M} \pm 1 \times 10^{-6} \text{ M}$. It is concluded that laser correlation spectroscopy is useful to perform a ultra high throughput screening for ligands diminishing the CuZnSOD/calcineurin interaction which simply can be monitored by a reduction of the autocorrelation time after addition of a potential drugs. It is possible to screen for suitable substances using substances available in chemical, peptide or natural compound screening libraries.

22. High Throughput BioAssay using recombinant calcineurin-A, recombinant calcineurin-B, calmodulin and RII-Fluophos (analytical assay to identify activators or inhibitors of calcineurin) - RII peptide was synthesized according to standard peptide synthesis protocols ((Blumenthal et al., 1988); Interactiva, Ulm, Germany). To generate a fluorescent labeled peptide which furthermore contains a phosphoester at Ser-15, amino acid residue Ser-15 was coupled with fluoresceine-phosphoamidit (FluoreDite Labeling Reagent, Perseptive Biosystems), which is usually used for labeling of nucleotides, to yield RII-Fluophos (Interactiva, Ulm, Germany). The expected molecular weight (2578.8 Dalton) was confirmed by mass spectrometry (2580.6 Dalton). The Fluophos-RII-peptide was not converted by calcineurin as was monitored by fluorescence spectrometrie (Biolumin 960 UV-VIS/fluorescence microplate reader). Therefore Fluophos-RII-peptide was used in laser fluorescence correlation spectroscopy as described in paragraph 20 except that λ excitation was 488 nm and λ emission was 520 nm. Furthermore, labeled CuZnSOD was substituted by 10 nM Fluophos-RII-peptide yielding a hydrodynamic radius corresponding to 4 kDa (expected 2.6 kDa). After calcineurin addition the molecular weight increases to 100.000 kDa and a binding

constant of $K_d = 0.6 \times 10^{-6}$ M is estimated. Binding constants were comparable between the six calcineurin isoforms/splicevariants. It is concluded that laser correlation spectroscopy is useful to perform a ultra high throughput screening for ligands directly substrate binding to calcineurin by simply monitoring the autocorrelation time after addition of potential drugs. By descriminating the binding properties of potential drugs to the six different heterodimer combinations (calcineurin-A-alpha1/calcineurin-B, calcineurin-A-alpha2/calcineurin-B, calcineurin-A-beta1/calcineurin-B, calcineurin-A-beta2/calcineurin-B, calcineurin-A-gamma1/calcineurin-B, calcineurin-A-gamma2/calcineurin-B) it is possible to identify tissue specific and therefore less toxic calcineurin inhibitors.

It is possible to combine the screening procedures described in paragraph 20 and 21 strategically: substances which are able to inhibit the calcineurin-CuZnSOD interaction (positive hit in paragraph 20) but failed to show an effect in procedure 21 (negative hit) are predominantly positive candidates for the therapeutical use in neurological disorders because a toxic immunosuppressive side effect is less probable. Substances which fail to inhibit the calcineurin-CuZnSOD interaction (negative hit) but show an effect in procedure 21 (positive hit) are predominantly positive candidates for immunosuppression. Substances effective in both procedures are likely to be toxic.

23. Cellular BioAssay using eucaryotic cells transfected with calcineurin-A-EGFP fusionprotein or CuZnSOD-EGFP fusion protein - PC12 cells stably transfected with CuZnSOD and calcineurin isoenzymes serve as a model for monitoring the effects of CuZnSOD or calcineurin overexpression in neuronal cells. CuZnSOD reportedly has been implicated to be involved

in the mediation of hypoxie tolerance, whereas calcineurin overexpression is associated with epileptogenesis, Parkinsonism or Alzheimers disease. It is possible to use theses cells subsequently to the identification of potential drugs in screening protocols 20 und 21. Toxicity of potential neuroprotective drugs and the effect on the subcellular distribution of calcineurin-isoforms or CuZnSOD, respectively can be monitored.

- 10 **24. Pull-Down-BioAssay using histidine tagged recombinant CuZnSOD to purify CuZnSOD interacting ligands (Preparative assay to isolate activators or inhibitors of CuZnSOD/calci-**
neurin interaction from biological sources) - Recombinant purified histidine tagged CuZnSOD in 50 mM sodiumphosphate
15 buffer pH 8.0 was attached to CuZn-NTA magnetic agarose beads by incubating 100 μ l beads suspension with 100 μ l CuZnSOD solution (0.3 μ g/ μ l) in 96 well microplates for 30 minutes at room temperature on a microplate shaker (600 rpm). CuZn-NTA magnetic beads were generated from Ni-NTA beads by applying
20 the same procedure as described under paragraph 5 (Ni-NTA magnetic agarose beads, Qiagen, Hilden, Germany). The microplate was placed on the 96 well magnet for 1 minute and the supernatant removed from the wells.
- 25 Cytosolic ligands were isolated as follows: 200 μ l interaction buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole pH 8.0, 0.1 % Tween-80) were added to the CuZn-NTA agarose beads/CuZnSOD containig wells and placed on the 96 well magnet to remove interaction buffer. 100 mg tissue, cells or
30 other biological specimen to be analyzed for CuZnSOD interacting ligands were homogenized in 200 μ l lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole pH 8.0, 0.1 % Tween-80) using a dounce homogenizer. The lysate was cleared by 30 min centrifugation at 10,000 g at 4 °C. The supernatant was

applied to the wells containing CuZn-NTA absorbed recombinant human CuZnSOD, mixed and incubated for 60 minutes at 0 °C. The microplate was placed on the 96 well magnet for 1 minute to remove the supernatant. After removal of the lysate the wells were washed twice by adding 200 µl interaction buffer. Elution of CuZnSOD and interacting ligands was achieved by addition of 100 µl elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole pH 8.0, 0.1 % Tween-80).

Membranous ligands were isolated as follows: denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH₂PO₄ pH 8.0, 0.1 % Tween-80) was added to the CuZn-NTA agarose beads/CuZnSOD containing wells and placed on the 96 well magnet to remove interaction buffer. The pellet from the procedure above was solubilized in 200 µl denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH₂PO₄ pH 8.0, 0.1 % Tween-80) for 60 minutes at room temperature. The solubilisate was cleared by 30 min centrifugation at 10,000 g at room temperature. The supernatant was applied to the wells containing CuZn-NTA absorbed recombinant human CuZnSOD, mixed and incubated for 60 minutes at room temperature. The microplate was placed on the 96 well magnet for 1 minute to remove the supernatant. The wells were washed once with 200 µl denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH₂PO₄ pH 8.0, 0.1 % Tween-80) and a second time with 200 µl denaturing wash buffer (8 M Urea, 100 mM NaH₂PO₄ pH 8.0, 0.1 % Tween-80). Elution of CuZnSOD and interacting ligands was achieved by addition of 100 µl denaturing elution buffer (8 M Urea, 100 mM NaH₂PO₄ pH 4.0, 0.1 % Tween-80).

30

To remove low molecular weight ligands for HPLC analysis, the eluates (cytosolic or membranous) were ultrafiltered through a 5 kDa membrane as described under 7. Low molecular weight ligands were separated on a preparative reverse phase HPLC

(UV detection at 200 nm). Homogeneity and molecular weight of UV detectable fractions were analyzed by mass spectrometry. High molecular weight ligands (ultrafiltration remainder) were separated on a 10% polyacrylamide gel and protein bands identified by sequencing or MALDI mass spectrometrie as described under 19. Interacting nucleic acid was analyzed by separating the membranous eluate on a 1 % agarose gel and staining with ethidium bromide. Fluorescent bands were extracted from the agarose (Qiagen gel extraktion kit, Qiagen, Hilden, Germany) subjected to digestion with RsaI and subcloned into RsaI treated pQE30 vector for DNA sequencing.

25. Pull-Down-BioAssay using histidine tagged recombinant calcineurin-A and calcineurin-B to purify calcineurin interacting ligands (Preparative assay to isolate activators or inhibitors of CuZnSOD/calcineurin interaction from biological sources) - Isolation and identification of calcineurin interacting ligands was performed analogous to paragraph 24 with the exception that recombinant calcineurin-A/B heterodimer was attached to Fe-NTA magnetic agarose beads which were prepared as described under paragraph 15. Furthermore six different heterodimer combinations were used (calcineurin-A-alpha1/calcineurin-B, calcineurin-A-alpha2/calcineurin-B, calcineurin-A-beta1/calcineurin-B, calcineurin-A-beta2/calcineurin-B, calcineurin-A-gamma1/calcineurin-B, calcineurin-A-gamma2/calcineurin-B) to descriminate between isoenzyme and splicevariant specific interaction partners.

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30

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- 15 35. Yu, L., Golbeck, J., Yao, J., and Rusnak, F. (1997). *Biochemistry* 36, 10727-10734.

Content of sequence listing

1. eukaryotic expression vector CuZnSOD-EGFP (CuZnSOD-
20 pEGFP) (DNA)
2. CuZnSOD (PRT)
3. EGFP (PRT)
4. eukaryotic expression vector EGFP-Calcineurin A alpha
(CNAa-pEGFP) (DNA)
- 25 5. EGFP (PRT)
6. Calcineurin A alpha (PRT)
7. eukaryotic expression vector EGFP-Calcineurin A beta
(CNAb-pEGFP) (DNA)
8. EGFP (PRT)
- 30 9. Calcineurin A beta (PRT)
10. eukaryotic expression vector EGFP-Calcineurin A gamma
(CNAg-pEGFP) (DNA)
11. EGFP (PRT)
12. Calcineurin A gamma (PRT)

13. prokaryotic expression vector His-CuZnSOD (CuZnSOD-pQE30) (DNA)
14. CuZnSOD (PRT)
15. prokaryotic expression vector CuZnSOD-His (CuZnSOD-pQE60) (DNA)
- 5 16. CuZnSOD (PRT)
17. prokaryotic expression vector His-Calcineurin A alpha1-Calcineurin B (CNAa1-pQE30) (DNA)
18. Calcineurin A alpha1 (PRT)
- 10 19. Calcineurin B (PRT)
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21. Calcineurin A alpha2 (PRT)
22. Calcineurin B (PRT)
- 15 23. prokaryotic expression vector His-Calcineurin A beta1-Calcineurin B (CNAb1-pQE30) (DNA)
24. Calcineurin A beta1 (PRT)
25. Calcineurin B (PRT)
26. prokaryotic expression vector His-Calcineurin A beta2-Calcineurin B (CNAb2-pQE30) (DNA)
- 20 27. Calcineurin A beta2 (PRT)
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- 25 30. Calcineurin A gamma1 (PRT)
31. Calcineurin B (PRT)
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- 30 34. Calcineurin B (PRT)
35. peptide RII (PRT)

5 Method for screening of modulators of calcineurin activity

Claims:

1. Method for screening of modulators of calcineurin activity, characterized in that an interaction between calcineurin
10 and superoxide dismutase is monitored, comprising the following steps
 - forming of a complex comprising at least calcineurin and superoxide dismutase under incubation with at least one potential modulator,
 - 15 - detecting the influence of the potential modulator by directly monitoring the complex formation and/or by monitoring the activity, especially the enzymatical activity of the complex.
- 20 2. Method according to claim 1, characterized in that the the superoxide dismutase is a Copper/Zinc-superoxide dismutase.
3. Method according to claim 1 or 2, characterized in that
25 forming of the complex is performed in the presence of the potential modulator.
4. Method according to claim 1 or 2, characterized in that the potential modulator is added after the complex has been
30 formed.
5. Method according to one of the preceding claims, characterized in that the monitoring is performed by detection of labels, especially fluorescent labels.

6. Method according to one of the preceding claims, characterized in that the calcineurin and/or the superoxide dismutase carry labels, especially fluorescent markers, wherein preferably the labels are enhanced green fluorescent protein.

7. Method according to claim 6, characterized in that calcineurin and/or superoxide dismutase are expressed as fluorescent proteins, particularly as fusion proteins together with enhanced green fluorescent protein.

8. Method according to one of the preceding claims, characterized in that the monitoring of complex formation is performed by laser fluctuation correlation spectroscopy.

9. Method according to one of the preceding claims, characterized in that calcineurin and superoxide dismutase are coexpressed in cells, especially in eukaryotic cells, and that the complex formation is performed within the cell.

10. Method according to one of the preceding claims, characterized in that calcineurin and/or superoxide dismutase are expressed in cells, especially in prokaryotic cells, and that calcineurin and/or superoxide dismutase are isolated and/or purified before the complex formation is performed.

11. Method according to claim 10, characterized in that purification of calcineurin is achieved by affinity chromatography, especially by ferro-nitrilotriacetat-metal affinity chromatography.

12. Method according to claim 10, characterized in that purification of superoxide dismutase is achieved by affinity

chromatography, especially by copper/zinc-nitrilotriacetat-metal affinity chromatography.

13. Method according to one of the preceding claims, characterized in that in the complex formation step additionally
5 calmodulin and/or calcium is added.

14. Method according to one of the preceding claims, characterized in that the monitoring of the enzymatical activity
10 is performed by analyzing the phosphatase activity of calcineurin.

15. Method according to claim 14, characterized in that the phosphatase activity is analyzed by the use of at least one
15 substrate, which preferably carries a label, especially a fluorescent label.

16. Method according to claim 15, characterized in that the substrate is a peptide, especially a peptide characterized by
20 the amino acid sequence

Asp - Leu - Asp - Val - Pro - Ile - Pro - Gly - Arg -
Phe - Asp - Arg - Arg - Val - Ser - Val - Ala - Ala -
Glu.

25 17. Method according to claim 15 or 16, characterized in that the substrate is a peptide containing a residue, especially a serine residue, labeled with fluoresceine.

18. Kit for screening of modulators of calcineurin activity
30 comprising

- calcineurin and/or a vector encoding for calcineurin and/or cells capable of expressing calcineurin, and
- superoxide dismutase and/or a vector encoding for

superoxide dismutase and/or cells capable of expressing calcineurin.

19. Expression vector, especially prokaryotic expression
5 vector, encoding calcineurin and/or superoxide dismutase with
at least one tag, especially a histidine tag.
20. Expression vector, especially eukaryotic expression
vector, encoding calcineurin and/or superoxide dismutase as
10 labeled protein, especially in combination with a fluorescent
protein, especially enhanced green fluorescent protein.
21. Expression vector according to claim 19 or 20 defined in
sequence listing SEQ ID NO 1, 4, 7, 10, 13, 15, 17, 20, 23,
15 26, 29, or 32.
22. Cells bearing at least one vector according to at least
one of claims 19 to 21.
- 20 23. Peptide defined in sequence listing SEQ ID NO 35, charac-
terized in that the serine residue is labeled with fluo-
resceine.

SEQUENCE LISTING

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<120> Method for screening of modulators of calcineurin activity

<130> A34157PCT

<140> PCT/EP99/05220

<141> 1999-07-22

<150> EP98113876

<151> 1998-07-22

<160> 35

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145					150					155					160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser
			165					170						175	

- 21 -

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
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210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
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<212> PRT

<213> Homo sapiens

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Ala Val Pro Phe Pro Pro Thr His Arg Leu Thr Ser Glu Glu Val Phe
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Asp Leu Asp Gly Ile Pro Arg Val Asp Val Leu Lys Asn His Leu Val
50 55 60

Lys Glu Gly Arg Val Asp Glu Glu Ile Ala Leu Arg Ile Ile Asn Glu
65 70 75 80

Gly Ala Ala Ile Leu Arg Arg Glu Lys Thr Met Ile Glu Val Glu Ala
85 90 95

Pro Ile Thr Val Cys Gly Asp Ile His Gly Gln Phe Phe Asp Leu Met
100 105 110

Lys Leu Phe Glu Val Gly Gly Ser Pro Ala Asn Thr Arg Tyr Leu Phe
115 120 125

Leu Gly Asp Tyr Val Asp Arg Gly Tyr Phe Ser Ile Glu Cys Val Leu
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Tyr Leu Trp Val Leu Lys Ile Leu Tyr Pro Ser Thr Leu Phe Leu Leu
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Arg Gly Asn His Glu Cys Arg His Leu Thr Glu Tyr Phe Thr Phe Lys
165 170 175

Gln Glu Cys Lys Ile Lys Tyr Ser Glu Arg Val Tyr Glu Ala Cys Met
180 185 190

Glu Ala Phe Asp Ser Leu Pro Leu Ala Ala Leu Leu Asn Gln Gln Phe
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Leu Cys Val His Gly Gly Leu Ser Pro Glu Ile His Thr Leu Asp Asp
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Ile Arg Arg Leu Asp Arg Phe Lys Glu Pro Pro Ala Phe Gly Pro Met

SUBSTITUTE SHEET (RULE 26)

- 22 -

225		230		235		240
Cys Asp Leu Leu Trp	Ser Asp Pro Ser	Glu Asp Phe Gly	Asn Glu Lys			
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Ser Gln Glu His Phe	Ser His Asn Thr	Val Arg Gly Cys	Ser Tyr Phe			
	260	265	270			
Tyr Asn Tyr Pro Ala	Val Cys Glu Phe	Leu Gln Asn Asn	Asn Leu Leu			
	275	280	285			
Ser Ile Ile Arg Ala	His Glu Ala Gln	Asp Ala Gly Tyr	Arg Met Tyr			
	290	295	300			
Arg Lys Ser Gln Thr	Thr Gly Phe Pro	Ser Leu Ile Thr	Ile Phe Ser			
	305	310	315			320
Ala Pro Asn Tyr Leu	Asp Val Tyr Asn	Asn Lys Ala Ala	Val Leu Lys			
	325	330	335			
Tyr Glu Asn Asn Val	Met Asn Ile Arg	Gln Phe Asn Cys	Ser Pro His			
	340	345	350			
Pro Tyr Trp Leu Pro	Asn Phe Met Asp	Val Phe Thr Trp	Ser Leu Pro			
	355	360	365			
Phe Val Gly Glu Lys	Val Thr Glu Met	Leu Val Asn Val	Leu Ser Ile			
	370	375	380			
Cys Ser Asp Asp Glu	Leu Met Thr Glu	Gly Glu Asp Gln	Phe Asp Gly			
	385	390	395			400
Ser Ala Ala Ala Arg	Lys Glu Ile Ile	Arg Asn Lys Ile	Arg Ala Ile			
	405	410	415			
Gly Lys Met Ala Arg	Val Phe Ser Val	Leu Arg Glu Glu	Ser Glu Ser			
	420	425	430			
Val Leu Thr Leu Lys	Gly Leu Thr Pro	Thr Gly Met Leu	Pro Ser Gly			
	435	440	445			
Val Leu Ala Gly Gly	Arg Gln Thr Leu	Gln Ser Ala Thr	Val Glu Ala			
	450	455	460			
Ile Glu Ala Glu Lys	Ala Ile Arg Gly	Phe Ser Pro Pro	His Arg Ile			
	465	470	475			480
Cys Ser Phe Glu Glu	Ala Lys Gly Leu	Asp Arg Ile Asn	Glu Arg Met			
	485	490	495			
Pro Pro Arg Lys Asp	Ala Val Gln Gln	Asp Gly Phe Asn	Ser Leu Asn			
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<212> DNA

<213> Homo sapiens

SUBSTITUTE SHEET (RULE 26)

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 acggtgggag gtctatataa gcagagctgg tttagtgaac cgtcagatcc gctagcgcta 600
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 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val
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 ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc 699
 Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser
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 gtg tcc ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg 747
 Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu
 30 35 40 45
 aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc 795
 Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
 50 55 60
 gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac 843
 Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp
 65 70 75
 cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac 891
 His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr
 80 85 90
 gtc cag gag cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc 939
 Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr
 95 100 105
 cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag 987
 Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu

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ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag				1035
Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys	130	135	140	
ctg gag tac aac tac aac agc cac aac gtc tat atc atg gcc gac aag				1083
Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys	145	150	155	
cag aag aac ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag				1131
Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu	160	165	170	
gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc				1179
Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile	175	180	185	
ggc gac ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag				1227
Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln	195	200	205	
tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg				1275
Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu	210	215	220	
ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg				1323
Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu	225	230	235	
tac aag tcc gga ctc aga tcc aga tcc atg tcc ggg agg cgc ttc cac				1371
Tyr Lys Ser Gly Leu Arg Ser Arg Ser Met Ser Gly Arg Arg Phe His	240	245	250	
ctc tcc acc acc gac cgc gtc atc aaa gct gtc ccc ttt cct cca acc				1419
Leu Ser Thr Thr Asp Arg Val Ile Lys Ala Val Pro Phe Pro Pro Thr	255	260	265	
caa cgg ctt act ttc aag gaa gta ttt gag aat ggg aaa cct aaa gtt				1467
Gln Arg Leu Thr Phe Lys Glu Val Phe Glu Asn Gly Lys Pro Lys Val	275	280	285	
gat gtt tta aaa aac cat ttg gta aag gaa gga cga ctg gaa gag gaa				1515
Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu Glu Glu Glu	290	295	300	
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Val Ala Leu Lys Ile Ile Asn Asp Gly Ala Ala Ile Leu Arg Gln Glu	305	310	315	
aag act atg ata gaa gta gat gct cca atc aca gta tgt ggt gat att				1611
Lys Thr Met Ile Glu Val Asp Ala Pro Ile Thr Val Cys Gly Asp Ile	320	325	330	
cat gga caa ttc ttt gac cta atg aag tta ttt gaa gtt gga gga tca				1659
His Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser	335	340	345	
cct agt aac aca cgc tac ctc ttt ctg ggt gac tat gtg gac aga ggc				1707
Pro Ser Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp Arg Gly	355	360	365	

tat ttc agt ata gag tgt gtg ctg tat tta tgg agt tta aag att aat	1755
Tyr Phe Ser Ile Glu Cys Val Leu Tyr Leu Trp Ser Leu Lys Ile Asn	
370 375 380	
cat ccc aaa aca ttg ttt ctg ctt cgg gga aat cat gaa tgc agg cat	1803
His Pro Lys Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg His	
385 390 395	
ctt aca gac tat ttc acc ttc aaa cag gaa tgt cga atc aaa tat tcg	1851
Leu Thr Asp Tyr Phe Thr Phe Lys Gln Glu Cys Arg Ile Lys Tyr Ser	
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gaa cag gtg tat gat gcc tgt atg gag aca ttt gac tgt ctt cct ctt	1899
Glu Gln Val Tyr Asp Ala Cys Met Glu Thr Phe Asp Cys Leu Pro Leu	
415 420 425	
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465 470 475	
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Ser Glu Asp Tyr Gly Asn Glu Lys Thr Leu Glu His Tyr Thr His Asn	
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Thr Val Arg Gly Cys Ser Tyr Phe Tyr Ser Tyr Pro Ala Val Cys Glu	
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Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His Glu Ala	
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Pro Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu Asp Val Tyr	
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Arg Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro Asn Phe Met	
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Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys Val Thr Glu	
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Met Leu Val Asn Val Leu Asn Ile Cys Ser Asp Asp Glu Leu Ile Ser	

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ggc aca ctc cct ctg ggc gtc ctc Gly Thr Leu Pro Leu Gly Val Leu Ser 670	tca gga ggc aag cag act atc gag Gly Gly Lys Gln Thr Ile Glu 675 680 685		2667
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 <213> Homo sapiens

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 35 40 45
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser

Pro Ala Phe Gly Pro Val Cys Asp Leu Leu Trp Ser Asp Pro Ser Glu
 225 230 235 240
 Asp Tyr Gly Asn Glu Lys Thr Leu Glu His Tyr Thr His Asn Thr Val
 245 250 255
 Arg Gly Cys Ser Tyr Phe Tyr Ser Tyr Pro Ala Val Cys Glu Phe Leu
 260 265 270
 Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His Glu Ala Gln Asp
 275 280 285
 Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Ala Thr Gly Phe Pro Ser
 290 295 300
 Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu Asp Val Tyr Asn Asn
 305 310 315 320
 Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val Met Asn Ile Arg Gln
 325 330 335
 Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro Asn Phe Met Asp Val
 340 345 350
 Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys Val Thr Glu Met Leu
 355 360 365
 Val Asn Val Leu Asn Ile Cys Ser Asp Asp Glu Leu Ile Ser Asp Asp
 370 375 380
 Glu Ala Glu Gly Ser Thr Thr Val Arg Lys Glu Ile Ile Arg Asn Lys
 385 390 395 400
 Ile Arg Ala Ile Gly Lys Met Ala Arg Val Phe Ser Ile Leu Arg Gln
 405 410 415
 Glu Ser Glu Ser Val Leu Thr Leu Lys Gly Leu Thr Pro Thr Gly Thr
 420 425 430
 Leu Pro Leu Gly Val Leu Ser Gly Gly Lys Gln Thr Ile Glu Thr Ala
 435 440 445
 Ile Arg Gly Phe Ser Leu Gln His Lys Ile Arg Ser Phe Glu Glu Ala
 450 455 460
 Arg Gly Leu Asp Arg Ile Asn Glu Arg Met Pro Pro Arg Lys Asp Ser
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<213> Homo sapiens

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<223> cathepsin-C cleavage site

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                Ala Thr Lys Ala Val Cys Val Leu
                  1                      5

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Lys Gly Asp Gly Pro Val Gln Gly Ile Ile Asn Phe Glu Gln Lys Glu
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Ser Asn Gly Pro Val Lys Val Trp Gly Ser Ile Lys Gly Leu Thr Glu
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ggc ctg cat gga ttc cat gtt cat gag ttt gga gat aat aca gca ggc 318
Gly Leu His Gly Phe His Val His Glu Phe Gly Asp Asn Thr Ala Gly
                45                      50                      55

tgt acc agt gca ggt cct cac ttt aat cct cta tcc aga aaa cac ggt 366
Cys Thr Ser Ala Gly Pro His Phe Asn Pro Leu Ser Arg Lys His Gly
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Gln

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<212> PRT

<213> Homo sapiens

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Ile	Ile	Asn	Phe	Glu	Gln	Lys	Glu	Ser	Asn	Gly	Pro	Val	Lys	Val	Trp
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

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Gly Ser Ile Lys Gly Leu Thr Glu Gly Leu His Gly Phe His Val His		
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Glu Phe Gly Asp Asn Thr Ala Gly Cys Thr Ser Ala Gly Pro His Phe		
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Asn Pro Leu Ser Arg Lys His Gly Gly Pro Lys Asp Glu Glu Arg His		
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Val Gly Asp Leu Gly Asn Val Thr Ala Asp Lys Asp Gly Val Ala Asp		
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Val Ser Ile Glu Asp Ser Val Ile Ser Leu Ser Gly Asp His Cys Ile		
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Ile Gly Arg Thr Leu Val Val His Glu Lys Ala Asp Asp Leu Gly Lys		
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 Ala Thr Lys Ala Val Cys Val Leu Lys Gly Asp Gly Pro Val Gln Gly
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 Ile Ile Asn Phe Glu Gln Lys Glu Ser Asn Gly Pro Val Lys Val Trp
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gga agc att aaa gga ctg act gaa ggc ctg cat gga ttc cat gtt cat 261
 Gly Ser Ile Lys Gly Leu Thr Glu Gly Leu His Gly Phe His Val His
 35 40 45

gag ttt gga gat aat aca gca ggc tgt acc agt gca ggt cct cac ttt 309
 Glu Phe Gly Asp Asn Thr Ala Gly Cys Thr Ser Ala Gly Pro His Phe
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aat cct cta tcc aga aaa cac ggt ggg cca aag gat gaa gag agg cat 357
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 Val Gly Asp Leu Gly Asn Val Thr Ala Asp Lys Asp Gly Val Ala Asp
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 Ala Cys Gly Val Ile Gly Ile Ala Gln
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 <213> Homo sapiens

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 35 40 45
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 50 55 60
 Phe Asn Pro Leu Ser Arg Lys His Gly Gly Pro Lys Asp Glu Glu Arg
 65 70 75 80
 His Val Gly Asp Leu Gly Asn Val Thr Ala Asp Lys Asp Gly Val Ala
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 Asp Val Ser Ile Glu Asp Ser Val Ile Ser Leu Ser Gly Asp His Cys
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 Ile Ile Gly Arg Thr Leu Val Val His Glu Lys Ala Asp Asp Leu Gly
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 <223> calcineurin B

<220>
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 domain), newly generated N-terminus exhibits
 protease activity

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 Arg Gly Ser His His His His His His Gly Ser Met Ser Glu Pro Lys
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 Gly Arg Leu Glu Glu Ser Val Ala Leu Arg Ile Ile Thr Glu Gly Ala
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 115 120 125

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Asn His Glu Cys Arg His Leu Thr Glu Tyr Phe Thr Phe Lys Gln Glu	
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Lys Leu Asp Arg Phe Lys Glu Pro Pro Ala Tyr Gly Pro Met Cys Asp	
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Ile Leu Trp Ser Asp Pro Leu Glu Asp Phe Gly Asn Glu Lys Thr Gln	
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Leu Gln Gln Asn Pro Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr
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Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Gly Val Ser
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Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe
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Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu
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Phe Gln Val Leu Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln
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Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Gly
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<212> PRT

<213> Homo sapiens

<400> 21

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Val	Pro	Phe	Pro	Pro	Ser	His	Arg	Leu	Thr	Ala	Lys	Glu	Val	Phe	Asp
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 Ala Ser Ile Leu Arg Gln Glu Lys Asn Leu Leu Asp Ile Asp Ala Pro
 85 90 95
 Val Thr Val Cys Gly Asp Ile His Gly Gln Phe Phe Asp Leu Met Lys
 100 105 110
 Leu Phe Glu Val Gly Gly Ser Pro Ala Asn Thr Arg Tyr Leu Phe Leu
 115 120 125
 Gly Asp Tyr Val Asp Arg Gly Tyr Phe Ser Ile Glu Cys Val Leu Tyr
 130 135 140
 Leu Trp Ala Leu Lys Ile Leu Tyr Pro Lys Thr Leu Phe Leu Leu Arg
 145 150 155 160
 Gly Asn His Glu Cys Arg His Leu Thr Glu Tyr Phe Thr Phe Lys Gln
 165 170 175
 Glu Cys Lys Ile Lys Tyr Ser Glu Arg Val Tyr Asp Ala Cys Met Asp
 180 185 190
 Ala Phe Asp Cys Leu Pro Leu Ala Ala Leu Met Asn Gln Gln Phe Leu
 195 200 205
 Cys Val His Gly Gly Leu Ser Pro Glu Ile Asn Thr Leu Asp Asp Ile
 210 215 220
 Arg Lys Leu Asp Arg Phe Lys Glu Pro Pro Ala Tyr Gly Pro Met Cys
 225 230 235 240
 Asp Ile Leu Trp Ser Asp Pro Leu Glu Asp Phe Gly Asn Glu Lys Thr
 245 250 255
 Gln Glu His Phe Thr His Asn Thr Val Arg Gly Cys Ser Tyr Phe Tyr
 260 265 270
 Ser Tyr Pro Ala Val Cys Glu Phe Leu Gln His Asn Asn Leu Leu Ser
 275 280 285
 Ile Leu Arg Ala His Glu Ala Gln Asp Ala Gly Tyr Arg Met Tyr Arg
 290 295 300
 Lys Ser Gln Thr Thr Gly Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala
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 Pro Asn Tyr Leu Asp Val Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr
 325 330 335
 Glu Asn Asn Val Met Asn Ile Arg Gln Phe Asn Cys Ser Pro His Pro
 340 345 350
 Tyr Trp Leu Pro Asn Phe Met Asp Val Phe Thr Trp Ser Leu Pro Phe
 355 360 365
 Val Gly Glu Lys Val Thr Glu Met Leu Val Asn Val Leu Asn Ile Cys
 370 375 380
 Ser Asp Asp Glu Leu Gly Ser Glu Glu Asp Gly Phe Asp Gly Ala Thr
 385 390 395 400

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 405 410 415

Lys Met Ala Arg Val Phe Ser Val Leu Arg Glu Glu Ser Glu Ser Val
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Leu Thr Leu Lys Gly Leu Thr Pro Thr Gly Met Leu Pro Ser Gly Val
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Leu Ser Gly Gly Lys Gln Thr Leu Gln Ser Ala Ile Lys Gly Phe Ser
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Pro Gln His Lys Ile Thr Ser Phe Glu Glu Ala Lys Gly Leu Asp Arg
 465 470 475 480

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

Leu Gln Gln Asn Pro Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr
 50 55 60

Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Gly Val Ser
 65 70 75 80

Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe
 85 90 95

Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu
 100 105 110

Phe Gln Val Leu Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln
 115 120 125

Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Gly
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Asp Gly Arg Ile Ser Phe Glu Glu Phe Cys Ala Val Val Gly Gly Leu
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Asp Ile His Lys Lys Met Val Val Asp Val
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Arg Gly Ser His His His His His His Gly Ser Ala Ala Pro Glu Pro
5 10 15
gcc cgg gct gca ccg ccc cca ccc ccg ccc ccg ccg ccc cct ccc ggg 213
Ala Arg Ala Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Gly
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gct gac cgc gtc gtc aaa gct gtc cct ttc ccc cca aca cat cgc ttg 261
Ala Asp Arg Val Val Lys Ala Val Pro Phe Pro Pro Thr His Arg Leu
35 40 45
aca tct gaa gaa gta ttt gat ttg gat ggg ata ccc agg gtt gat gtt 309
Thr Ser Glu Glu Val Phe Asp Leu Asp Gly Ile Pro Arg Val Asp Val
50 55 60 65
ctg aag aac cac ttg gtg aaa gaa ggt cga gta gat gaa gaa att gcg 357
Leu Lys Asn His Leu Val Lys Glu Gly Arg Val Asp Glu Glu Ile Ala
70 75 80
ctt aga att atc aat gag ggt gct gcc atc ctt cgg aga gag aaa acc 405
Leu Arg Ile Ile Asn Glu Gly Ala Ala Ile Leu Arg Arg Glu Lys Thr
85 90 95
atg ata gaa gta gaa gct cca atc aca gtg tgt ggt gac atc cat ggc 453
Met Ile Glu Val Glu Ala Pro Ile Thr Val Cys Gly Asp Ile His Gly
100 105 110
caa ttt ttt gat ctg atg aaa ctt ttt gaa gta gga gga tca cct gct 501
Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser Pro Ala

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agc aca tta ttt ctt ctg aga ggc aac cat gaa tgc aga cac ctt act Ser Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg His Leu Thr 165 170 175			645
gaa tat ttt acc ttt aag cag gaa tgt aaa att aag tat tcg gaa aga Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr Ser Glu Arg 180 185 190			693
gtc tat gaa gct tgt atg gaa gct ttt gat agt ttg cct ctt gct gca Val Tyr Glu Ala Cys Met Glu Ala Phe Asp Ser Leu Pro Leu Ala Ala 195 200 205			741
ctt tta aac caa cag ttt ctt tgt gtt cat ggt gga ctt tca cca gaa Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly Gly Leu Ser Pro Glu 210 215 220 225			789
ata cac aca ctg gat gat att agg aga tta gat aga ttc aaa gag cca Ile His Thr Leu Asp Asp Ile Arg Arg Leu Asp Arg Phe Lys Glu Pro 230 235 240			837
cct gca ttt gga cca atg tgt gac ttg tta tgg tcc gat cct tct gaa Pro Ala Phe Gly Pro Met Cys Asp Leu Leu Trp Ser Asp Pro Ser Glu 245 250 255			885
gat ttt gga aat gaa aaa tca cag gaa cat ttt agt cac aat aca gtt Asp Phe Gly Asn Glu Lys Ser Gln Glu His Phe Ser His Asn Thr Val 260 265 270			933
cga gga tgt tct tat ttt tat aac tat cca gca gtg tgt gaa ttt ttg Arg Gly Cys Ser Tyr Phe Tyr Asn Tyr Pro Ala Val Cys Glu Phe Leu 275 280 285			981
caa aac aat aat ttg tta tcg att att aga gct cat gaa gct caa gat Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His Glu Ala Gln Asp 290 295 300 305			1029
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 Val Asn Val Leu Ser Ile Cys Ser Asp Asp Glu Leu Met Thr Glu Gly
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gaa gac cag ttt gat ggt tca gct gca gcc cgg aaa gaa atc ata aga 1365
 Glu Asp Gln Phe Asp Gly Ser Ala Ala Ala Arg Lys Glu Ile Ile Arg
 405 410 415

aac aaa att cga gca att ggc aag atg gca aga gtc ttc tct gtt ctc 1413
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 Ser Ala Thr Val Glu Ala Ile Glu Ala Glu Lys Ala Ile Arg Gly Phe
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tct cca cca cat aga atc tgc agt ttt gaa gag gca aag ggt ttg gat 1605
 Ser Pro Pro His Arg Ile Cys Ser Phe Glu Glu Ala Lys Gly Leu Asp
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agg atc aat gag aga atg cca cct cgg aaa gat gct gta cag caa gat 1653
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 Gly Phe Asn Ser Leu Asn Thr Ala His Ala Thr Glu Asn His Gly Thr
 515 520 525

ggc aac cat act gcc cag tga ttaactaggg taccgccggg aaagaggaga 1752
 Gly Asn His Thr Ala Gln
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gat ttg gac aat tct ggt tct ttg agt gtg gaa gag ttc atg tct ctr 1898
 Asp Leu Asp Asn Ser Gly Ser Leu Ser Val Glu Glu Phe Met Ser Xaa
 570 575 580

cct gag tta caa cag aat ccy tta gta cag cga gta ata gat ata ttc 1946
 Pro Glu Leu Gln Gln Asn Xaa Leu Val Gln Arg Val Ile Asp Ile Phe
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gac aca gat ggg aat gga gaa gta gac ttt aaa gar ttc att gag ggm 1994
 Asp Thr Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Xaa

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gat ggr gat gga aga ata tcc ttt gaa gaa ttc tgt gct gtt gta ggy Asp Xaa Asp Gly Arg Ile Ser Phe Glu Glu Phe Cys Ala Val Val Xaa 680 685 690			2234
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 35 40 45
 Leu Thr Ser Glu Glu Val Phe Asp Leu Asp Gly Ile Pro Arg Val Asp
 50 55 60
 Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Val Asp Glu Glu Ile
 65 70 75 80
 Ala Leu Arg Ile Ile Asn Glu Gly Ala Ala Ile Leu Arg Arg Glu Lys
 85 90 95
 Thr Met Ile Glu Val Glu Ala Pro Ile Thr Val Cys Gly Asp Ile His
 100 105 110
 Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser Pro
 115 120 125
 Ala Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp Arg Gly Tyr
 130 135 140
 Phe Ser Ile Glu Cys Val Leu Tyr Leu Trp Val Leu Lys Ile Leu Tyr
 145 150 155 160
 Pro Ser Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg His Leu
 165 170 175
 Thr Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr Ser Glu
 180 185 190
 Arg Val Tyr Glu Ala Cys Met Glu Ala Phe Asp Ser Leu Pro Leu Ala
 195 200 205
 Ala Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly Gly Leu Ser Pro
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<212> PRT

<213> Homo sapiens

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 35 40 45

Leu Gln Gln Asn Xaa Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr
 50 55 60

Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Xaa Val Ser
 65 70 75 80

Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe
 85 90 95

Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu
 100 105 110

Phe Gln Xaa Xaa Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln
 115 120 125

Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Xaa
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Asp Gly Arg Ile Ser Phe Glu Glu Phe Cys Ala Val Val Xaa Gly Leu
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Asp Ile His Lys Lys Met Val Val Asp Val
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<210> 26

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<212> DNA

<213> Homo sapiens

<220>

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<222> (115)..(1776)

<223> histidine tagged calcineurin A beta2

<220>

<221> misc_feature

<222> (1777)..(1814)

<223> ribosomal binding site, multiple cloning site 2

<220>

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<222> (1815)..(2327)

<223> calcineurin B

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Met
1

aga gga tcg cat cac cat cac cat cac gga tcc gcc gcc ccg gag ccg 165
Arg Gly Ser His His His His His His Gly Ser Ala Ala Pro Glu Pro
5 10 15

gcc cgg gct gca ccg ccc cca ccc ccg ccc ccg ccg ccc cct ccc ggg 213
Ala Arg Ala Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Gly
20 25 30

gct gac cgc gtc gtc aaa gct gtc cct ttc ccc cca aca cat cgc ttg 261
Ala Asp Arg Val Val Lys Ala Val Pro Phe Pro Pro Thr His Arg Leu
35 40 45

aca tct gaa gaa gta ttt gat ttg gat ggg ata ccc agg gtt gat gtt 309
Thr Ser Glu Glu Val Phe Asp Leu Asp Gly Ile Pro Arg Val Asp Val
50 55 60 65

ctg aag aac cac ttg gtg aaa gaa ggt cga gta gat gaa gaa att gcg 357
Leu Lys Asn His Leu Val Lys Glu Gly Arg Val Asp Glu Glu Ile Ala
70 75 80

ctt aga att atc aat gag ggt gct gcc atc ctt cgg aga gag aaa acc 405
Leu Arg Ile Ile Asn Glu Gly Ala Ala Ile Leu Arg Arg Glu Lys Thr
85 90 95

atg ata gaa gta gaa gct cca atc aca gtg tgt ggt gac atc cat ggc 453
Met Ile Glu Val Glu Ala Pro Ile Thr Val Cys Gly Asp Ile His Gly
100 105 110

caa ttt ttt gat ctg atg aaa ctt ttt gaa gta gga gga tca cct gct 501
Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser Pro Ala
115 120 125

aat aca cga tac ctt ttt ctt ggc gat tat gtg gac aga ggt tat ttt 549
Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp Arg Gly Tyr Phe
130 135 140 145

agt ata gag cat gtt cta ggc act gaa gac ata tcg att aat cct cac 597
Ser Ile Glu His Val Leu Gly Thr Glu Asp Ile Ser Ile Asn Pro His
150 155 160

aat aat att aat gag tgt gtc tta tat tta tgg gtt ctg aag att cta 645
Asn Asn Ile Asn Glu Cys Val Leu Tyr Leu Trp Val Leu Lys Ile Leu
165 170 175

tac cca agc aca tta ttt ctt ctg aga ggc aac cat gaa tgc aga cac 693
Tyr Pro Ser Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg His
180 185 190

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Leu Thr Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr Ser
195 200 205

gaa aga gtc tat gaa gct tgt atg gaa gct ttt gat agt ttg cct ctt 789
Glu Arg Val Tyr Glu Ala Cys Met Glu Ala Phe Asp Ser Leu Pro Leu
210 215 220 225

gct gca ctt tta aac caa cag ttt ctt tgt gtt cat ggt gga ctt tca 837
Ala Ala Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly Gly Leu Ser

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tct gaa gat ttt gga aat gaa aaa tca cag gaa cat ttt agt cac aat Ser Glu Asp Phe Gly Asn Glu Lys Ser Gln Glu His Phe Ser His Asn 275 280 285			981
aca gtt cga gga tgt tct tat ttt tat aac tat cca gca gtg tgt gaa Thr Val Arg Gly Cys Ser Tyr Phe Tyr Asn Tyr Pro Ala Val Cys Glu 290 295 300 305			1029
ttt ttg caa aac aat aat ttg tta tcg att att aga gct cat gaa gct Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His Glu Ala 310 315 320			1077
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Gly Phe Ser Pro Pro His Arg Ile Cys Ser Phe Glu Glu Ala Lys Gly	
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ttg gat agg atc aat gag aga atg cca cct cgg aaa gat gct gta cag	1701
Leu Asp Arg Ile Asn Glu Arg Met Pro Pro Arg Lys Asp Ala Val Gln	
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caa gat ggt ttc aat tct ctg aac acc gca cat gcc act gag aac cac	1749
Gln Asp Gly Phe Asn Ser Leu Asn Thr Ala His Ala Thr Glu Asn His	
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Gly Thr Gly Asn His Thr Ala Gln	
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Met Gly Asn Glu Ala Ser Tyr Pro Leu Glu Met	
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Cys Ser His Phe Asp Xaa Asp Glu Ile Lys Arg Leu Gly Lys Arg Phe	
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Lys Lys Xaa Asp Leu Asp Asn Ser Gly Ser Leu Ser Val Glu Glu Phe	
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Ile Glu Xaa Val Ser Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys	
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Leu Arg Phe Ala Phe Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile	
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Ser Asn Gly Glu Leu Phe Gln Xaa Xaa Lys Met Met Val Gly Asn Asn	
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Leu Lys Asp Thr Gln Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn	
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<211> 553

<212> PRT

<213> Homo sapiens

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 35 40 45
 Leu Thr Ser Glu Glu Val Phe Asp Leu Asp Gly Ile Pro Arg Val Asp
 50 55 60
 Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Val Asp Glu Glu Ile
 65 70 75 80
 Ala Leu Arg Ile Ile Asn Glu Gly Ala Ala Ile Leu Arg Arg Glu Lys
 85 90 95
 Thr Met Ile Glu Val Glu Ala Pro Ile Thr Val Cys Gly Asp Ile His
 100 105 110
 Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser Pro
 115 120 125
 Ala Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp Arg Gly Tyr
 130 135 140
 Phe Ser Ile Glu His Val Leu Gly Thr Glu Asp Ile Ser Ile Asn Pro
 145 150 155 160
 His Asn Asn Ile Asn Glu Cys Val Leu Tyr Leu Trp Val Leu Lys Ile
 165 170 175
 Leu Tyr Pro Ser Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg
 180 185 190
 His Leu Thr Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr
 195 200 205
 Ser Glu Arg Val Tyr Glu Ala Cys Met Glu Ala Phe Asp Ser Leu Pro
 210 215 220
 Leu Ala Ala Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly Gly Leu
 225 230 235 240
 Ser Pro Glu Ile His Thr Leu Asp Asp Ile Arg Arg Leu Asp Arg Phe
 245 250 255
 Lys Glu Pro Pro Ala Phe Gly Pro Met Cys Asp Leu Leu Trp Ser Asp
 260 265 270
 Pro Ser Glu Asp Phe Gly Asn Glu Lys Ser Gln Glu His Phe Ser His
 275 280 285
 Asn Thr Val Arg Gly Cys Ser Tyr Phe Tyr Asn Tyr Pro Ala Val Cys
 290 295 300
 Glu Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His Glu
 305 310 315 320
 Ala Gln Asp Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Thr Thr Gly
 325 330 335
 Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu Asp Val
 340 345 350
 Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val Met Asn

- 67 -

355		360		365
Ile Arg Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro Asn Phe				
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Met Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys Val Thr				
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Glu Met Leu Val Asn Val Leu Ser Ile Cys Ser Asp Asp Glu Leu Met				
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Thr Glu Gly Glu Asp Gln Phe Asp Gly Ser Ala Ala Ala Arg Lys Glu				
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Ile Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys Met Ala Arg Val Phe				
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Ser Val Leu Arg Glu Glu Ser Glu Ser Val Leu Thr Leu Lys Gly Leu				
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Thr Pro Thr Gly Met Leu Pro Ser Gly Val Leu Ala Gly Gly Arg Gln				
	465		470	475 480
Thr Leu Gln Ser Ala Thr Val Glu Ala Ile Glu Ala Glu Lys Ala Ile				
	485		490	495
Arg Gly Phe Ser Pro Pro His Arg Ile Cys Ser Phe Glu Glu Ala Lys				
	500		505	510
Gly Leu Asp Arg Ile Asn Glu Arg Met Pro Pro Arg Lys Asp Ala Val				
	515		520	525
Gln Gln Asp Gly Phe Asn Ser Leu Asn Thr Ala His Ala Thr Glu Asn				
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His Gly Thr Gly Asn His Thr Ala Gln				
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<211> 170

<212> PRT

<213> Homo sapiens

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Asp Asn Ser Gly Ser Leu Ser Val Glu Glu Phe Met Ser Xaa Pro Glu				
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Leu Gln Gln Asn Xaa Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr				
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Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Xaa Val Ser				
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Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe				
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SUBSTITUTE SHEET (RULE 26)

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Arg Gly Ser His His His His His His Gly Ser Met Ser Gly Arg Arg
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ttc cac ctc tcc acc acc gac cgc gtc atc aaa gct gtc ccc ttt cct 213
Phe His Leu Ser Thr Thr Asp Arg Val Ile Lys Ala Val Pro Phe Pro
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cca acc caa cgg ctt act ttc aag gaa gta ttt gag aat ggg aaa cct 261
Pro Thr Gln Arg Leu Thr Phe Lys Glu Val Phe Glu Asn Gly Lys Pro
      35                      40                      45

aaa gtt gat gtt tta aaa aac cat ttg gta aag gaa gga cga ctg gaa 309
Lys Val Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu Glu
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Gln Glu Lys Thr Met Ile Glu Val Asp Ala Pro Ile Thr Val Cys Gly	
85 90 95	
gat att cat gga caa ttc ttt gac cta atg aag tta ttt gaa gtt gga	453
Asp Ile His Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly	
100 105 110	
gga tca cct agt aac aca cgc tac ctc ttt ctg ggt gac tat gtg gac	501
Gly Ser Pro Ser Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp	
115 120 125	
aga ggc tat ttc agt ata gag tgt gtg ctg tat tta tgg agt tta aag	549
Arg Gly Tyr Phe Ser Ile Glu Cys Val Leu Tyr Leu Trp Ser Leu Lys	
130 135 140 145	
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Ile Asn His Pro Lys Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys	
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Arg His Leu Thr Asp Tyr Phe Thr Phe Lys Gln Glu Cys Arg Ile Lys	
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Tyr Ser Glu Gln Val Tyr Asp Ala Cys Met Glu Thr Phe Asp Cys Leu	
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Pro Leu Ala Ala Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly Gly	
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Cys Glu Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His	
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Glu Ala Gln Asp Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Ala Thr	
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 Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe Arg Ile
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<212> PRT

<213> Homo sapiens

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Pro	Lys	Val	Asp	Val	Leu	Lys	Asn	His	Leu	Val	Lys	Glu	Gly	Arg	Leu
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Lys	Ile	Asn	His	Pro	Lys	Thr	Leu	Phe	Leu	Leu	Arg	Gly	Asn	His	Glu
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Cys	Arg	His	Leu	Thr	Asp	Tyr	Phe	Thr	Phe	Lys	Gln	Glu	Cys	Arg	Ile
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Gly Met Ser Pro Glu Ile Thr Ser Leu Asp Asp Ile Arg Lys Leu Asp		
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Arg Phe Thr Glu Pro Pro Ala Phe Gly Pro Val Cys Asp Leu Leu Trp		
225	230	235
Ser Asp Pro Ser Glu Asp Tyr Gly Asn Glu Lys Thr Leu Glu His Tyr		
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Thr His Asn Thr Val Arg Gly Cys Ser Tyr Phe Tyr Ser Tyr Pro Ala		
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Val Cys Glu Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala		
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Thr Gly Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu		
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Asp Val Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val		
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Met Asn Ile Arg Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro		
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Asn Phe Met Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys		
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Val Thr Glu Met Leu Val Asn Val Leu Asn Ile Cys Ser Asp Asp Glu		
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Ile Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys Met Ala Arg Val Phe		
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Ser Phe Glu Glu Ala Arg Gly Leu Asp Arg Ile Asn Glu Arg Met Pro		
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- 75 -

His Ser

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 35 40 45
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 50 55 60
 Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Gly Val Ser
 65 70 75 80
 Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe
 85 90 95
 Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu
 100 105 110
 Phe Gln Val Leu Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln
 115 120 125
 Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Gly
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 Asp Ile His Lys Lys Met Val Val Asp Val
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 <221> misc feature
 <222> (1690)..(1731)
 <223> ribosomal binding site, multiple cloning site 2

<220>
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 <222> (1732)..(2244)

<223> calcineurin B

<220>

<221> variation

<222> (1474)..(1503)

<223> splicevariant: substitution with ACAGTAGAAG
CGGTAGAGGC CCGGGAAGCC, interaction domain with
cytoskeleton, death-domain homolog, stomatin
homolog

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Met
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Arg Gly Ser His His His His His His Gly Ser Met Ser Gly Arg Arg
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Phe His Leu Ser Thr Thr Asp Arg Val Ile Lys Ala Val Pro Phe Pro
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cca acc caa cgg ctt act ttc aag gaa gta ttt gag aat ggg aaa cct 261
Pro Thr Gln Arg Leu Thr Phe Lys Glu Val Phe Glu Asn Gly Lys Pro
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Lys Val Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu Glu
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Glu Glu Val Ala Leu Lys Ile Ile Asn Asp Gly Ala Ala Ile Leu Arg
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caa gag aag act atg ata gaa gta gat gct cca atc aca gta tgt ggt 405
Gln Glu Lys Thr Met Ile Glu Val Asp Ala Pro Ile Thr Val Cys Gly
85 90 95

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115 120 125

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Arg Gly Tyr Phe Ser Ile Glu Cys Val Leu Tyr Leu Trp Ser Leu Lys
130 135 140 145

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150 155 160

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165 170 175

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/L. 99/05220

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/53 C12N15/55 C12N15/62 C12N15/70
 C12N15/85 C12N9/16 C12N9/02 C07K14/47 C12Q1/02
 G01N33/50 G01N33/573 A61K38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE, JEAN-PYO (1) ET AL: "Calcineurin activity in PC12 cells expressing a human ALS-associated SOD-1 mutant." SOCIETY FOR NEUROSCIENCE ABSTRACTS, (1997) VOL. 23, NO. 1-2, PP. 554. MEETING INFO.: 27TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, PART 1 NEW ORLEANS, LOUISIANA, USA OCTOBER 25-30, 1997 , XP000862971 Abstract no. 215.12; abstract --- -/-	1-4,9, 14,18-22

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

7 December 1999

Date of mailing of the international search report

20. 12. 99

Name and mailing address of the ISA

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Hornig, H

INTERNATIONAL SEARCH REPORT

International Application No

PCT/L. 99/05220

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X	X. WANG ET AL.: "Superoxide dismutase protects calcineurin from inactivation" NATURE, vol. 383, 3 October 1996 (1996-10-03), pages 434-437, XP002124884 MACMILLAN JOURNALS LTD., LONDON, UK cited in the application the whole document ---	1-4
X	A. MONDRAGON ET AL.: "Overexpression and purification of human calcineurin alpha from Escherichia coli and assessment of catalytic functions of residues surrounding the binuclear metal center" BIOCHEMISTRY, vol. 36, 1997, pages 4934-4942, XP002124885 AM. CHEM. SOC., WASHINGTON, DC, US cited in the application the whole document ---	19-22
X	O. ELROY-STEIN ET AL.: "Overproduction of human Cu/Zn-superoxide dismutase in transfected cells. extenuation of paraquat-mediated cytotoxicity and enhancement of lipid peroxidation" EMBO J., vol. 5, no. 3, March 1986 (1986-03), pages 615-622, XP000862989 OXFORD UNIVERSITY PRESS, GB; the whole document ---	19-22
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International Application No

PCT/Eur 99/05220

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199431 Derwent Publications Ltd., London, GB; Class B04, AN 1994-251696 XP002098865 & JP 06 181778 A (TAKEDA CHEM IND LTD), 5 July 1994 (1994-07-05) abstract ---	19-22
X	D. GUERINI AND C.B. KLEE: "Cloning of human calcineurin A: Evidence for two isozymes and identification of a polyproline structural domain" PROC. NATL. ACAD. SCI., vol. 86, December 1989 (1989-12), pages 9183-9187, XP002124888 NATL. ACAD. SCI., WASHINGTON, DC, US; the whole document ---	19-22
X	GUERINI D ET AL: "ISOLATION AND SEQUENCE OF A CDNA CLONE FOR HUMAN CALCINEURIN B, THE CA2+-BINDING SUBUNIT OF THE CA2+/CALMODULIN-STIMULATED PROTEIN PHOSPHATASE" DNA, US, NEW YORK, NY, vol. 8, no. 9, page 675-682 XP002062997 the whole document ---	19-22
X	T. MURAMATSU AND R.L. KINCAID ET AL.: "molecular cloning and chromosomal mapping of the human gene for the testis-specific catalytic subunit of calmodulin-dependent protein phosphatase (Calcineurin A)" BIOCHEM. AND BIOPHYS. RES. COMMUNICATIONS, vol. 188, no. 1, 15 October 1992 (1992-10-15), pages 265-271, XP002124889 ACADEMIC PRESS, NEW YORK, US the whole document ---	21,22
X	F. GAMONET AND G. J.-M. LAQUIN: "The Saccharomyces cerevisiae LYS7 gene is involved in oxidative stress protection" EUR. J. BIOCHEM., vol. 251, no. 3, February 1998 (1998-02), pages 716-723, XP000857423 SPRINGER, BERLIN, D page 719, left-hand column, line 1 - line 16 page 720, left-hand column, line 24 -right-hand column, line 1 --- -/--	19,20

INTERNATIONAL SEARCH REPORT

International Application No

PCT/LR 99/05220

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D.K. BLUMENTHAL ET AL.: "Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (type II) by calmodulin-dependent phosphatase" J. BIOL. CHEM., vol. 261, no. 18, 25 June 1986 (1986-06-25), pages 8140-8145, XP002124890 AM. SOC. BIOCHEM. MOL.BIOL.,INC.,BALTIMORE,US the whole document ---	23
A	P.M. STEMMER ET AL.: "Factors responsible for the Ca++-dependent inactivation of calcineurin in brain" FEBS LETTERS, vol. 374, 1995, pages 237-240, XP002124891 ELSEVIER, AMSTERDAM, NL cited in the application the whole document ---	
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A	TATLOCK J H ET AL: "Structure-based design of novel calcineurin (PP2B) inhibitors" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS,GB,OXFORD, vol. 7, no. 8, page 1007-1012 XP004136174 ISSN: 0960-894X the whole document -----	

INTERNATIONAL SEARCH REPORT

In relation on patent family members

International Application No

PCT/LR 99/05220

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WO 9616172	A	30-05-1996	US 5807693 A US 5744354 A US 5871945 A AU 4513396 A BR 9506540 A CA 2181826 A CN 1148407 A CZ 9602136 A EP 0748379 A FI 962932 A HU 75826 A JP 9509849 T NO 963049 A PL 315627 A SK 95096 A US 5629163 A	15-09-1998 28-04-1998 16-02-1999 17-06-1996 07-10-1997 30-05-1996 23-04-1997 14-05-1997 18-12-1996 20-09-1996 28-05-1997 07-10-1997 23-09-1996 25-11-1996 09-07-1998 13-05-1999
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