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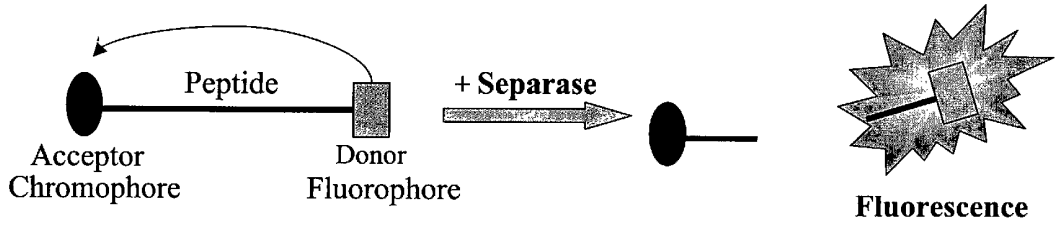
(54) Title: METHOD FOR IDENTIFYING COMPOUNDS MODULATING SISTER CHROMATID SEPARATION



(57) Abstract: Screening methods for identifying separase inhibitors based on active forms of separase and compounds identified in such methods.

Method for identifying compounds modulating sister chromatid separation

The invention relates to compounds influencing mitosis and meiosis in eukaryotic cells and methods for identifying such compounds. In particular, the invention largely relates to the treatment and prevention of human conditions by modulating sister chromatid segregation.

A key prerequisite for the successful division of one cell into two is the duplication and subsequent segregation of the cellular genome into the two forming daughter cells. Duplication of the genome by DNA replication occurs during synthesis (S) phase of the cell cycle, whereas segregation of the duplicated DNA takes place much later during anaphase of mitosis. During S phase, replicated DNA molecules remain physically attached to each other, a phenomenon called cohesion, until they are separated in anaphase. At the beginning of mitosis, the cellular DNA is condensed into chromosomes, in which each of the two replicated DNA molecules are microscopically visible as sister chromatids. To allow the segregation of sister chromatids to the forming daughter cells in anaphase, the cohesion that is holding sisters together has to be dissolved. This process is mediated by a protease, called separin or separase, that is cleaving a complex of chromosomal cohesion proteins (the cohesin complex) that is required to hold sister chromatids together. This cleavage reaction liberates sisters from each other so that they can be pulled towards opposite poles of the diving cells by the spindle apparatus (reviewed by Nasmyth et al., 2000).

Inhibition of cohesin cleavage by separase in experimental systems such as budding yeast or human cultured cells inhibits sister chromatid separation and thus prevents the formation of viable daughter cells (Uhlmann et al., 1999; Uhlmann et al., 2000; Hauf et al., 2001).

WO 00/48627 suggests a method for identifying compounds which exert their effect by directly modulating, in particular by inhibiting separase's proteolytic activity, i.e. by being protease inhibitors specific for separase. (In the following, if not otherwise stated, "separase" stands for "human separase".)

The screening method for identifying compounds that have the ability of modulating sister chromatid separation in plant or animal cells as described in WO00/48627 comprises incubating separase, in the presence of the substrate(s) for its proteolytic activity and optionally its co-factor(s), with test compounds and determining the modulating effect of the test compounds on the proteolytic activity of the separase.

It was an object of the invention to gain further insight into the mechanism of sister chromatid separation, in particular to elucidate the mechanism by which separase exerts its proteolytic activity, which has been shown to be involved in this process. The mechanism of separase activation provides the basis for developing improved enzymatic assays to identify modulators, in particular inhibitors of separase, in order to provide drugs that exert their effect by modulating, in particular inhibiting, sister chromatid segregation.

The identification of small molecule inhibitors of separase requires enzyme assays in which the protease activity of separase can be directly measured. Preferably, such assays are adaptable to high throughput formats so that large libraries of chemical compounds can be tested for their ability to inhibit separase. The previously reported experiments demonstrating that separase is associated with a protease activity were performed with separase isolated in

small scale by immunoprecipitation or affinity chromatography from either yeast or human cells (Uhlmann et al., 2000; Waizenegger et al., 2000). Along these lines, WO00/48627 suggests to use full-length separase, preferably in recombinant form, for performing protease assays.

The protease activity of separase is tightly regulated during the cell cycle, ensuring that the ability of separase to cleave cohesin and thereby to dissolve sister chromatid cohesion is not activated before the transition from metaphase to anaphase. Work in budding yeast and human cells has shown that prior to anaphase separase is inhibited by a protein called securin (Ciosk et al., 1998; Uhlmann et al., 1999; Waizenegger et al., 2000). Securin binds to separase until securin is ubiquitinated by the anaphase-promoting complex and subsequently degraded by the 26S proteasome shortly before the onset of anaphase. The destruction of securin is thought to activate separase. In human cells, separase itself is cleaved at the same time as securin is destroyed, resulting in at least two C-terminal cleavage products called p55 and p60 (Waizenegger et al., 2000). These results suggest that the activity of separase is controlled by both securin destruction and separase cleavage. It is not known however and cannot be concluded from these results, which form of the separase, i.e. the full-length form or the cleavage products of separase, represent the active form of the protease or whether and in which way securin has a potential to contribute to the activation of separase.

To establish a separase protease assay suitable for high throughput format it is essential to know which form of separase represents the active enzyme and how the active form of separase can be obtained.

The present invention provides the first evidence that the cleaved forms of separase represent the active protease and that N- and C-terminal cleavage products of separase remain physically associated. The present invention further provides evidence that securin inhibits separase by directly binding to it.

Securin binding to separase could either directly block the access of substrates to the active site of separase or it could keep separase in a conformation in which its active site is not accessible to substrates. The present invention further provides evidence that it is the autocatalytic reaction, i.e. the reaction in which separase cleaves itself, which is responsible for the cleavage of separase into its active form. Finally, the present invention shows that active forms of tagged recombinant full-length separase can be obtained and that fluorogenic peptide substrates are useful to measure the protease activity of separase.

It was previously reported that active human separase can be obtained by immunoprecipitating separase from human cell extracts (Waizenegger et al., 2000). Briefly, antibodies specific for the C-terminus of separase coupled to beads are incubated in lysates obtained from human cultured cells (for example of the line HeLa) which have previously been arrested in mitosis by treatment with microtubule poisons such as nocodazole. After removal of the cell lysate the antibody beads with bound separase are incubated in mitotic Xenopus laevis egg extracts which are able to ubiquitinate and degrade the securin protein which is bound to separase when it is immunoprecipitated from mitotic human cell lysates. During the incubation in mitotic Xenopus extracts the majority of securin is degraded, but in addition a major portion of human separase is also cleaved. After washing away the Xenopus egg extracts the antibody beads with bound separase are incubated with either purified cohesin complexes or with recombinant SCC1, which is the subunit of the cohesin complex that is cleaved by separase. The activity of separase is then monitored by analyzing the cleavage of SCC1, which can be analyzed by SDS gel electrophoresis and subsequent immunoblotting with antibodies to SCC1. If recombinant radioactively labeled SCC1 is used the gels can also be analyzed by autoradiography or Phosphorimaging.

In the experiments of the invention (Example 1), it was found that securin is able to bind to separase. It was shown that securin can bind to the cleaved form of separase as well as to the full-length separase. It was found that securin binding to separase inhibits the protease activity of separase (Fig. 1).

To further study the mechanism of separase activation it was tested if two different peptide inhibitors developed to inhibit separase from budding yeast are able to inhibit the protease activity of human separase. It was found that the concentration of these peptide derivatives required to inhibit human separase was similar to the concentration needed to inhibit separase from budding yeast (compare Fig. 2, upper panel and WO 00/48627, Uhlmann et al., 2000). It was further observed that the formation of the p55 cleavage product of human separase was largely inhibited by both peptides at the same concentration at which the ability of separase to cleave SCC1 was inhibited The obtained results suggest that separase activity itself is required for separase cleavage, i.e. that separase cleavage occurs autocatalytically.

A peptide inhibitors developed on the basis of the cleavage recognition site of human SCC1, "DREIMR", was also able to inhibit separase at a similar concentration as the above mentioned peptide inhibitors (Fig. 3).

It was further tested which form of separase represents the active protease. It was found that the peptide inhibitor Bio-SVEQGR-amk bound exclusively to the cleaved forms of human separase when the peptide derivative was added to separase after its activation in mitotic Xenopus extracts. This observation shows that the active site of separase is accessible to Bio-SVEQGR-amk in the cleaved, but not in the residual amount of full-length separase to which securin is still bound (Fig. 4). These results suggest that the cleaved forms of separase represent active forms of the protease.

The obtained results also suggest that the full-length form of separase is also transiently active, presumably once its bound inhibitor securin has been destroyed, but that this form is normally labile because it is further processed into the cleaved forms by autocleavage.

It was shown that securin inhibits separase by directly or indirectly blocking the access of substrates to the active site of separase (Fig 4D). It was further shown directly that separase cleavage can occur autocatalytically in trans and two cleavage sites in human separase were mapped (Fig. 8).

To test if the N- and C-terminal fragments of separase remain non-covalently associated after cleavage, tagged forms of separase were generated. During the course of these experiments it was recognized that recombinant separase expressed from the published cDNA sequence (KIAA 0165) was not the full-length human enzyme but instead a fragment lacking the N-terminus of separase. The 5'-end of the human separase cDNA that encodes the missing N-terminus was therefore cloned. Subsequently, N-terminally FLAG-tagged full-length separase was transiently expressed in HeLa cells and isolated by immunoprecipitation with an anti-FLAG antibody. The immunoprecipitates were incubated in mitotic Xenopus egg extracts to allow securin destruction and separase cleavage. The finding that both C-and N-terminal separase fragments could be detected in the immunoprecipitates, suggested that the N- and C-terminal separase fragments remain associated after cleavage (Fig. 6).

Together, the obtained results suggest the model for the activation of human separase that is shown in Figure 7. According to this model, full-length separase (p200) is associated with securin and represent the inactive state of the protease. Upon proteolysis of securin a labile full-length form of separase exists. The cleaved forms of separase stay physically associated with the N-terminal fragment(s) and represent the stabile, active enzyme.

The findings of the present invention show that protease assays for identifying separase inhibitors can be based on an active form of human separase, which is present in the cleaved forms of separase. These forms have the advantage to be more stable than the complete separase molecule and are thus expected to be better suitable for being employed in a high throughput format assay.

In order to obtain one or more active, stable forms of separase, the following steps can be taken:

Recombinant forms of the p55 and p60 cleavage products (Waizenegger et al., 2000), or potential other active cleavage products of separase, are produced in suitable expression systems, purified and tested for their ability to cleave SCC1, or a fragment thereof that contains the separase cleavage site, in vitro. Standard expression systems such as E. coli, budding yeast, Baculovirus infected Sf9 and Hi5 insect cells and transfected mammalian, e.g. human cells can be used. For purification, standard biochemical protocols can be used, e.g. those described in WO00/48627 for obtaining separase.

If p55 or p60 (or another, natural or synthetic, C-terminal fragment of human separase) alone or in combination with another fragment is sufficient for separase activity, the respective fragment (or a combination of fragments) can be employed in the protease assay (as described in WO00/48627) as a substitute for the full-length separase molecule.

Since the N- and C-terminal cleavage products of separase remain associated with each other (see above, Figure 6) it is also possible that both the N- and the corresponding C-terminal fragments will be required to obtain active recombinant separase. The two or more fragments can either be expressed individually, purified and then mixed together, or they can be co-expressed in expression systems as listed above, and the obtained complexes containing both N- and C-terminal fragments are purified. All of these forms, e.g. all

combinations of C-terminal and corresponding N-terminal fragments, will then be tested for their ability to cleave SCC1 in vitro. If any of the complexes described above yields human separase activity, the respective complex of separase fragments can be employed in the proteolytic assay in the same manner as a C-terminal fragment by itself, as described above.

If the above-described approach using complexes comprising various separase fragments does not exhibit human separase activity, full-length human separase is expressed in expression systems as listed above, the recombinant protein is isolated and activated, e.g. by incubation in mitotic Xenopus egg extracts, to induce its activation by cleavage.

In parallel, complexes of separase and securin can be generated to investigate whether the binding of securin to separase may not only inhibit separase but may also be required for its subsequent activation. To test this possibility, either recombinant securin is added to recombinant separase after their individual expression and purification, or securin and separase are co-expressed in expression systems as above. All forms of separase, i. e. full-length separase, separase fragments or combinations of fragments, with and without transiently bound securin, are tested for their ability to cleave SCC1 in vitro after incubation of the different forms of separase in mitotic Xenopus extracts.

In the case that these experiments result in the finding that securin is required for human separase activity, the assay is performed by employing the respective form of separase (fragment(s)) in combination with securin. The separase (fragment) is activated in the presence of securin in cell extracts, e.g. Xenopus laevis cell extracts. Preferably, as for the other assay components, securin, or a fragment thereof that proves to be sufficient for activation of separase (if not stated otherwise, "securin" also stands for an active fragment thereof) is employed in recombinant form, based on the cDNA sequence (Lee

et al., 1999; Zhang et al, 1999). In order to obtain a protein complex for the assay, the separase (fragment) and securin can either be expressed and purified separately and then combined or they can be co-expressed and co-purified; as described above.

The results of Figure 6 shows that the latter approach is feasible. Flag-tagged separase was coexpressed with myc-tagged securin in HeLa cells. Coexpression with securin lead to a higher yield of separase. Mitotically actived separase immunoprecipitates were able to cleave SCC1.

Once the active form(s) of human separase have been obtained by one or more of the methods described above, synthetic peptide substrates for separase are designed and synthesized that allow the simple detection of protease activity in high throughput format, e.g. by fluorogenic methods. The proteolytic assays suitable for this purpose have been described in WO00/48627. By way of example, substrate peptides containing the separase recognition sequence (see WO00/48627) that carry a C-terminal fluorophore such as a 7-amino-4-methyl-coumarin group (AMC) are synthesized by standard methods. The cleavage of AMC (or other fluorophore groups used) results in a rise in fluorescence which can be measured fluorometrically. In an experiment of the present invention, the activity of mitotically activated immunoprecipitates of separase (Waizenegger et al., 2000) was measured fluorometrically by using AMC-coupled peptides based on the cleavage recognition sites of human separase, "SFEILR". The result of this experiment provides the basis for the development of a screening assay for identifying separase inhibitors. For conducting this assay in the high throughput mode, compounds, e.g. from chemical or natural product libraries, can be tested for their ability to inhibit the cleavage of fluorogenic peptide substrates by the active form(s) of human separase, which is preferably employed in the screen in recombinant form.

Similarly, the approaches as described above can be used to determine the active forms of separase from other eukaryotic organisms, to generate these forms as recombinant active proteins and to establish screening method for identifying inhibitors of these enzymes.

The present invention relates to methods for identifying a compound that has the ability of modulating sister chromatid separation by inhibiting the proteolytic activity of separase, characterized in that an active separase in the form of

a) one or more separase fragment(s), optionally upon activation in the presence of securin, or

b) full-length separase upon activation in the presence of securin,

is incubated in the presence of a separase substrate, with a test compound and that the modulating effect of the test compounds on the proteolytic activity of the separase is determined.

Any variation of the proteolytic screening assay method of the invention, e.g. carried out with one or more separase fragments, in the presence or absence of securin, can be carried out according to standard methods, in particular as described in WO 00/48627:
Various assay methods for identifying protease inhibitors that are useful in the present invention and are amenable to automation in a high-throughput format have been described, e.g. the radiometric method described by Cerretani et al., 1999, for hepatitis C virus NS3 protease, the method based on fluorescence quenching described by Ambrose et al., 1998, or by Taliani et al., 1996, the microtiter colorimetric assay fot the HIV-1 protease described by Stebbins and Debouck, 1997, the fluorescence polarization assay described by Levine et al., 1997 (reviewed by Jolley, 1996), the method using immobilized peptide substrates described by Singh et al., 1996, the assay

used for studying the inhibition of cathepsin G, using biotinylated and cysteine-modified peptides described by Brown et al., 1994. A further example for a suitable assay is based on the phenomenonon of fluorescence resonance energy transfer (FRET), as described by Gershkovich et al., 1996 or by Matayoshi et al., 1990. Additional examples for assays that may be used in the present invention for a high-throughput screening method to identify inhibitors of separase activity were described by Gray et al., 1994, Murray et al., 1993, Sarubbi et al., 1991.

Fluorescent or radioactive labels and the other reagents for carrying out the enzymatic reaction on a high-throughput scale are commercially available and can be employed according to supplier's instructions (e.g. Molecular Probes, Wallac). The specific assay design depends on various parameters, e.g. on the size of the substrate used. In the case of using a short peptide, the fluorescence quenching or the fluorescence resonance energy transfer methods are preferred examples for suitable assay technologies.

The fluorescence quenching (Resonance Energy Transfer „RET") assay relies on synthetic substrates which are capable of direct, continuous signal generation that is proportional to the extent of substrate hydrolysis. The substrate peptide carries a fluorescent donor near one end and an acceptor near the other end. The fluorescence of the substrate is initially quenched by intramolecular RET between donor and acceptor. Upon cleavage of the substrate by the protease the cleavage products are released from RET quenching and the a fluorescence proportional to the amount of cleaved substrate can be detected. In Example 9, this type of assay is exemplified by use of AMC; which serves as a donor fluorophore and in the case of the separase-specific peptide substrates the amino acid bonds of the peptides function as acceptor chromophores.

An assay of this type may be also carried out as follows: the solution of the labeled substrate (e.g. the peptide labeled with 4-[[4′-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) at the one end and with 5-[(2′-aminoethyl)amino]naphtalenesulfonic acid (EDANS) at the other end or labeled with benzyloxycarbonyl at the one end and with 4-aminomethylcoumarin at the other end) in assay buffer is pipetted into each well of black 96-well microtiter plates. After addition of the test substances in the defined concentration, the separase activity containing solution is added to the wells. After incubation under conditions and for a period of time sufficient for the proteolytic cleavage reaction, e.g. for 1 hour at room temperature, the fluorescence is measured in a fluorometer at the excitation wavelength, e.g. at 340 nm, and at the emission wavelength, e.g. at 485 nm.

In the case of using the FRET assay, labeling pairs that are suitable for the method of the invention are commercially availabe, e.g. Europium (Eu) and Allophycocyanin (APC), Eu and Cy5, Eu and PE (Wallac, Turku, Finland).

The compounds identified in the above methods have the ability to interfere with sister chromatid separation by modulating the proteolytic activity of separase.

The present invention also relates to compounds which act as inhibitors of separase for use in human therapy, in particular cancer therapy.

In a further aspect, the invention relates to a pharmaceutical composition which contains, as the active ingredient, one or more compounds which interfere with or modulate sister chromatid separation by inhibiting separase activity.

In a preferred embodiment, the invention comprises pharmaceutically active compounds and their use in therapy, which are small chemical molecules that

have been identified as separase inhibitors in the screening method of the invention.

As an alternative to identifying small molecules in a screening method, separase inhibitors can be obtained starting from the recombinant active separase. In this approach, synthetic peptide derivatives, exemplified by derivatives of SVEQGR, DREIMR, SFEILR or EWELLR (e.g. Bio-SVEQGR-amk) can be used as the structural basis to develop peptidomimetic molecules that inhibit separase. For inhibitors of human separase, the cleavage the cleavage sequence of human SCC1 or human separase can preferably be used. The assays described above using recombinant active separase and peptide substrates, e.g. fluorogenic peptides, can be used to optimize such compounds.

Inhibitors of human separase activity identified in the screening methods of the invention or based on rational inhibitor design can be used as cytotoxic therapeutics for the treatment of diseases that are caused by uncontrolled cell proliferation, such as cancers, leukaemias, or cardiac restenosis. Species specific inhibitors of separase from eukaryotic pathogenic microorganisms can be used to treat infectious diseases caused by such microorganisms, for example infections caused by pathogenic fungi or diseases caused by parasites such as Leishmania species.

To address how separase inhibition affects cell cycle progression, RNA interference experiments can be used to knock out separase expression in human cultured cells, according to known methods, as described, e.g. by Elbashir et al, 2001.

Influencing the process of sister chromatid separation may be also beneficial in preventing birth defects caused by missegration of chromosomes in human

meioses. For example, since cases of human aneuploidy such as Down's syndrome may be caused by premature separation of sister chromatids (Griffin, 1996), the use of a drug that inhibits separase activity might be able to reduce precocious sister separation and thereby the incidence of aneuploidy in human fetuses.

Thus, in a further aspect, the invention relates to separase inhibitors for the prevention of birth defects caused by missegration of chromosomes in human meioses.

The efficacy of compounds identified as separase inhibitors in the method of the invention, can be tested for *in vivo* efficacy either on yeast cells or in mammalian cells. Effective compounds should block (or at least in some way interfere with) sister chromatid separation, which can be measured, e.g. by using CenV-GFP in yeast, as described by Ciosk et al., 1998, or standard cytological techniques in mammalian cells. Compounds effective in tumor therapy should be either cytostatic or cytotoxic. Substances whose potential for therapeutic use has been confirmed in such secondary screens can be further tested for their effect on tumor cells.

To test the inhibition of tumor cell proliferation, primary human tumor cells are incubated with the compound identified in the screen and the inhibition of tumor cell proliferation is tested by conventional methods, e.g. bromo-desoxy-uridine or ^{3}H incorporation. Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumor animal models and used for the therapy of tumors.

Toxicity and therapeutic efficacy of the compounds identified as drug candidates by the method of the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the IC_{50}, LD_{50}, the ED_{50}. The data obtained are used for determining the human dose range, which will also depend on the dosage

form (tablets, capsules, aerosol sprays, ampules, etc.) and the administration route (oral, buccal, nasal, paterental or rectal). A pharmaceutical composition containing the compound as the active ingredient can be formulated in conventional manner using one or more physologically active carriers and excipients. Methods for making such formulations can be found in manuals, e.g. "Remington Pharmaceutical Sciences".

Separase inhibitors may also be useful in applications which aim at the deliberate polyploidisation of plant cells for crop development. In yeast, it has been shown that inhibition of separase activity prevents chromosome separation without blocking cell cycle progression and therefore gives rise to cells with increased ploidy. Inhibitors that block separase's protease activity could therefore be used to increase the ploidy of any eukaryotic cell, including all plant cells. Increasing the ploidy of plant cells is useful for 1) producing larger plants, 2) for increasing the ploidy of breeding stocks, and 3) for generating fertile hybrids.

Therefore, the present invention relates, in a further aspect, to separase inhibitors for the treatment of plant cells for increasing their ploidy.

To identify separase inhibitors that are useful for the above-mentioned agricultural purposes, the screening method of the invention can be easily adapted by employing plant components, i.e. a plant separase and a plant homolog of SCC1. Sequence homologs of plant separase and SCC1 are present in databases, e.g. of the Arabidopsis thaliana genome.

Separase inhibitors which impair sister chromatid separation may also be used in cytological analyses of chromosomes, for example, in medical diagnoses of chromosome structure.

Brief description of the Figures:

Fig. 1: Securin acts as an inhibitor for separase

Fig. 2: Yeast peptides inhibit proteolytic activity of human separase in similar concentration as they inhibit yeast separase and influence the processing of human separase

Fig.3: Human peptides inhibit proteolytic activity of human separase in a similar concentration as the yeast peptides do

Fig. 4: Addition of yeast peptides at different stages during the activation of separase suggests that the cleaved forms are the active forms of separase and securin binding blocks access of peptide substrates to the active site of separase

Fig. 5: Mitotically activated separase has autocatalytic activity

Fig. 6: N- and C-terminal cleavage products of human separase stay associated after mitotic cleavage and ectopically expressed separase is able to cleave human SCC1

Fig. 7: Working model for the activation of human separase

Fig. 8: Mapping of the cleavage sites in human separase

Fig. 9: A Principle of fluorogenic separase inhibitor screening assay

B: Processing of a separase peptide substrate by trypsin

Fig. 10: In vitro assay using activated separase bound to microbeads

Fig. 11: Transcleavage separase screening assay showing inhibiting effect of small molecular compounds

Materials and methods:

SCC1 in vitro cleavage assay

Inactive human separase (separase) immunoprecipitates were activated in mitotic Xenopus egg extracts (for details see Waizenegger et al., 2000). 30 microliter beads were incubated with 40 µg bacterially expressed wildtype securin, destruction box deleted form of securin (Gmachl et al., 2000) or BSA diluted in XB + 1 mM DTT for 40 min at RT. Subsequently the beads were washed with XB + 1 mM DTT and with TBS + 0.5 M NaCl + 1 mM DTT + 0.5% TWEEN20, followed by one wash with XB + 1mM DTT. To control the rebinding of securin an aliquot of 5 µl was taken per assay and subsequently analysed by immunoblotting with antibodies against separase and securin.

The beads were used for the SCC1 in vitro cleavage assay: 20 µl beads were mixed with 30 µl of the following SCC1 in vitro translation mix (SCC1myc IVT + 1 µl PLK-GST + 0.3 µl 1 M $MgCl_2$, 0.3 µl 100 mM ATP, 0.12 µl 250 mM EGTA, 13.78 µl XB + 1mM DTT) and incubated at 22° C and 1200 rpm. 5 µl were taken per time point, the reaction was stopped by addition of SDS loading buffer. Samples were analyzed by immunoblotting with mouse monoclonal antibodies against myc (9E10).

Modifications:

A. Mitotically activated separase immunoprecipitates were preincubated with yeast peptides (Uhlmann et al., 2000) before they were used in the SCC1-cleavage assay. Biotin-SVEQGR-amk or Biotin-SVEQGR-cmk were used at

different concentration (0.1, 1, 10, 100, 1000 μM in XB + 0.5 mM DTT). Separase immunoprecipitates were incubated for 10 minutes, 22° C and 1200 rpm. The SCC1 in vitro cleavage assay was performed after washing once with XB + 0.5 mM DTT (see above). Human peptides, Biotin-DREIMR-amk or DREIMR-amk, were diluted and used as above described for the yeast peptides.

B. Separase immunoprecipitates were incubated with 100 μM Biotin-SVEQGR-amk or with DMSO prior mitotic activation. Incubation for 10 minutes, 22° C and 1000 rpm. After washing twice in XB + 1mM DTT the activation was performed in mitotic Xenopus egg extracts.

Interphase Xenopus egg extracts were driven into mitosis in the presence of 1 mM Biotin-SVEQGR-amk or DMSO. Those mitotic Xenopus egg extracts were used to activate a batch of separase immunoprecipitates.

Biotin labeled peptides were detected via immunoblotting according to Faleiro et al., 1997.

C. Mitotically activated separase immunoprecipitates were preincubated with either 8 μg recombinant securin or with 8 μg of a C-terminal truncated version of cyclinB in 100 μl XB+ 1 mM DTT for 30 min at 22° C and 1250 rpm. Subsequently the beads were washed twice with TBS + 0.5 M NaCl + 1 mM DTT + 0.5 % Tween 20 and twice with XB + 1 mM DTT. Thereafter beads were incubated with 100μl of 100 μM Bio-DREIMR-amk in XB + 1 mM DTT. After 10 min incubation at 22°C and 1250 rpm beads were again washed and analyzed by immunoblotting.

D. A separase construct tagged with one myc epitope at its C-terminus was in vitro transcribed and translated and then used as a substrate for separase bound to beads. The reaction mix was supplemented as described above for

the SCC1 cleavage assay. The myc tag allowed the discrimination between separase as a substrate and as an enzyme.

Example 1

Securin acts as an inhibitor for separase

To test if securin inhibits separase by directly binding to it and if it is still able to bind and inhibit the cleaved form of separase human securin was expressed in E. coli and purified. When the recombinant securin was added to separase that had been isolated by immunoprecipitation and had been activated in mitotic Xenopus extracts as above it was observed that securin was able to bind to separase (Figure 1A). Importantly, the amount of securin that bound to separase was at least as high as the amount of securin that had been bound to separase originally before securin was degraded by incubating the separase immunoprecipitates in mitotic Xenopus extracts, although the majority of separase had been cleaved during the incubation in the Xenopus extract. This result shows that securin can bind to the cleaved form of separase as well as to the full-length separase.

When the cleaved form of separase to which recombinant securin had been bound was incubated with recombinant SCC1, no cleavage of SCC1 was detected (Figure 1B), demonstrating that recombinant securin is able to inhibit the protease activity of separase by directly binding to it. Because the majority of separase used in this experiment was present in its cleaved form these observations suggest that it is the cleaved form of separase that is the active protease which cleaves SCC1, unless it is inhibited by the rebinding of securin. Identical results were obtained with two different forms of securin, the wildtype

protein (WT-securin) and a form that can not be destroyed by ubiquitin-dependent proteolysis (DB-securin; Figure 1A and B).

Fig. 1A: Separase immunoprecipitates (separase IP) obtained from Nocodazole arrested HeLa cells and bound by antibodies against the C-terminus of separase coupled to beads were activated in mitotic Xenopus egg extracts (separase $IP^{mitotic}$). Mitotically activated separase immunoprecipitates were either incubated in buffer, wildtype securin, destruction box deleted securin or in BSA. Aliquots were taken and analysed by immunoblotting with antibodies against separase and securin.

Fig.1B: Activated separase immunprecipitates which were either incubated with buffer, wildtype securin, destruction box deleted securin or BSA were incubated SCC1-myc reaction mix. Aliquots were taken at indicated timepoints and analysed by immunoblotting with antibodies against myc. Cleaved SCC1 is marked by arrows.

Example 2

Yeast peptides inhibit proteolytic activity of human separase in similar concentration as they inhibit yeast separase and influence the processing of human separase

To further study the mechanism of separase activation it was tested if two different peptide inhibitors developed to inhibit separase from budding yeast (Uhlmann et al., 2000) are able to inhibit the protease activity of human separase. These inhibitors are synthetic peptides containing the cleavage site of budding yeast SCC1, "SVEQGR", where the last arginine residue represents the P1 site after which separase cleaves. The C-terminus of this peptide is either modified to a chloromethyl ketone (cmk) or to an acyloxymethyl ketone (amk). Both peptide derivatives are coupled to biotin moieties at their N-termini. The two inhibitors are therefore called Bio-SVEQGR-cmk and Bio-SVEQGR-amk. When these inhibitors were added to human separase that had been isolated by immunoprecipitation and had been activated in mitotic Xenopus extracts as above, it was observed that both inhibitors are able to block the ability of separase to cleave SCC1 (Figure 2A). The concentration of these peptide derivatives required to inhibit human separase was similar to the concentration needed to inhibit separase from budding yeast (compare Fig. 2, upper panel and WO 00/48627, Uhlmann et al., 2000). It was further observed in this experiment that the formation of the p55 cleavage product of human separase was largely inhibited by both peptides at the same concentration at which the ability of separase to cleave SCC1 was inhibited (Figure 2B).

Fig. 2A: The structure of the yeast peptides

Fig. 2B: Separase immunoprecipitates obtained from nocodazole arrested HeLa cells bound by antibodies against the C-terminus of separase coupled to beads were activated in mitotic Xenopus egg extracts. Subsequently samples were incubated with indicated concentrations of yeast peptides (Biotin-SVEQGR-cmk or Biotin-SVEQGR–amk). After a short wash samples were mixed with SCC1-myc reaction mix for 1 hour. Samples were analysed by immunoblotting with antibodies against myc. Cleaved SCC1 is marked by an arrow.

Fig. 2C: The samples (see B) were immunblotted with antibodies against separase.

Example 3

Human peptides inhibit proteolytic activity of human separase in a similar concentration as the yeast peptides inhibit yeast and human separase

Separase immunoprecipitates obtained from nocodazole-arrested HeLa cells bound by antibodies against the C-terminus of separase coupled to beads were activted in mitotic Xenopus egg extracts. Subsequently samples were incubated with the indicated concentrations of human peptides (DREIMR-amk or Biotin-DREIMR-amk). After washing samples were mixed with SCC1-myc reaction mix for 1 hour. Samples were analysed by immunoblotting with antibodies against myc. For control a sample was treated with the same concentration of DMSO which was used for the solubilization of the peptides (DMSO). The SCC1-myc reaction mix was loaded as an input control (SCC1-myc input). It was found that peptide inhibitors developed on the basis of the cleavage recognition site of human SCC1, "DREIMR", were able to inhibit separase at a similar concentration as the peptide inhibitors derived from yeast (Fig. 3).

Fig. 3A shows the structure of the human peptide

Fig. 3B shows that human peptide derivatives inhibit the SCC1 cleavage activity of separase. Full length SCC1 is indicated by an arrowhead, cleaved SCC1 is marked by an arrow.

Example 4

Addition of yeast inhibiting peptides at different stages during the activation of separase suggests that the cleaved forms are the active forms of separase and that securin blocks access of substrates to the active site of separase

To further test which form of separase represents the active protease, the ability of Bio-SVEQGR-amk, the more effective one of the two yeast peptide inhibitors, to bind to different forms of separase, was tested. Previous work has shown that the peptide derivatives inhibit separase by covalently binding to an active site cysteine residue within separase (Uhlmann et al., 2000). These binding reactions can be directly visualized by separating the separase-inhibitor conjugate by SDS gel electrophoresis and by subsequently labeling the biotin moiety on the peptide derivative by streptavidin (Uhlmann et al., 2000). By using this method it was found that Bio-SVEQGR-amk bound exclusively to the cleaved forms of human separase when the peptide derivative was added to separase after its activation in mitotic Xenopus extracts (Figure 4B, lane 3i). This treatment inhibited SCC1 cleavage by separase (Figure 4C, lane 3i). This observation shows that the active site of separase is accessible to Bio-SVEQGR-amk in the cleaved, but not in the residual amount of full-length separase, confirming the conclusion from the securin addback experiments that the cleaved forms of separase represent active forms of the protease.

When Bio-SVEQGR-amk was added to human separase immunoprecipitates before separase had been activated in mitotic Xenopus extracts, only the small amount of p60 that is already present in these immunoprecipitates was labeled by the peptides, whereas full-length separase was not (Figure 4B, lane 1i), further confirming that the active site of separase is only accessible in the cleaved forms. When the peptide inhibitor was washed away before the separase was subsequently incubated in mitotic Xenopus extracts separase could be activated normally to cleave SCC1 (Figure 4C, lane 1i). This result suggests that the presence of securin prevents the binding of Bio-SVEQGR-amk to the active site of separase, implying that securin inhibits separase by directly or indirectly blocking the access of substrates to the active site of separase.

When the activation of human separase immunoprecipitates in mitotic Xenopus extracts was carried out in the presence of Bio-SVEQGR-amk both the cleaved forms of separase and full-length separase were covalently labeled with the peptide (Figure 4B, lane 2i) and separase was unable to cleave SCC1 (Figure 4C, lane 2i). This observation suggests that the full-length form of separase is also transiently active, presumably once its bound inhibitor securin has been destroyed, but that this form is normally labile because it is further processed into the cleaved forms by autocleavage. It was further shown that binding of recombinant securin to cleaved active separase prevented the binding of peptide inhibitors to the active site of separase (Fig. 4D). This result suggests that securin inhibits separase by either directly or indirectly blocking the access of substrates to the active site of separase.

Fig. 4A: Separase immunoprecipitates (separase IP) obtained from Nocodazole arrested HeLa cells bound by antibodies against the C-terminus of separase coupled to beads were activated in mitotic Xenopus egg extracts

(separase $IP^{mitotic}$). Aliquots were analysed by immunoblotting with antibodies against separase and securin.

Fig. 4B: Separase IPs (see A) were either preincubated with Biotin-SVEQGR-amk (preinc. with inh. peptide) or with DMSO (preinc. with DMSO), subsequently washed and aliquots were taken. Thereafter they were incubated in mitotic Xenopus egg extracts and washed again. Samples were taken for analysis (1i, 1c). Separase IPs were activated either in mitotic Xenopus egg extracts which were driven into mitosis in the presence of Biotin-SVEQGR-amk or DMSO, subsequently washed and aliquots were taken for analysis (2i, 2c). Already mitotically activated separase IPs (see A) were incubated with Biotin-SVEQGR-amk or DMSO, thereafter washed and aliquots were taken for analysis (3i, 3c). All samples were analysed by immunoblotting with avidin.

Fig. 4C: Samples 1i, 1c, 2i, 2c, 3i and 3c (see B) were mixed with SCC1-myc reaction mix for 1 hour and analysed by immunoblotting with antibodies against myc. Arrows indicate the first and second SCC1 cleavage product.

Fig. 4D: Activated separase immunoprecipitates bound by antibodies against the C-terminus of separase to beads were first incubated with recombinant securin or, for control, with recombinant truncated cyclinB. After washing the precipitates were incubated with the human peptide inhibitor. Thereafter, the immunoprecipitates were again washed and analyzed by immunoblotting.

Example 5

a) Cloning of full length human separase

The missing N-terminal part of human separase was amplified from a HeLa cDNA library by polymerase chain reaction using the following primers:

5'primer: $^{5'}$GGCCAATTGATATCATGAGGAGCTTCAAAAGAG$^{3'}$ (SEQ ID NO:3)

3'primer: $^{5'}$CAACTGTCCACTAGTTGGGTCAGG$^{3'}$ (SEQ ID NO:4)

The resulting DNA fragment was inserted via EcoRV and SpeI into the existing truncated form of human separase (KIAA 0165). The complete coding sequence of human separase is shown in SEQ ID NO: 1, the amino acid sequence is shown in SEQ ID NO:2.

b) Preparation of recombinant human separase

Human separase was N-terminally tagged with a Flag epitope and transiently transfected in HeLa cells either in a single transfection or in a cotransfection with human securin which was C-terminally tagged with a myc epitope. For control HeLa cells were also transiently transfected with securin-myc. After 24 hours transfection 330 nM nocodazole was added for 18 hours. Cells were harvested, washed with PBS and cell extracts were generated as described in Waizenegger et al. 2000. These cell extracts were used to immunoprecipitate exogenous Flag-separase with mouse anti-Flag antibodies bound to sepharose. These immunoprecipitates were then incubated in mitotic Xenopus egg extracts and reisolated. The immunoprecipitates and the mitotically activated immunoprecipitates were analysed by immunoblotting with mouse antibodies against separase (7A6), securin (mouse serum) and Flag (M2, Stratagene). The mitotically activated immunoprecipitates were analysed for their activity (see above: SCC1 in vitro cleavage assay).

Example 6

Mitotically activated separase has autocatalytic activity

To test if separase has autocatalytic activity, a myc-tagged form of separase obtained by in vitro transcription and translation was used as a substrate. The transcription-translation reaction was carried out in the presence of ^{35}S-labeled methionine and cysteine, resulting in radiolabeled translation products. The in vitro translated separase was stable when incubated with separase immunoprecipitates which were incubated in interphase Xenopus egg extracts but in vitro translated separase was cleaved when it was incubated with active separase obtained by incubation of separase immunoprecipitates in mitotic Xenopus egg extracts (Figure 5). These results show that separase cleavage can occur autocatalytically in trans. This allows to distinguish between separase acting as an enzyme and separase serving as a substrate.

Figure 5 shows that only upon mitotic activation of separase separase-myc is autocatalytically cleaved.

Fig. 5A: Separase immunoprecipitates (separase IP) obtained from Nocodazole arrested HeLa cells bound by antibodies against the C-terminus of separase coupled to beads were either incubated in mitotic (separase IP^{mitotic}) or in interphase Xenopus egg extracts (separase IP^{interphase}). Aliquots were analysed by immunoblotting with antibodies against separase and securin.

Fig. 5B: Separase immunoprecipitates were incubated either in a mitotic or interphase Xenopus egg extracts and then mixed with recombinant separase-myc reaction mix. At indicated time points samples were taken and analysed by immunoblotting with antibodies against myc.

Example 7

N-and C-terminally cleavage products of separase remain physically associated and tagged recombinant human separase is active

To test if the N- and C-terminal fragments of separase remain non-covalently associated after cleavage, tagged forms of separase were generated.

N-terminally FLAG-tagged full-length separase was transiently expressed in HeLa cells as described in Example 5B and isolated by immunoprecipitation with an anti-FLAG antibody. The immunoprecipitates were incubated in mitotic Xenopus egg extracts to allow securin destruction and separase cleavage (Figure 6A). After re-isolation the immunoprecipitates were immunoblotted with anti-separase antibodies, anti-securin antibodies or anti-FLAG antibodies. Both C-and N-terminal separase fragments could be detected in the immunoprecipitates, suggesting that the N- and C-terminal separase fragments remain associated after cleavage (Figure 6A).

Fig. 6A: HeLa cells were transiently transfected with FLAG-separase, FLAG-separase and securin-myc or only with securin-myc. Mitotic extracts were performed from these cells and used for immunoprecipitation with anti-FLAG antibodies bound to sepharose (IP). The immunoprecipitates were activated in mitotic Xenopus egg extracts (IP^m). The immunoprecipitates were analysed by immunoblotting with antibodies against securin, separase (7A6) and FLAG.

Fig. 6B: Aliquots of above described immunoprecipitates were incubated with SCC1-myc reaction mix. At indicated time points samples were withdrawn and analysed by immunoblotting with antibodies against myc. Arrows indicate SCC1-myc cleavage products.

Example 8

Mapping of the separase recognition sites

The cleavage sites in human separase were mapped by a method that has been previously used to map cleavage sites in SCC1 (WO00/48627). Briefly, truncated versions of the human separase cDNA were generated by polymerase chain reactions, and the resulting cDNAs were used directly for coupled in vitro transcription-translation reactions by using rabbit reticulocyte lysates. The transcription-translation reactions were carried out in the presence of ^{35}S-labeled methionine and cysteine, resulting in radiolabeled translation products. These were then separated by SDS gel electrophoresis side by side with the in vitro cleavage products of mitotically activated human separase immunoprecipitates which were detected by immunoblotting. The comparison of the electrophoretic mobility of a series of deletion mutants with the mobility of the in vitro cleavage products narrows down the regions of cleavage to about 10 amino acid residues. Because separase cleavage sites in all known organisms cleave after the sequence EXXR (where X represents any amino acid residue; WO 00/48627; Uhlmann et al., 1999; Buonomo et al., 2000; Hauf et al., 2001) it is assumed that SFEILR^{1506} and EWELLR^{1535} represent two of the separase cleavage sites.

N-terminally truncated separase cDNA was generated by polymerase chain reaction. The 5'primers contain a sequence with the T7 polymerase binding site

1. start aa 1487

$^{5'}$GAATTCTAATACGACTCACTATAGGATCCATGATCCCTGAGGAAGAACTG ACTG$^{3'}$ (SEQ ID NO:5)

2. start aa 1507
$^{5'}$GAATTCTAATACGACTCACTATAGGATCCATGTCTGACGGGGAAGACTC AGCCTC$^{3'}$ (SEQ ID NO:6)

3. start aa 1536
$^{5'}$GAATTCTAATACGACTCACTATAGGATCCATGGATTCCAGCAAGAAGAAG CTGCCC$^{3'}$ (SEQ ID NO:7)

The following 3'primer was used:

$^{5'}$TTATTACCGCAGAGAGACAGGCAAGCC$^{3'}$ (SEQ ID NO:8)

The PCR products were in vitro transcribed and translated with the TNT system (Promega). The recombinant products, which start with an exogenous methionine at the indicated amino acids obtained, were loaded side by side with in vitro cleaved separase on a SDS-PAGE. For immunoblotting the mouse anti-separase antibody (7A6) was used. The results are shown in Fig. 8.

Example 9

Assay for identifying separase inhibitors using a fluorogenic peptid substrate
In order to establish a robust screening assay (based on liquid phase fluorescence energy transfer) for identifying inhibitors of recombinant human separase, four peptide substrates (1: SFEILR-AMC, 2: SFEILRG-AMC, 3:EWELLR-AMC and 4: DREIMR-AMC) were synthesized. These peptides are linked to AMC (7-Amido-4-methylcoumarin), a fluorogenic group, which has been described for proteolytic assays, such as for trypsin (Zimmerman et al., 1977) and cathepsin B (Barrett and Kirschke, 1981). AMC serves as a donor fluorophore and in the case of the separase-specific peptide substrates the

amino acid bonds of the peptides function as acceptor chromophores (Fig. 9A). The peptide substrates are cleaved at the P1´-AMC junction; by processing the peptide-AMC bond the unquenched AMC is set free and can be monitored as increasing fluorescence. The designed peptide substrates represent the intramolecular cis-cleavage site in separase itself (peptides 1 - 3) and the intermolecular trans-cleavage site in cohesin respectively (peptide 4). Since all these peptides contain an Arg at the P1´ site, the peptides could be easily tested by utilizing trypsin (specific recognition site at P1´: Arg or Lys). Except for peptide 2, which contains an additional Gly between P1´ (Arg) and the AMC residue, all peptide substrates could be efficiently cleaved (Fig. 9B) by trypsin as follows:
Trypsin solution (Gibco 043-90317 FU) was diluted 1:1000 in Hepes buffer containing 20mM Herpes (pH: 7.7), 100mM KCl, 1mM $MgCl_2$, 0.1mM $CaCl_2$ and 1mM DTT (freshly added). 1 µl of peptide 1 (4mg/ml in DMSO) was added, mixed and measured in a Hitachi f-2000 fluorescence spectrophotometer (Ex: 355 and Em: 460nm). A typical kinetics is shown in Fig. 9B.

Due to the complex activation procedure of recombinant human separase, a bead-suspension with coupled and activated separase had to be used for the establishment of a separase assay (activation of separase is described in Materials and Methods). The separase assay was performed as follows: ~ 500 µl of separase bead suspension was diluted with 1800 µl Hepes buffer. 30 µl of the diluted suspension was applied per well of a 96 "Packard OptiPlate black" plate. Additionally, 70 µl Hepes buffer and 1 µl of a LMW-compound stock solution (5mg/ml in DMSO) were added per well. After 10 minutes of pre-incubation at room temperature the reaction was initialized by adding 1 µl of the peptide 1 (4mg/ml in DMSO). The reaction was monitored in a Fluoroskan II 96-well reader (Ex: 355nm, Em: 460nm) at room temperature (Fig. 10). As a

control, measurement was performed in the presence of 2 µl DMSO per 100 µl reaction volume whereas for inhibition a cleavage peptide linked to an AMK (acyl-oxymethyl ketone) residue, which was described in WO 00/48627, was used. The AMK residue serves as a broad-spectrum inhibitor for many cysteine and serine proteases (for details see Beynon and Bond, 1989). In parallel the activity of the separase preparations bound to the beads was tested in an in vitro assay demonstrating the cleavage of cohesin (for details see Example 2, 3, Fig. 10). From a compound pool, 51 compounds that were shown to be potential protease inhibitors in preliminary experiments, were selected and tested for their ability to interfere with the activity of human separse. Out of these compounds, nine were found to inhibit separase activity within the same range as the AMK peptide (Fig. 11).

*

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Claims

1. A method for identifying a compound that has the ability of modulating sister chromatid separation by inhibiting the proteolytic activity of separase, characterized in that an active separase in the form of

   a) one or more separase fragment(s), optionally upon activation in the presence of securin, or

   b) the full-length separase upon activation in the presence of securin,

   is incubated in the presence of a separase substrate, with a test compound and that the modulating effect of the test compound on the proteolytic activity of the active separase is determined.

2. The method of claim 1, wherein the active separase is human.

3. The method of claim 1 or 2, wherein the active separase (fragment) is activated in a mitotic cell extract in the presence of securin.

4. The method of claim 3, wherein the mitotic cell extract has been obtained from Xenopus laevis eggs.

5. The method of claim, wherein the separase substrate is peptide that carries a fluorogenic group, which upon processing of the peptide results in a change in fluorescence and that the change in fluorescence is correlated with the separase activity.

6. The method of claim 5, where*in the separase substrate is a peptide selected from peptides containing the sequence DREIMR, SFEILR or EWELLR.

7. A peptide selected from peptides containing the sequence DREIMR, SFEILR or EWELLR or a derivative thereof.

8. The peptide of claim 7 or a derivative thereof for the the treatment of cancer.

9. Pharmaceutical composition containing, as the active ingredient, the peptide(derivative) of claim 7.

10. An inhibitor of separase which has been identified in the method of claim 1 for human therapy.

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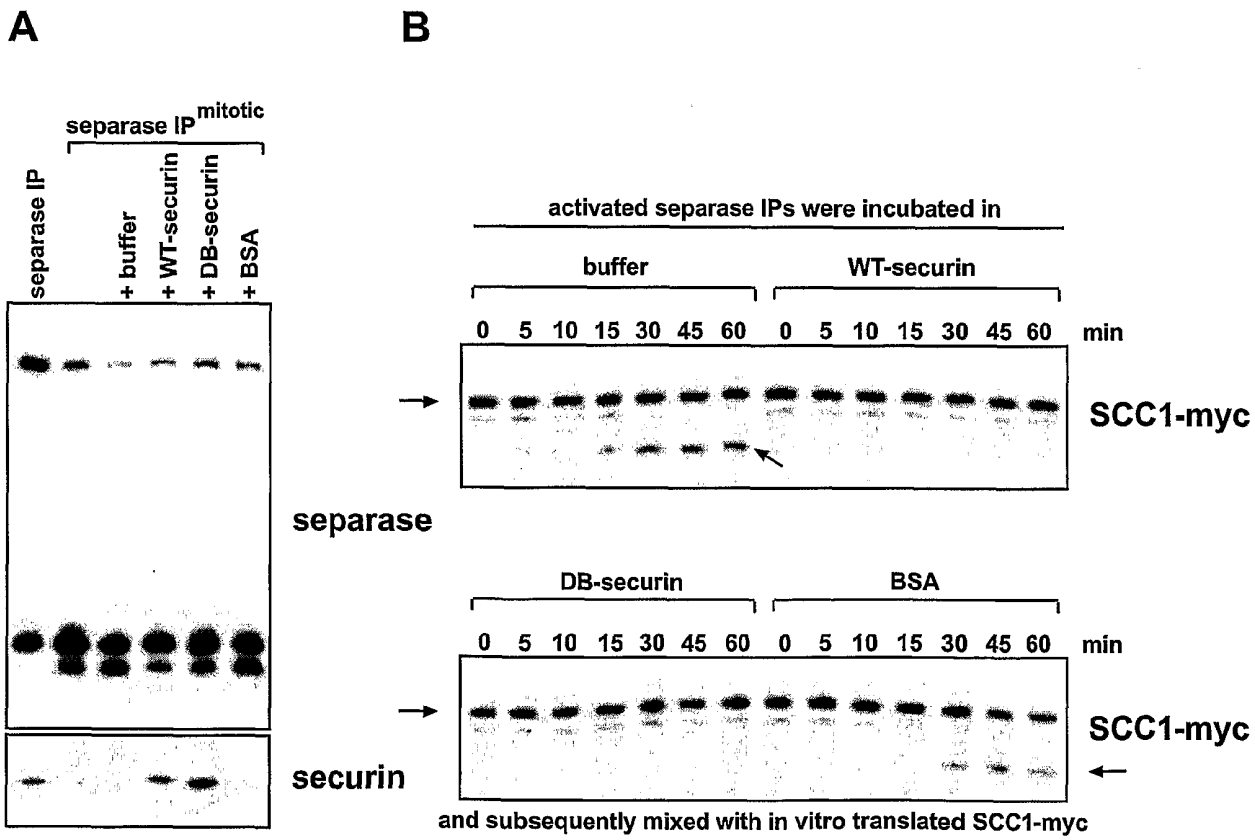
Fig. 1


A
B
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separase IP
+ buffer
+ WT-securin
+ DB-securin
+ BSA
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securin
activated separase IPs were incubated in
buffer
WT-securin
0 5 10 15 30 45 60 0 5 10 15 30 45 60 min
SCC1-myc
DB-securin
BSA
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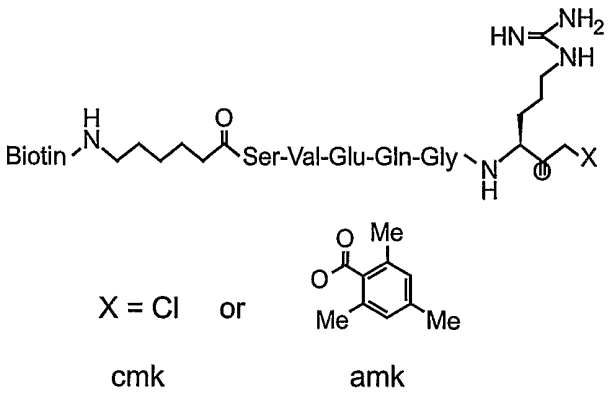
and subsequently mixed with in vitro translated SCC1-myc

2/14

Fig. 2

A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amk

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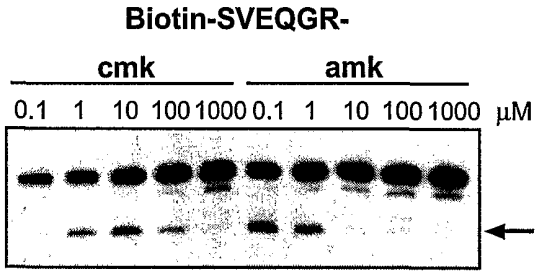
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amk
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C

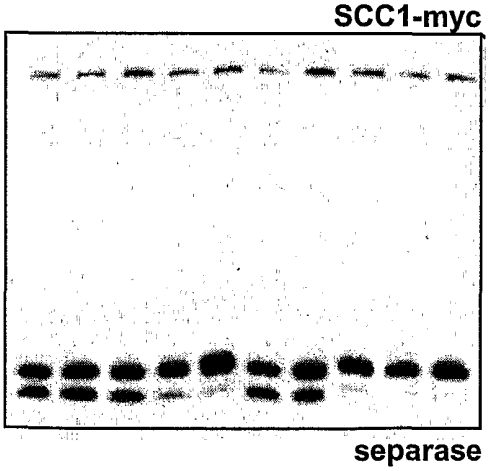
SCC1-myc
separase

Fig. 3

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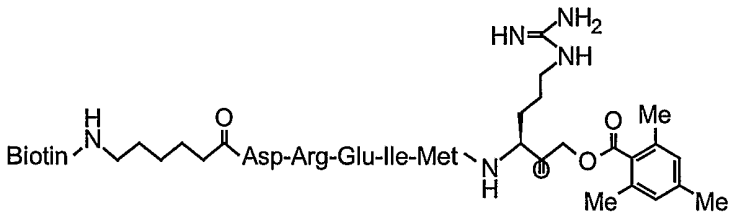
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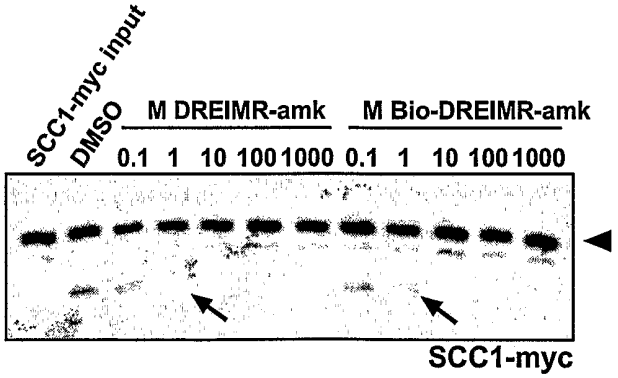
B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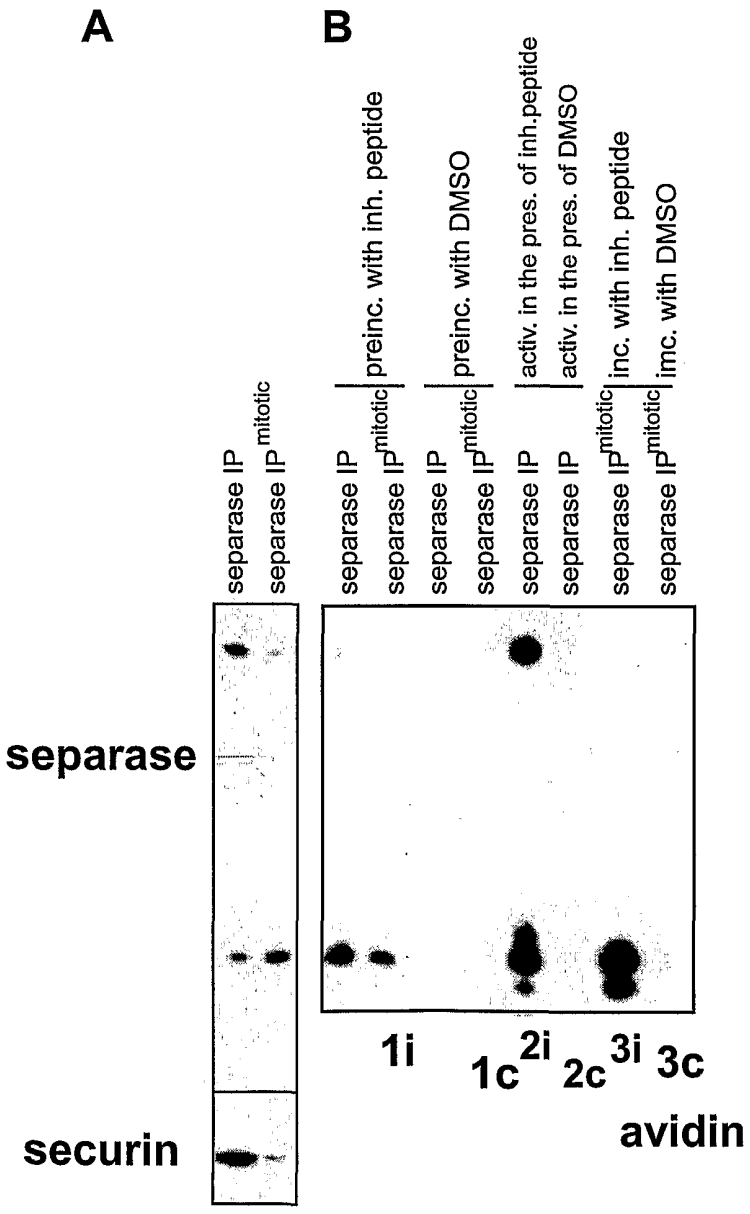
SCC1-myc

Fig. 4A, B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B
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separase IP mitotic
separase
securin
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separase IP
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separase IP
activ. in the pres. of inh.peptide
separase IP
activ. in the pres. of DMSO
separase IP mitotic
inc. with inh. peptide
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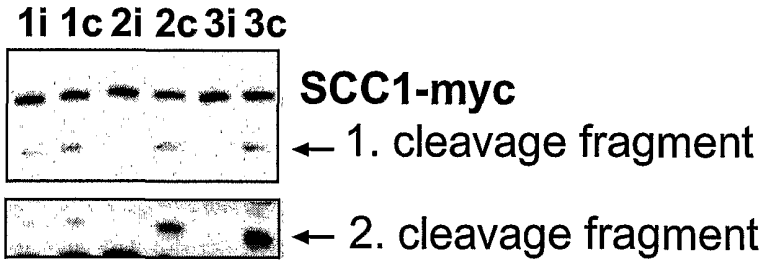
imc. with DMSO
1i
1c
2i
2c
3i
3c
avidin

Fig. 4C

C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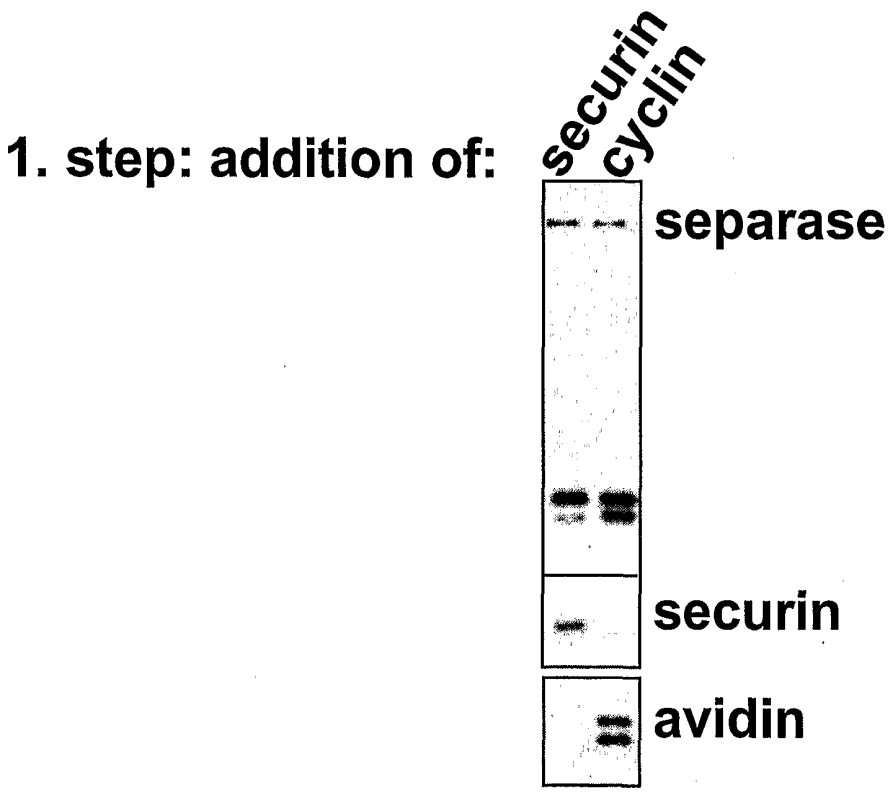
← 1. cleavage fragment
← 2. cleavage fragment

Fig. 4D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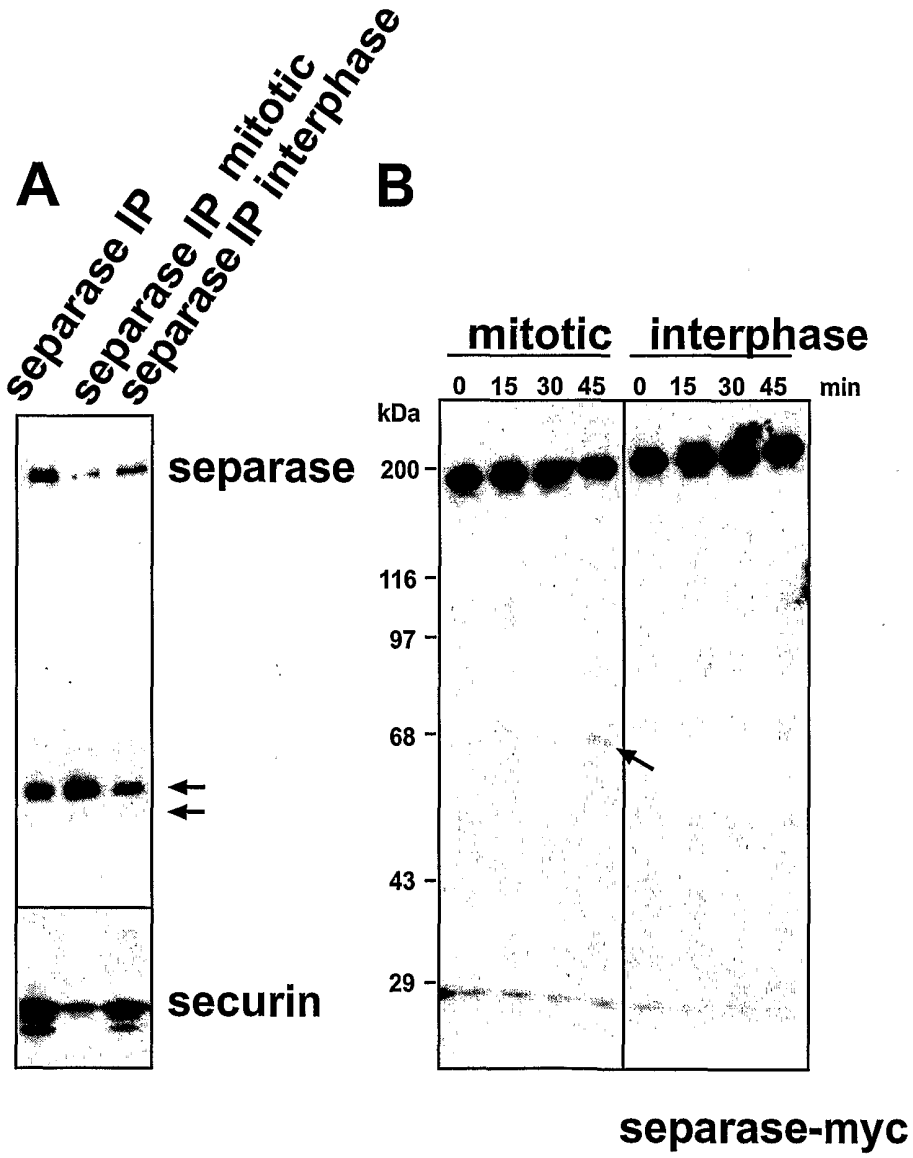
cyclin
separase
securin
avidin

Fig. 5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separase IP mitotic
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B
mitotic
interphase
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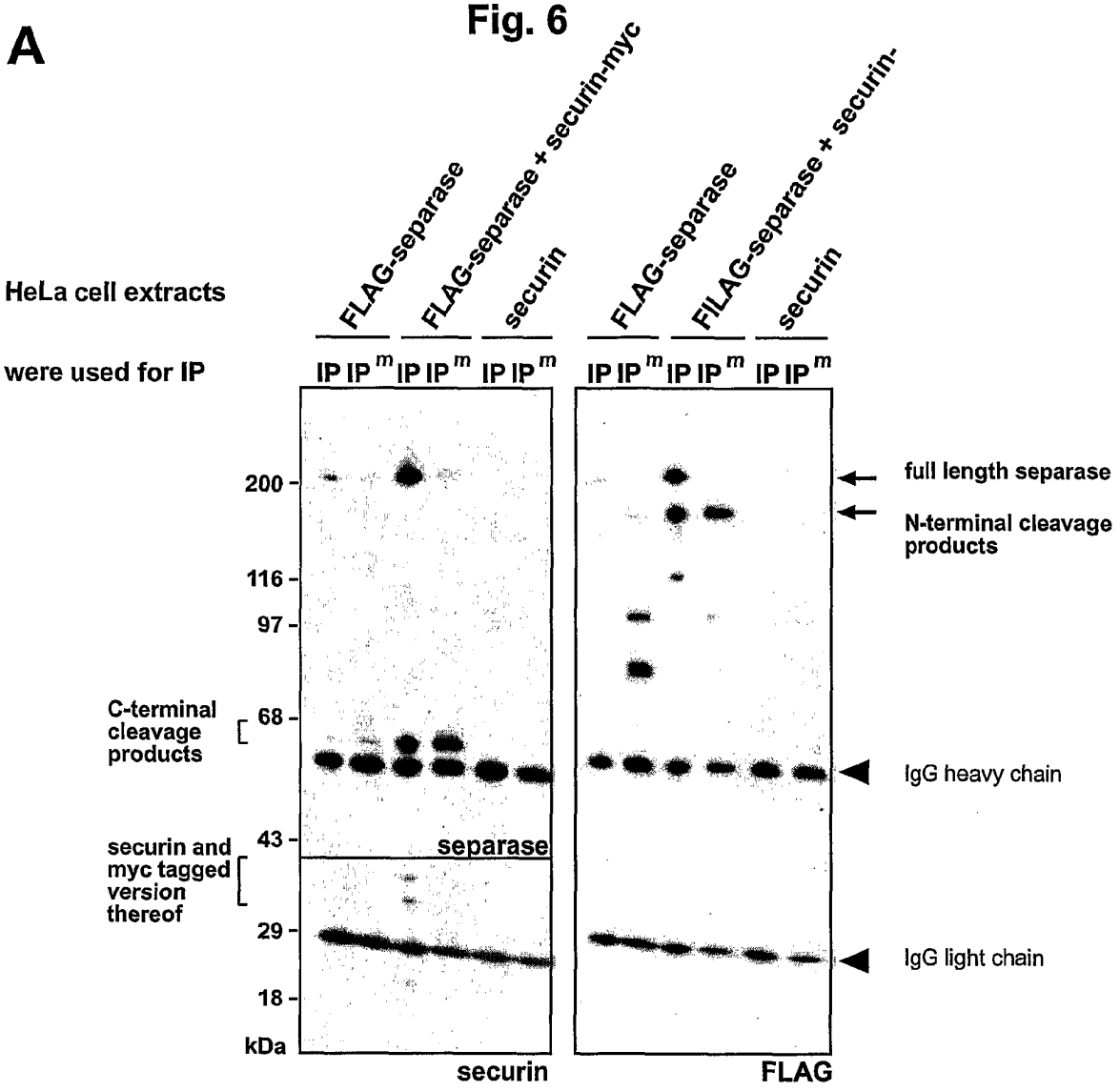
kDa
200
116
97
68
43
29
separase-myc

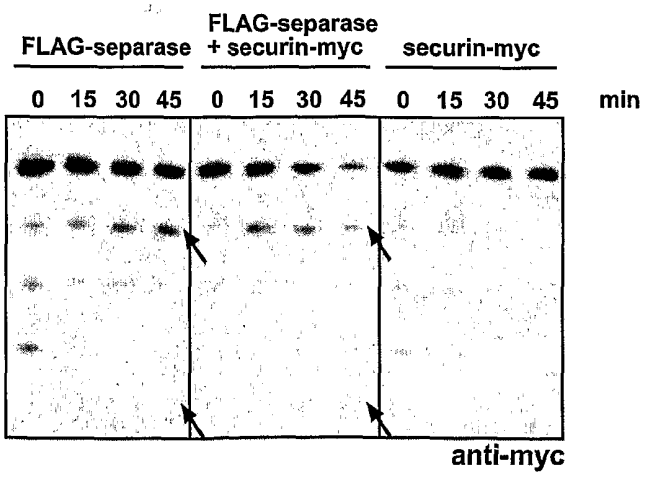
8/14

Fig. 6

A


HeLa cell extracts
were used for IP
FLAG-separase
FLAG-separase + securin-myc
securin
FLAG-separase
FILAG-separase + securin-
securin
IP IP m IP IP m IP IP m
IP IP m IP IP m IP IP m
200
116
97
68
43
29
18
kDa
C-terminal cleavage products
securin and myc tagged version thereof
separase
securin
FLAG
full length separase
N-terminal cleavage products
IgG heavy chain
IgG light chain

B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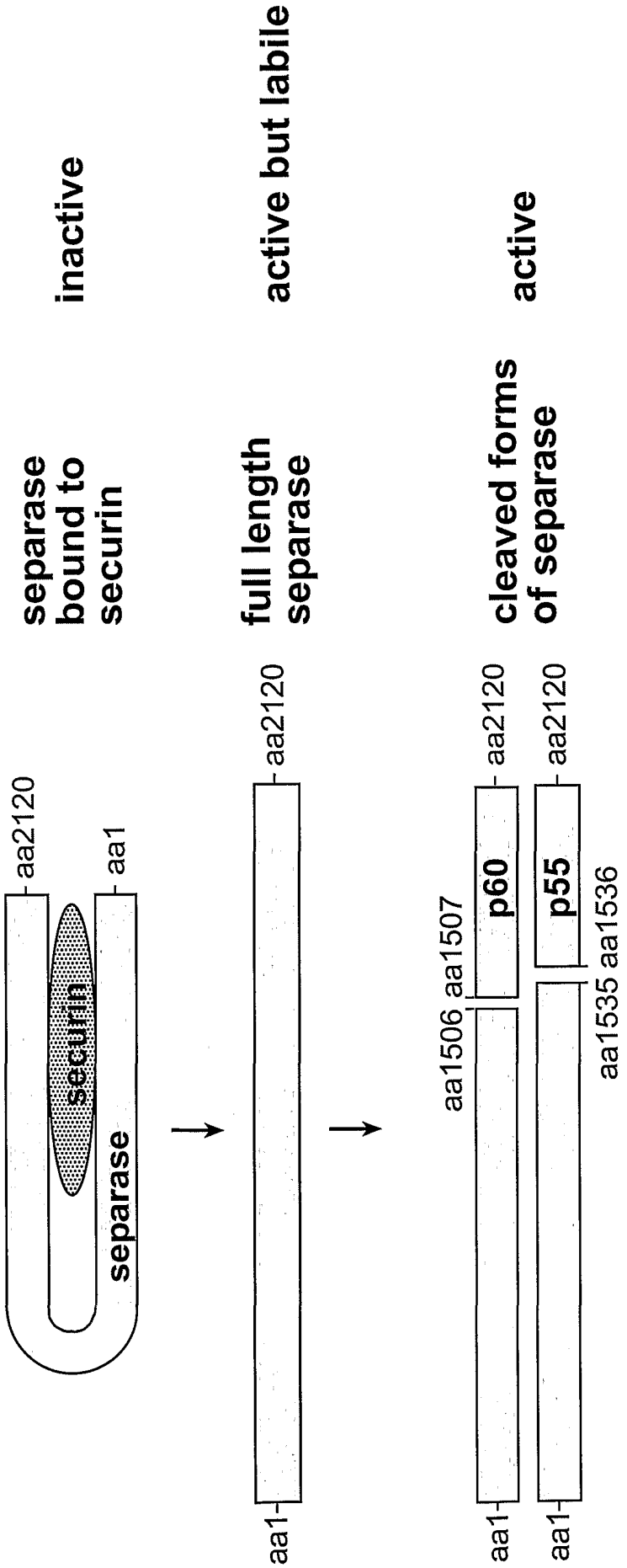
FLAG-separase + securin-myc
securin-myc
0 15 30 45 0 15 30 45 0 15 30 45
min
anti-myc

Fig. 7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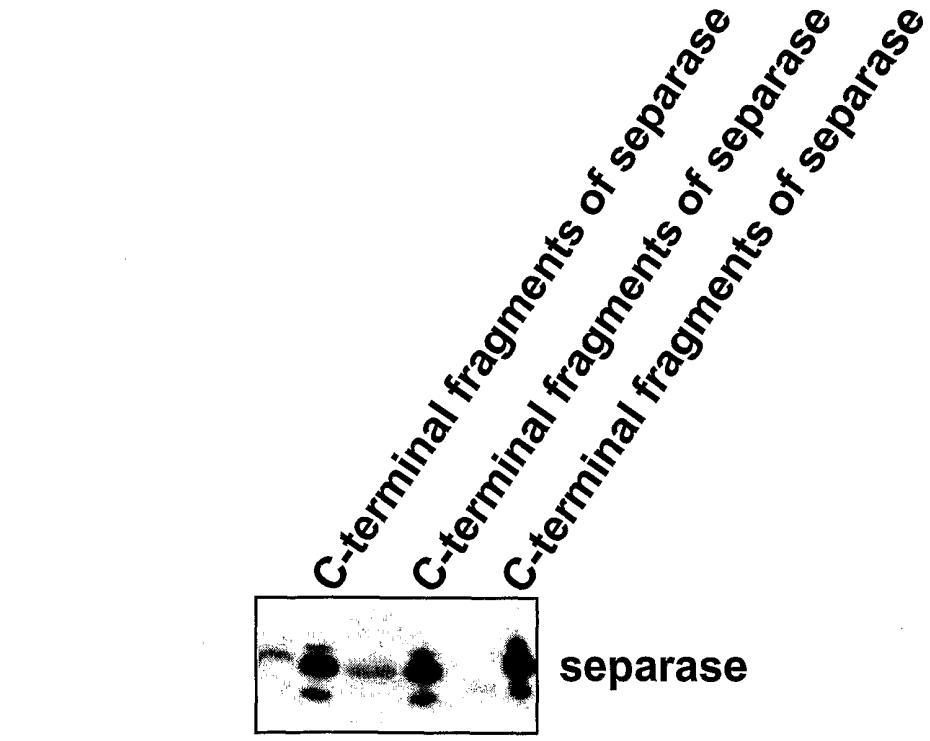
inactive
full length separase
active but labile
cleaved forms of separase
active
aa2120
aa1
securin
separase
aa2120
aa1
aa2120
aa1506
aa1507
p60
aa1
aa2120
p55
aa1535
aa1536
aa1

10/14

Fig. 8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C-terminal fragments of separase
C-terminal fragments of separase
separase
start at aa: 1487 1507 1536

Fig. 9 A

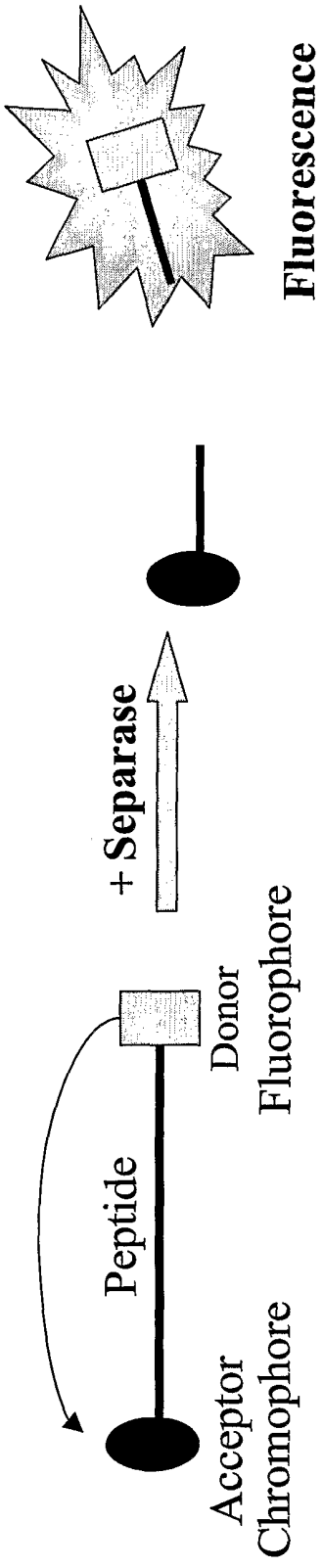
Fluorescence
+ Separase
Donor Fluorophore
Peptide
Acceptor Chromophore

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Fig. 9 B

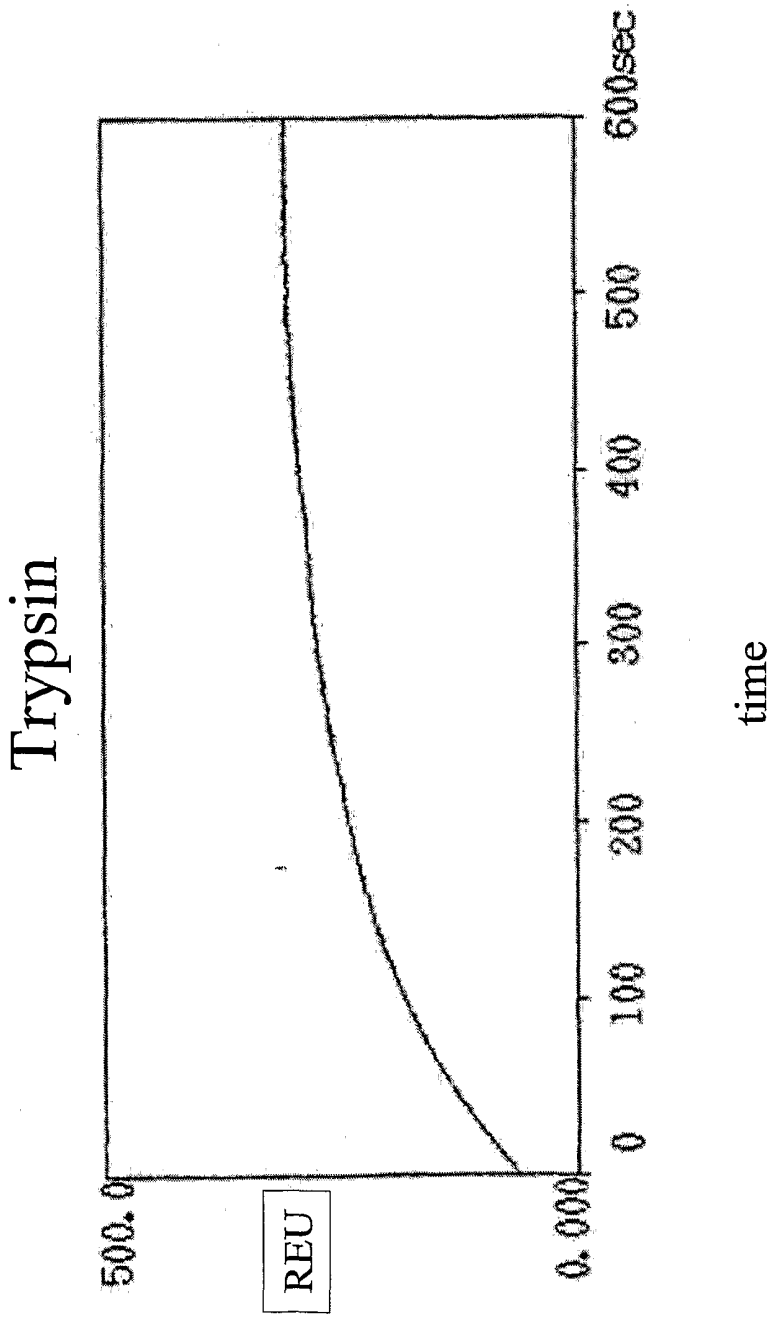

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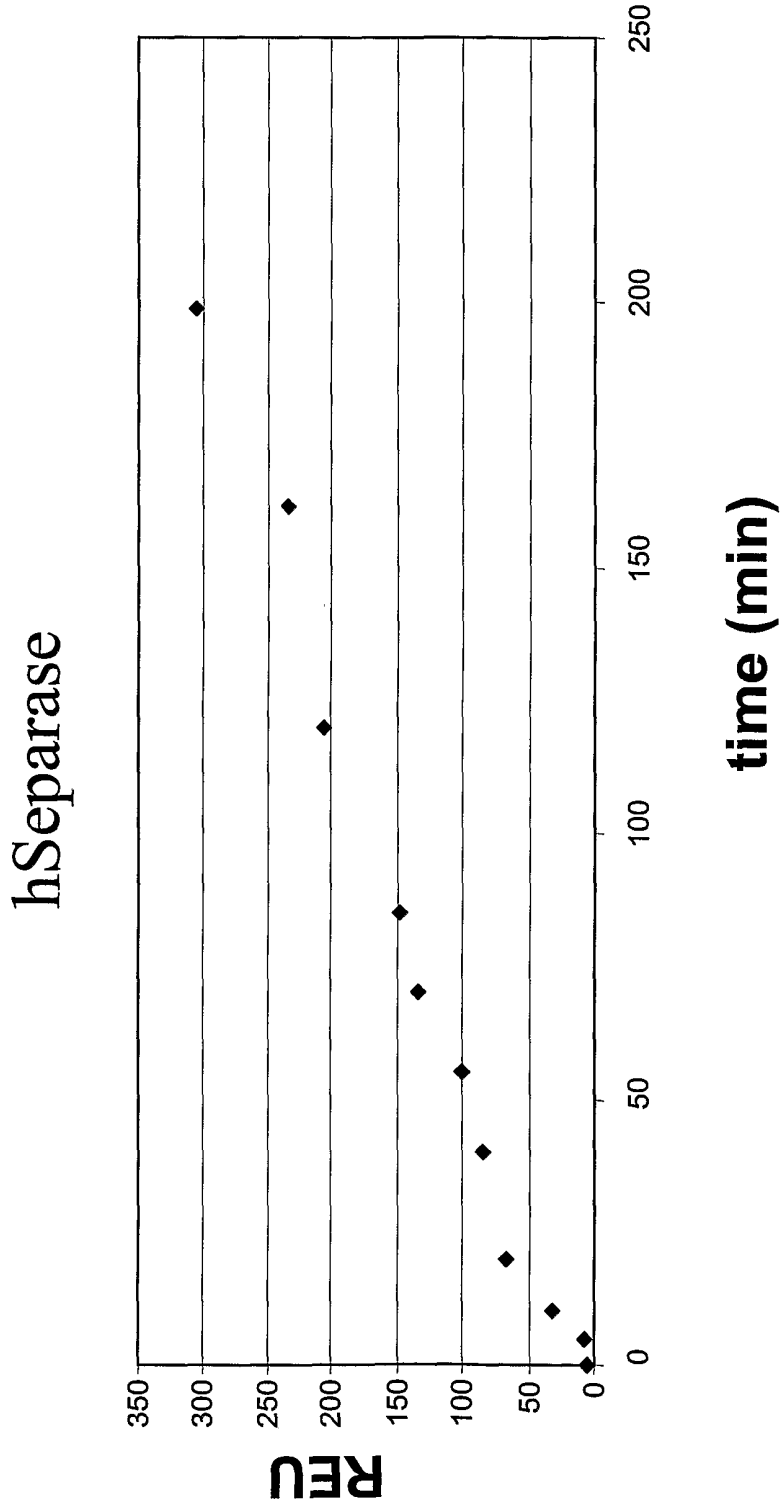
Fig. 10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REU
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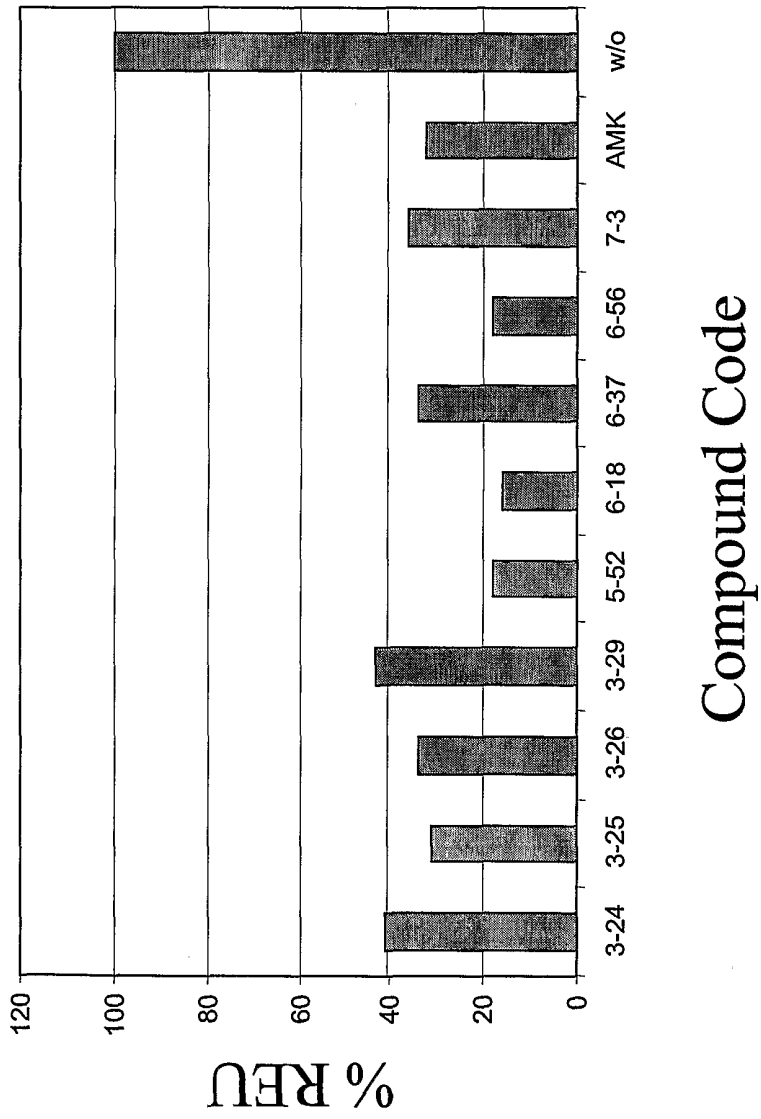
100
150
200
250
300
350
0
50
100
150
200
250

14/14

Fig. 11


% REU
120
100
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w/o
Compound Code

SEQUENCE LISTING

<110> Boehringer Ingelheim International GmbH

<120> Method for identifying compounds that modulate sister chromatid separation

<130> Case 14-060

<140>
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ccttttccct agctctccgg tgtc atg agg agc ttc aaa aga gtc aac ttt 171
Met Arg Ser Phe Lys Arg Val Asn Phe
1 5

ggg act ctg cta agc agc cag aag gag gct gaa gag ttg ctg ccc gac 219
Gly Thr Leu Leu Ser Ser Gln Lys Glu Ala Glu Glu Leu Leu Pro Asp
10 15 20 25

ttg aag gag ttc ctg tcc aac cct cca gct ggt ttt ccc agc agc cga 267
Leu Lys Glu Phe Leu Ser Asn Pro Pro Ala Gly Phe Pro Ser Ser Arg
30 35 40

tct gat gct gag agg aga caa gct tgt gat gcc atc ctg agg gct tgc 315
Ser Asp Ala Glu Arg Arg Gln Ala Cys Asp Ala Ile Leu Arg Ala Cys
45 50 55

aac cag cag ctg act gct aag cta gct tgc cct agg cat ctg ggg agc 363
Asn Gln Gln Leu Thr Ala Lys Leu Ala Cys Pro Arg His Leu Gly Ser
60 65 70

ctg ctg gag ctg gca gag ctg gcc tgt gat ggc tac tta gtg tct acc 411
Leu Leu Glu Leu Ala Glu Leu Ala Cys Asp Gly Tyr Leu Val Ser Thr
75 80 85

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cca cag cgt cct ccc ctc tac ctg gaa cga att ctc ttt gtc tta ctg   459
Pro Gln Arg Pro Pro Leu Tyr Leu Glu Arg Ile Leu Phe Val Leu Leu
 90                  95                 100                 105

cgg aat gct gct gca caa gga agc cca gag gtc aca ctc cgc ctt gct   507
Arg Asn Ala Ala Ala Gln Gly Ser Pro Glu Val Thr Leu Arg Leu Ala
                110                 115                 120

cag ccc ctc cat gcc tgc ttg gtg cag tgc tct cgc gag gct gct ccc   555
Gln Pro Leu His Ala Cys Leu Val Gln Cys Ser Arg Glu Ala Ala Pro
            125                 130                 135

cag gac tat gag gcc gtg gct cgg ggc agc ttt tct ctg ctt tgg aag   603
Gln Asp Tyr Glu Ala Val Ala Arg Gly Ser Phe Ser Leu Leu Trp Lys
        140                 145                 150

ggg gca gaa gcc ctg ttg gaa cgg cga gct gca ttt gca gct cgg ctg   651
Gly Ala Glu Ala Leu Leu Glu Arg Arg Ala Ala Phe Ala Ala Arg Leu
    155                 160                 165

aag gcc ttg agc ttc cta gta ctc ttg gag gat gaa agt acc cct tgt   699
Lys Ala Leu Ser Phe Leu Val Leu Leu Glu Asp Glu Ser Thr Pro Cys
170                 175                 180                 185

gag gtt cct cac ttt gct tct cca aca gcc tgt cga gcg gta gct gcc   747
Glu Val Pro His Phe Ala Ser Pro Thr Ala Cys Arg Ala Val Ala Ala
                190                 195                 200

cat cag cta ttt gat gcc agt ggc cat ggt cta aat gaa gca gat gct   795
His Gln Leu Phe Asp Ala Ser Gly His Gly Leu Asn Glu Ala Asp Ala
            205                 210                 215

gat ttc cta gat gac ctg ctc tcc agg cac gtg atc aga gcc ttg gtg   843
Asp Phe Leu Asp Asp Leu Leu Ser Arg His Val Ile Arg Ala Leu Val
        220                 225                 230

ggt gag aga ggg agc tct tct ggg ctt ctt tct ccc cag agg gcc ctc   891
Gly Glu Arg Gly Ser Ser Ser Gly Leu Leu Ser Pro Gln Arg Ala Leu
    235                 240                 245

tgc ctc ttg gag ctc acc ttg gaa cac tgc cgt cgc ttt tgc tgg agc   939
Cys Leu Leu Glu Leu Thr Leu Glu His Cys Arg Arg Phe Cys Trp Ser
250                 255                 260                 265

cgc cac cat gac aaa gcc atc agc gca gtg gag aag gct cac agt tac   987
Arg His His Asp Lys Ala Ile Ser Ala Val Glu Lys Ala His Ser Tyr
                270                 275                 280

cta agg aac acc aat cta gcc cct agc ctt cag cta tgt cag ctg ggg   1035
Leu Arg Asn Thr Asn Leu Ala Pro Ser Leu Gln Leu Cys Gln Leu Gly
            285                 290                 295

gtt aag ctg ctg cag gtt ggg gag gaa gga cct cag gca gtg gcc aag   1083
Val Lys Leu Leu Gln Val Gly Glu Glu Gly Pro Gln Ala Val Ala Lys
        300                 305                 310

ctt ctg atc aag gca tca gct gtc ctg agc aag agt atg gag gca cca   1131
Leu Leu Ile Lys Ala Ser Ala Val Leu Ser Lys Ser Met Glu Ala Pro
    315                 320                 325
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tca ccc cca ctt cgg gca ttg tat gag agc tgc cag ttc ttc ctt tca   1179
Ser Pro Pro Leu Arg Ala Leu Tyr Glu Ser Cys Gln Phe Phe Leu Ser
330                 335                 340                 345

ggc ctg gaa cga ggc acc aag agg cgc tat aga ctt gat gcc att ctg   1227
Gly Leu Glu Arg Gly Thr Lys Arg Arg Tyr Arg Leu Asp Ala Ile Leu
                350                 355                 360

agc ctc ttt gct ttt ctt gga ggg tac tgc tct ctt ctg cag cag ctg   1275
Ser Leu Phe Ala Phe Leu Gly Gly Tyr Cys Ser Leu Leu Gln Gln Leu
            365                 370                 375

cgg gat gat ggt gtg tat ggg ggc tcc tcc aag caa cag cag tct ttt   1323
Arg Asp Asp Gly Val Tyr Gly Gly Ser Ser Lys Gln Gln Gln Ser Phe
        380                 385                 390

ctt cag atg tac ttt cag gga ctt cac ctc tac act gtg gtg gtt tat   1371
Leu Gln Met Tyr Phe Gln Gly Leu His Leu Tyr Thr Val Val Val Tyr
    395                 400                 405

gac ttt gcc caa ggc tgt cag ata gtt gat ttg gct gac ctg acc caa   1419
Asp Phe Ala Gln Gly Cys Gln Ile Val Asp Leu Ala Asp Leu Thr Gln
410                 415                 420                 425

cta gtg gac agt tgt aaa tct acc gtt gtc tgg atg ctg gag gcc tta   1467
Leu Val Asp Ser Cys Lys Ser Thr Val Val Trp Met Leu Glu Ala Leu
                430                 435                 440

gag ggc ctg tcg ggc caa gag ctg acg gac cac atg ggg atg acc gct   1515
Glu Gly Leu Ser Gly Gln Glu Leu Thr Asp His Met Gly Met Thr Ala
            445                 450                 455

tct tac acc agt aat ttg gcc tac agc ttc tat agt cac aag ctc tat   1563
Ser Tyr Thr Ser Asn Leu Ala Tyr Ser Phe Tyr Ser His Lys Leu Tyr
        460                 465                 470

gcc gag gcc tgt gcc atc tct gag ccg ctc tgt cag cac ctg ggt ttg   1611
Ala Glu Ala Cys Ala Ile Ser Glu Pro Leu Cys Gln His Leu Gly Leu
    475                 480                 485

gtg aag cca ggc act tat ccc gag gtg cct cct gag aag ttg cac agg   1659
Val Lys Pro Gly Thr Tyr Pro Glu Val Pro Pro Glu Lys Leu His Arg
490                 495                 500                 505

tgc ttc cgg cta caa gta gag agt ttg aag aaa ctg ggt aaa cag gcc   1707
Cys Phe Arg Leu Gln Val Glu Ser Leu Lys Lys Leu Gly Lys Gln Ala
                510                 515                 520

cag ggc tgc aag atg gtg att ttg tgg ctg gca gcc ctg caa ccc tgt   1755
Gln Gly Cys Lys Met Val Ile Leu Trp Leu Ala Ala Leu Gln Pro Cys
            525                 530                 535

agc cct gaa cac atg gct gag cca gtc act ttc tgg gtt cgg gtc aag   1803
Ser Pro Glu His Met Ala Glu Pro Val Thr Phe Trp Val Arg Val Lys
        540                 545                 550

atg gat gcg gcc agg gct gga gac aag gag cta cag cta aag act ctg   1851
Met Asp Ala Ala Arg Ala Gly Asp Lys Glu Leu Gln Leu Lys Thr Leu
    555                 560                 565

cga gac agc ctc agt ggc tgg gac ccg gag acc ctg gcc ctc ctg ctg   1899
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Arg Asp Ser Leu Ser Gly Trp Asp Pro Glu Thr Leu Ala Leu Leu Leu
570                 575                 580                 585

agg gag gag ctg cag gcc tac aag gcg gtg cgg gcc gac act gga cag   1947
Arg Glu Glu Leu Gln Ala Tyr Lys Ala Val Arg Ala Asp Thr Gly Gln
                590                 595                 600

gaa cgc ttc aac atc atc tgt gac ctc ctg gag ctg agc ccc gag gag   1995
Glu Arg Phe Asn Ile Ile Cys Asp Leu Leu Glu Leu Ser Pro Glu Glu
            605                 610                 615

aca cca gcc ggg gcc tgg gca cga gcc acc cac ctg gta gaa ctg gct   2043
Thr Pro Ala Gly Ala Trp Ala Arg Ala Thr His Leu Val Glu Leu Ala
        620                 625                 630

cag gtg ctc tgc tac cac gac ttt acg cag cag acc aac tgc tct gct   2091
Gln Val Leu Cys Tyr His Asp Phe Thr Gln Gln Thr Asn Cys Ser Ala
    635                 640                 645

ctg gat gct atc cgg gaa gcc ctg cag ctt ctg gac tct gtg agg cct   2139
Leu Asp Ala Ile Arg Glu Ala Leu Gln Leu Leu Asp Ser Val Arg Pro
650                 655                 660                 665

gag gcc cag gcc aga gat cag ctt ctg gac gat aaa gca cag gcc ttg   2187
Glu Ala Gln Ala Arg Asp Gln Leu Leu Asp Asp Lys Ala Gln Ala Leu
                670                 675                 680

ctg tgg ctt tac atc tgt act ctg gaa gcc aaa ata cag gaa ggt atc   2235
Leu Trp Leu Tyr Ile Cys Thr Leu Glu Ala Lys Ile Gln Glu Gly Ile
            685                 690                 695

gag cgg gat cgg aga gcc cag gcc cct ggt aac ttg gag gaa ttt gaa   2283
Glu Arg Asp Arg Arg Ala Gln Ala Pro Gly Asn Leu Glu Glu Phe Glu
        700                 705                 710

gtc aat gac ctg aac tat gaa gat aaa ctc cag gaa gat cgt ttc cta   2331
Val Asn Asp Leu Asn Tyr Glu Asp Lys Leu Gln Glu Asp Arg Phe Leu
    715                 720                 725

tac agt aac att gcc ttc aac ctg gct gca gat gct gct cag tcc aaa   2379
Tyr Ser Asn Ile Ala Phe Asn Leu Ala Ala Asp Ala Ala Gln Ser Lys
730                 735                 740                 745

tgc ctg gac caa gcc ctg gcc ctg tgg aag gag ctg ctt aca aag ggg   2427
Cys Leu Asp Gln Ala Leu Ala Leu Trp Lys Glu Leu Leu Thr Lys Gly
                750                 755                 760

cag gcc cca gct gta cgg tgt ctc cag cag aca gca gcc tca ctg cag   2475
Gln Ala Pro Ala Val Arg Cys Leu Gln Gln Thr Ala Ala Ser Leu Gln
            765                 770                 775

atc cta gca gcc ctc tac cag ctg gtg gca aag ccc atg cag gct ctg   2523
Ile Leu Ala Ala Leu Tyr Gln Leu Val Ala Lys Pro Met Gln Ala Leu
        780                 785                 790

gag gtc ctc ctg ctg cta cgg att gtc tct gag aga ctg aag gac cac   2571
Glu Val Leu Leu Leu Leu Arg Ile Val Ser Glu Arg Leu Lys Asp His
    795                 800                 805

tcg aag gca gct ggc tcc tcc tgc cac atc acc cag ctc ctc ctg acc   2619
Ser Lys Ala Ala Gly Ser Ser Cys His Ile Thr Gln Leu Leu Leu Thr
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810                 815                 820                 825

ctc ggc tgt ccc agc tat gcc cag tta cac ctg gaa gag gca gca tcg   2667
Leu Gly Cys Pro Ser Tyr Ala Gln Leu His Leu Glu Glu Ala Ala Ser
                830                 835                 840

agc ctg aag cat ctc gat cag act act gac aca tac ctg ctc ctt tcc   2715
Ser Leu Lys His Leu Asp Gln Thr Thr Asp Thr Tyr Leu Leu Leu Ser
            845                 850                 855

ctg acc tgt gat ctg ctt cga agt caa ctc tac tgg act cac cag aag   2763
Leu Thr Cys Asp Leu Leu Arg Ser Gln Leu Tyr Trp Thr His Gln Lys
        860                 865                 870

gtg acc aag ggt gtc tct ctg ctg ctg tct gtg ctt cgg gat cct gcc   2811
Val Thr Lys Gly Val Ser Leu Leu Leu Ser Val Leu Arg Asp Pro Ala
    875                 880                 885

ctc cag aag tcc tcc aag gct tgg tac ttg ctg cgt gtc cag gtc ctg   2859
Leu Gln Lys Ser Ser Lys Ala Trp Tyr Leu Leu Arg Val Gln Val Leu
890                 895                 900                 905

cag ctg gtg gca gct tac ctt agc ctc ccg tca aac aac ctc tca cac   2907
Gln Leu Val Ala Ala Tyr Leu Ser Leu Pro Ser Asn Asn Leu Ser His
                910                 915                 920

tcc ctg tgg gag cag ctc tgt gcc caa ggc tgg cag aca cct gag ata   2955
Ser Leu Trp Glu Gln Leu Cys Ala Gln Gly Trp Gln Thr Pro Glu Ile
            925                 930                 935

gct ctc ata gac tcc cat aag ctc ctc cga agc atc atc ctc ctg ctg   3003
Ala Leu Ile Asp Ser His Lys Leu Leu Arg Ser Ile Ile Leu Leu Leu
        940                 945                 950

atg ggc agt gac att ctc tca act cag aaa gca gct gtg gag aca tcg   3051
Met Gly Ser Asp Ile Leu Ser Thr Gln Lys Ala Ala Val Glu Thr Ser
    955                 960                 965

ttt ttg gac tat ggt gaa aat ctg gta caa aaa tgg cag gtt ctt tca   3099
Phe Leu Asp Tyr Gly Glu Asn Leu Val Gln Lys Trp Gln Val Leu Ser
970                 975                 980                 985

gag gtg ctg agc tgc tca gag aag ctg gtc tgc cac ctg ggc cgc ctg   3147
Glu Val Leu Ser Cys Ser Glu Lys Leu Val Cys His Leu Gly Arg Leu
                990                 995                1000

ggt agt gtg agt gaa gcc aag gcc ttt tgc ttg gag gcc cta aaa ctt   3195
Gly Ser Val Ser Glu Ala Lys Ala Phe Cys Leu Glu Ala Leu Lys Leu
           1005                1010                1015

aca aca aag ctg cag ata cca cgc cag tgt gcc ctg ttc ctg gtg ctg   3243
Thr Thr Lys Leu Gln Ile Pro Arg Gln Cys Ala Leu Phe Leu Val Leu
       1020                1025                1030

aag ggc gag ctg gag ctg gcc cgc aat gac att gat ctc tgt cag tcg   3291
Lys Gly Glu Leu Glu Leu Ala Arg Asn Asp Ile Asp Leu Cys Gln Ser
   1035                1040                1045

gac ctg cag cag gtt ctg ttc ttg ctt gag tct tgc aca gag ttt ggt   3339
Asp Leu Gln Gln Val Leu Phe Leu Leu Glu Ser Cys Thr Glu Phe Gly
1050                1055                1060                1065
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Gly Val Thr Gln His Leu Asp Ser Val Lys Lys Val His Leu Gln Lys
               1070                1075                1080

ggg aag cag cag gcc cag gtc ccc tgt cct cca cag ctc cca gag gag   3435
Gly Lys Gln Gln Ala Gln Val Pro Cys Pro Pro Gln Leu Pro Glu Glu
           1085                1090                1095

gag ctc ttc cta aga ggc cct gct cta gag ctg gtg gcc act gtg gcc   3483
Glu Leu Phe Leu Arg Gly Pro Ala Leu Glu Leu Val Ala Thr Val Ala
       1100                1105                1110

aag gag cct ggc ccc ata gca cct tct aca aac tcc tcc cca gtc ttg   3531
Lys Glu Pro Gly Pro Ile Ala Pro Ser Thr Asn Ser Ser Pro Val Leu
   1115                1120                1125

aaa acc aag ccc cag ccc ata ccc aac ttc ctg tcc cat tca ccc acc   3579
Lys Thr Lys Pro Gln Pro Ile Pro Asn Phe Leu Ser His Ser Pro Thr
1130                1135                1140                1145

tgt gac tgc tcg ctc tgc gcc agc cct gtc ctc aca gca gtc tgt ctg   3627
Cys Asp Cys Ser Leu Cys Ala Ser Pro Val Leu Thr Ala Val Cys Leu
               1150                1155                1160

cgc tgg gta ttg gtc acg gca ggg gtg agg ctg gcc atg ggc cac caa   3675
Arg Trp Val Leu Val Thr Ala Gly Val Arg Leu Ala Met Gly His Gln
           1165                1170                1175

gcc cag ggt ctg gat ctg ctg cag gtc gtg ctg aag ggc tgt cct gaa   3723
Ala Gln Gly Leu Asp Leu Leu Gln Val Val Leu Lys Gly Cys Pro Glu
       1180                1185                1190

gcc gct gag cgc ctc acc caa gct ctc caa gct tcc ctg aat cat aaa   3771
Ala Ala Glu Arg Leu Thr Gln Ala Leu Gln Ala Ser Leu Asn His Lys
   1195                1200                1205

aca ccc ccc tcc ttg gtt cca agc ctc ttg gat gag atc ttg gct caa   3819
Thr Pro Pro Ser Leu Val Pro Ser Leu Leu Asp Glu Ile Leu Ala Gln
1210                1215                1220                1225

gca tac aca ctg ttg gca ctg gag ggc ctg aac cag cca tca aac gag   3867
Ala Tyr Thr Leu Leu Ala Leu Glu Gly Leu Asn Gln Pro Ser Asn Glu
               1230                1235                1240

agc ctg cag aag gtt cta cag tca ggg ctg aag ttt gta gca gca cgg   3915
Ser Leu Gln Lys Val Leu Gln Ser Gly Leu Lys Phe Val Ala Ala Arg
           1245                1250                1255

ata ccc cac cta gag ccc tgg cga gcc agc ctg ctc ttg att tgg gcc   3963
Ile Pro His Leu Glu Pro Trp Arg Ala Ser Leu Leu Leu Ile Trp Ala
       1260                1265                1270

ctc aca aaa cta ggt ggc ctc agc tgc tgt act acc caa ctt ttt gca   4011
Leu Thr Lys Leu Gly Gly Leu Ser Cys Cys Thr Thr Gln Leu Phe Ala
   1275                1280                1285

agc tcc tgg ggc tgg cag cca cca tta ata aaa agt gtc cct ggc tca   4059
Ser Ser Trp Gly Trp Gln Pro Pro Leu Ile Lys Ser Val Pro Gly Ser
1290                1295                1300                1305
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aag tta gcc tct gct ccc ctg agc ctc aat aat acc tct cag aaa ggt   4155
Lys Leu Ala Ser Ala Pro Leu Ser Leu Asn Asn Thr Ser Gln Lys Gly
          1325                1330                1335

ctg gaa ggt aga gga ctg ccc tgc aca cct aaa ccc cca gac cgg atc   4203
Leu Glu Gly Arg Gly Leu Pro Cys Thr Pro Lys Pro Pro Asp Arg Ile
      1340                1345                1350

agg caa gct ggc cct cat gtc ccc ttc acg gtg ttt gag gaa gtc tgc   4251
Arg Gln Ala Gly Pro His Val Pro Phe Thr Val Phe Glu Glu Val Cys
  1355                1360                1365

cct aca gag agc aag cct gaa gta ccc cag gcc ccc agg gta caa cag   4299
Pro Thr Glu Ser Lys Pro Glu Val Pro Gln Ala Pro Arg Val Gln Gln
1370              1375                1380                1385

aga gtc cag acg cgc ctc aag gtg aac ttc agt gat gac agt gac ttg   4347
Arg Val Gln Thr Arg Leu Lys Val Asn Phe Ser Asp Asp Ser Asp Leu
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gaa gac cct gtc tca gct gag gcc tgg ctg gca gag gag cct aag aga   4395
Glu Asp Pro Val Ser Ala Glu Ala Trp Leu Ala Glu Glu Pro Lys Arg
          1405                1410                1415

cgg ggc act gct tcc cgg ggc cgg ggg cga gca agg aag ggc ctg agc   4443
Arg Gly Thr Ala Ser Arg Gly Arg Gly Arg Ala Arg Lys Gly Leu Ser
      1420                1425                1430

cta aag acg gat gcc gtg gtt gcc cca ggt agt gcc cct ggg aac cct   4491
Leu Lys Thr Asp Ala Val Val Ala Pro Gly Ser Ala Pro Gly Asn Pro
  1435                1440                1445

ggc ctg aat ggc agg agc cgg agg gcc aag aag gtg gca tca aga cat   4539
Gly Leu Asn Gly Arg Ser Arg Arg Ala Lys Lys Val Ala Ser Arg His
1450              1455                1460                1465

tgt gag gag cgg cgt ccc cag agg gcc agt gac cag gcc agg cct ggc   4587
Cys Glu Glu Arg Arg Pro Gln Arg Ala Ser Asp Gln Ala Arg Pro Gly
              1470                1475                1480

cct gag atc atg agg acc atc cct gag gaa gaa ctg act gac aac tgg   4635
Pro Glu Ile Met Arg Thr Ile Pro Glu Glu Glu Leu Thr Asp Asn Trp
          1485                1490                1495

aga aaa atg agc ttt gag atc ctc agg ggc tct gac ggg gaa gac tca   4683
Arg Lys Met Ser Phe Glu Ile Leu Arg Gly Ser Asp Gly Glu Asp Ser
      1500                1505                1510

gcc tca ggt ggg aag act cca gct ccg ggc cct gag gca gct tct gga   4731
Ala Ser Gly Gly Lys Thr Pro Ala Pro Gly Pro Glu Ala Ala Ser Gly
  1515                1520                1525

gaa tgg gag ctg ctg agg ctg gat tcc agc aag aag aag ctg ccc agc   4779
Glu Trp Glu Leu Leu Arg Leu Asp Ser Ser Lys Lys Lys Leu Pro Ser
1530              1535                1540                1545

cca tgc cca gac aag gag agt gac aag gac ctt ggt cct cgg ctc cag   4827
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Pro Cys Pro Asp Lys Glu Ser Asp Lys Asp Leu Gly Pro Arg Leu Gln
                1550                1555                1560

ctc ccc tca gcc ccc gta gcc act ggt ctt tct acc ctg gac tcc atc   4875
Leu Pro Ser Ala Pro Val Ala Thr Gly Leu Ser Thr Leu Asp Ser Ile
            1565                1570                1575

tgt gac tcc ctg agt gtt gct ttc cgg ggc att agt cac tgt cct cct   4923
Cys Asp Ser Leu Ser Val Ala Phe Arg Gly Ile Ser His Cys Pro Pro
        1580                1585                1590

agt ggg ctc tat gcc cac ctc tgc cgc ttc ctg gcc ttg tgc ctg ggc   4971
Ser Gly Leu Tyr Ala His Leu Cys Arg Phe Leu Ala Leu Cys Leu Gly
    1595                1600                1605

cac cgg gat cct tat gcc act gct ttc ctt gtc acc gag tct gtc tcc   5019
His Arg Asp Pro Tyr Ala Thr Ala Phe Leu Val Thr Glu Ser Val Ser
1610                1615                1620                1625

atc acc tgt cgc cac cag ctg ctc acc cac ctc cac aga cag ctc agc   5067
Ile Thr Cys Arg His Gln Leu Leu Thr His Leu His Arg Gln Leu Ser
                1630                1635                1640

aag gcc cag aag cac cga gga tca ctt gaa ata gca gac cag ctg cag   5115
Lys Ala Gln Lys His Arg Gly Ser Leu Glu Ile Ala Asp Gln Leu Gln
            1645                1650                1655

ggg ctg agc ctt cag gag atg cct gga gat gtc ccc ctg gcc cgc atc   5163
Gly Leu Ser Leu Gln Glu Met Pro Gly Asp Val Pro Leu Ala Arg Ile
        1660                1665                1670

cag cgc ctc ttt tcc ttc agg gct ttg gaa tct ggc cac ttc ccc cag   5211
Gln Arg Leu Phe Ser Phe Arg Ala Leu Glu Ser Gly His Phe Pro Gln
    1675                1680                1685

cct gaa aag gag agt ttc cag gag cgc ctg gct ctg atc ccc agt ggg   5259
Pro Glu Lys Glu Ser Phe Gln Glu Arg Leu Ala Leu Ile Pro Ser Gly
1690                1695                1700                1705

gtg act gtg tgt gtg ttg gcc ctg gcc acc ctc cag ccc gga acc gtg   5307
Val Thr Val Cys Val Leu Ala Leu Ala Thr Leu Gln Pro Gly Thr Val
                1710                1715                1720

ggc aac acc ctc ctg ctg acc cgg ctg gaa aag gac agt ccc cca gtc   5355
Gly Asn Thr Leu Leu Leu Thr Arg Leu Glu Lys Asp Ser Pro Pro Val
            1725                1730                1735

agt gtg cag att ccc act ggc cag aac aag ctt cat ctg cgt tca gtc   5403
Ser Val Gln Ile Pro Thr Gly Gln Asn Lys Leu His Leu Arg Ser Val
        1740                1745                1750

ctg aat gag ttt gat gcc atc cag aag gca cag aaa gag aac agc agc   5451
Leu Asn Glu Phe Asp Ala Ile Gln Lys Ala Gln Lys Glu Asn Ser Ser
    1755                1760                1765

tgt act gac aag cga gaa tgg tgg aca ggg cgg ctg gca ctg gac cac   5499
Cys Thr Asp Lys Arg Glu Trp Trp Thr Gly Arg Leu Ala Leu Asp His
1770                1775                1780                1785

agg atg gag gtt ctc atc gct tcc cta gag aag tct gtg ctg ggc tgc   5547
Arg Met Glu Val Leu Ile Ala Ser Leu Glu Lys Ser Val Leu Gly Cys
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                1790                1795                1800

tgg aag ggg ctg ctg ctg ccg tcc agt gag gag ccc ggc cct gcc cag   5595
Trp Lys Gly Leu Leu Leu Pro Ser Ser Glu Glu Pro Gly Pro Ala Gln
           1805                1810                1815

gag gcc tcc cgc cta cag gag ctg cta cag gac tgt ggc tgg aaa tat   5643
Glu Ala Ser Arg Leu Gln Glu Leu Leu Gln Asp Cys Gly Trp Lys Tyr
       1820                1825                1830

cct gac cgc act ctg ctg aaa atc atg ctc agt ggt gcc ggt gcc ctc   5691
Pro Asp Arg Thr Leu Leu Lys Ile Met Leu Ser Gly Ala Gly Ala Leu
   1835                1840                1845

acc cct cag gac att cag gcc ctg gcc tac ggg ctg tgc cca acc cag   5739
Thr Pro Gln Asp Ile Gln Ala Leu Ala Tyr Gly Leu Cys Pro Thr Gln
1850                1855                1860                1865

cca gag cga gcc cag gag ctc ctg aat gag gca gta gga cgt cta cag   5787
Pro Glu Arg Ala Gln Glu Leu Leu Asn Glu Ala Val Gly Arg Leu Gln
               1870                1875                1880

ggc ctg aca gta cca agc aat agc cac ctt gtc ttg gtc cta gac aag   5835
Gly Leu Thr Val Pro Ser Asn Ser His Leu Val Leu Val Leu Asp Lys
           1885                1890                1895

gac ttg cag aag ctg ccg tgg gaa agc atg ccc agc ctc caa gca ctg   5883
Asp Leu Gln Lys Leu Pro Trp Glu Ser Met Pro Ser Leu Gln Ala Leu
       1900                1905                1910

cct gtc acc cgg ctg ccc tcc ttc cgc ttc cta ctc agc tac tcc atc   5931
Pro Val Thr Arg Leu Pro Ser Phe Arg Phe Leu Leu Ser Tyr Ser Ile
   1915                1920                1925

atc aaa gag tat ggg gcc tcg cca gtg ctg agt caa ggg gtg gat cca   5979
Ile Lys Glu Tyr Gly Ala Ser Pro Val Leu Ser Gln Gly Val Asp Pro
1930                1935                1940                1945

cga agt acc ttc tat gtc ctg aac cct cac aat aac ctg tca agc aca   6027
Arg Ser Thr Phe Tyr Val Leu Asn Pro His Asn Asn Leu Ser Ser Thr
               1950                1955                1960

gag gag caa ttt cga gcc aat ttc agc agt gaa gct ggc tgg aga gga   6075
Glu Glu Gln Phe Arg Ala Asn Phe Ser Ser Glu Ala Gly Trp Arg Gly
           1965                1970                1975

gtg gtt ggg gag gtg cca aga cct gaa cag gtg cag gaa gcc ctg aca   6123
Val Val Gly Glu Val Pro Arg Pro Glu Gln Val Gln Glu Ala Leu Thr
       1980                1985                1990

aag cat gat ttg tat atc tat gca ggg cat ggg gct ggt gcc cgc ttc   6171
Lys His Asp Leu Tyr Ile Tyr Ala Gly His Gly Ala Gly Ala Arg Phe
   1995                2000                2005

ctt gat ggg cag gct gtc ctg cgg ctg agc tgt cgg gca gtg gcc ctg   6219
Leu Asp Gly Gln Ala Val Leu Arg Leu Ser Cys Arg Ala Val Ala Leu
2010                2015                2020                2025

ctg ttt ggc tgt agc agt gcg gcc ctg gct gtg cat gga aac ctg gag   6267
Leu Phe Gly Cys Ser Ser Ala Ala Leu Ala Val His Gly Asn Leu Glu
               2030                2035                2040
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ggg gct ggc atc gtg ctc aag tac atc atg gct ggt tgc ccc ttg ttt   6315
Gly Ala Gly Ile Val Leu Lys Tyr Ile Met Ala Gly Cys Pro Leu Phe
          2045                2050                2055

ctg ggt aat ctc tgg gat gtg act gac cgc gac att gac cgc tac acg   6363
Leu Gly Asn Leu Trp Asp Val Thr Asp Arg Asp Ile Asp Arg Tyr Thr
      2060                2065                2070

gaa gct ctg ctg caa ggc tgg ctt gga gca ggc cca ggg gcc ccc ctt   6411
Glu Ala Leu Leu Gln Gly Trp Leu Gly Ala Gly Pro Gly Ala Pro Leu
   2075                2080                2085

ctc tac tat gta aac cag gcc cgc caa gct ccc cga ctc aag tat ctt   6459
Leu Tyr Tyr Val Asn Gln Ala Arg Gln Ala Pro Arg Leu Lys Tyr Leu
2090                2095                2100                2105

att ggg gct gca cct ata gcc tat ggc ttg cct gtc tct ctg cgg taa   6507
Ile Gly Ala Ala Pro Ile Ala Tyr Gly Leu Pro Val Ser Leu Arg
               2110                2115                2120

ccccatggag ctgtcttatt gatgctagaa gcctcataac tgttctacct ccaaggttag 6567

atttaatcct taggataact cttttaaagt gattttcccc agtgttttat atgaaacatt 6627

tccttttgat ttaacctcag tataataaag atacatcatt t                     6668
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<210> 2
<211> 2120
<212> PRT
<213> Homo sapiens

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<400> 2
Met Arg Ser Phe Lys Arg Val Asn Phe Gly Thr Leu Leu Ser Ser Gln
  1               5                  10                  15

Lys Glu Ala Glu Glu Leu Leu Pro Asp Leu Lys Glu Phe Leu Ser Asn
             20                  25                  30

Pro Pro Ala Gly Phe Pro Ser Ser Arg Ser Asp Ala Glu Arg Arg Gln
         35                  40                  45

Ala Cys Asp Ala Ile Leu Arg Ala Cys Asn Gln Gln Leu Thr Ala Lys
     50                  55                  60

Leu Ala Cys Pro Arg His Leu Gly Ser Leu Leu Glu Leu Ala Glu Leu
 65                  70                  75                  80

Ala Cys Asp Gly Tyr Leu Val Ser Thr Pro Gln Arg Pro Pro Leu Tyr
                 85                  90                  95

Leu Glu Arg Ile Leu Phe Val Leu Leu Arg Asn Ala Ala Ala Gln Gly
            100                 105                 110

Ser Pro Glu Val Thr Leu Arg Leu Ala Gln Pro Leu His Ala Cys Leu
        115                 120                 125

Val Gln Cys Ser Arg Glu Ala Ala Pro Gln Asp Tyr Glu Ala Val Ala
    130                 135                 140
```

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Arg Gly Ser Phe Ser Leu Leu Trp Lys Gly Ala Glu Ala Leu Leu Glu
145                 150                 155                 160

Arg Arg Ala Ala Phe Ala Ala Arg Leu Lys Ala Leu Ser Phe Leu Val
                165                 170                 175

Leu Leu Glu Asp Glu Ser Thr Pro Cys Glu Val Pro His Phe Ala Ser
            180                 185                 190

Pro Thr Ala Cys Arg Ala Val Ala Ala His Gln Leu Phe Asp Ala Ser
        195                 200                 205

Gly His Gly Leu Asn Glu Ala Asp Ala Asp Phe Leu Asp Asp Leu Leu
    210                 215                 220

Ser Arg His Val Ile Arg Ala Leu Val Gly Glu Arg Gly Ser Ser Ser
225                 230                 235                 240

Gly Leu Leu Ser Pro Gln Arg Ala Leu Cys Leu Leu Glu Leu Thr Leu
                245                 250                 255

Glu His Cys Arg Arg Phe Cys Trp Ser Arg His His Asp Lys Ala Ile
            260                 265                 270

Ser Ala Val Glu Lys Ala His Ser Tyr Leu Arg Asn Thr Asn Leu Ala
        275                 280                 285

Pro Ser Leu Gln Leu Cys Gln Leu Gly Val Lys Leu Leu Gln Val Gly
    290                 295                 300

Glu Glu Gly Pro Gln Ala Val Ala Lys Leu Leu Ile Lys Ala Ser Ala
305                 310                 315                 320

Val Leu Ser Lys Ser Met Glu Ala Pro Ser Pro Pro Leu Arg Ala Leu
                325                 330                 335

Tyr Glu Ser Cys Gln Phe Phe Leu Ser Gly Leu Glu Arg Gly Thr Lys
            340                 345                 350

Arg Arg Tyr Arg Leu Asp Ala Ile Leu Ser Leu Phe Ala Phe Leu Gly
        355                 360                 365

Gly Tyr Cys Ser Leu Leu Gln Gln Leu Arg Asp Asp Gly Val Tyr Gly
    370                 375                 380

Gly Ser Ser Lys Gln Gln Gln Ser Phe Leu Gln Met Tyr Phe Gln Gly
385                 390                 395                 400

Leu His Leu Tyr Thr Val Val Val Tyr Asp Phe Ala Gln Gly Cys Gln
                405                 410                 415

Ile Val Asp Leu Ala Asp Leu Thr Gln Leu Val Asp Ser Cys Lys Ser
            420                 425                 430

Thr Val Val Trp Met Leu Glu Ala Leu Glu Gly Leu Ser Gly Gln Glu
        435                 440                 445

Leu Thr Asp His Met Gly Met Thr Ala Ser Tyr Thr Ser Asn Leu Ala
    450                 455                 460

Tyr Ser Phe Tyr Ser His Lys Leu Tyr Ala Glu Ala Cys Ala Ile Ser
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465                 470                 475                 480

Glu Pro Leu Cys Gln His Leu Gly Leu Val Lys Pro Gly Thr Tyr Pro
                485                 490                 495

Glu Val Pro Pro Glu Lys Leu His Arg Cys Phe Arg Leu Gln Val Glu
            500                 505                 510

Ser Leu Lys Lys Leu Gly Lys Gln Ala Gln Gly Cys Lys Met Val Ile
        515                 520                 525

Leu Trp Leu Ala Ala Leu Gln Pro Cys Ser Pro Glu His Met Ala Glu
    530                 535                 540

Pro Val Thr Phe Trp Val Arg Val Lys Met Asp Ala Ala Arg Ala Gly
545                 550                 555                 560

Asp Lys Glu Leu Gln Leu Lys Thr Leu Arg Asp Ser Leu Ser Gly Trp
                565                 570                 575

Asp Pro Glu Thr Leu Ala Leu Leu Leu Arg Glu Glu Leu Gln Ala Tyr
            580                 585                 590

Lys Ala Val Arg Ala Asp Thr Gly Gln Glu Arg Phe Asn Ile Ile Cys
        595                 600                 605

Asp Leu Leu Glu Leu Ser Pro Glu Glu Thr Pro Ala Gly Ala Trp Ala
    610                 615                 620

Arg Ala Thr His Leu Val Glu Leu Ala Gln Val Leu Cys Tyr His Asp
625                 630                 635                 640

Phe Thr Gln Gln Thr Asn Cys Ser Ala Leu Asp Ala Ile Arg Glu Ala
                645                 650                 655

Leu Gln Leu Leu Asp Ser Val Arg Pro Glu Ala Gln Ala Arg Asp Gln
            660                 665                 670

Leu Leu Asp Asp Lys Ala Gln Ala Leu Leu Trp Leu Tyr Ile Cys Thr
        675                 680                 685

Leu Glu Ala Lys Ile Gln Glu Gly Ile Glu Arg Asp Arg Arg Ala Gln
    690                 695                 700

Ala Pro Gly Asn Leu Glu Glu Phe Glu Val Asn Asp Leu Asn Tyr Glu
705                 710                 715                 720

Asp Lys Leu Gln Glu Asp Arg Phe Leu Tyr Ser Asn Ile Ala Phe Asn
                725                 730                 735

Leu Ala Ala Asp Ala Ala Gln Ser Lys Cys Leu Asp Gln Ala Leu Ala
            740                 745                 750

Leu Trp Lys Glu Leu Leu Thr Lys Gly Gln Ala Pro Ala Val Arg Cys
        755                 760                 765

Leu Gln Gln Thr Ala Ala Ser Leu Gln Ile Leu Ala Ala Leu Tyr Gln
    770                 775                 780

Leu Val Ala Lys Pro Met Gln Ala Leu Glu Val Leu Leu Leu Leu Arg
785                 790                 795                 800
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Ile Val Ser Glu Arg Leu Lys Asp His Ser Lys Ala Ala Gly Ser Ser
                805                 810                 815

Cys His Ile Thr Gln Leu Leu Leu Thr Leu Gly Cys Pro Ser Tyr Ala
            820                 825                 830

Gln Leu His Leu Glu Glu Ala Ala Ser Ser Leu Lys His Leu Asp Gln
        835                 840                 845

Thr Thr Asp Thr Tyr Leu Leu Leu Ser Leu Thr Cys Asp Leu Leu Arg
    850                 855                 860

Ser Gln Leu Tyr Trp Thr His Gln Lys Val Thr Lys Gly Val Ser Leu
865                 870                 875                 880

Leu Leu Ser Val Leu Arg Asp Pro Ala Leu Gln Lys Ser Ser Lys Ala
                885                 890                 895

Trp Tyr Leu Leu Arg Val Gln Val Leu Gln Leu Val Ala Ala Tyr Leu
            900                 905                 910

Ser Leu Pro Ser Asn Asn Leu Ser His Ser Leu Trp Glu Gln Leu Cys
        915                 920                 925

Ala Gln Gly Trp Gln Thr Pro Glu Ile Ala Leu Ile Asp Ser His Lys
    930                 935                 940

Leu Leu Arg Ser Ile Ile Leu Leu Leu Met Gly Ser Asp Ile Leu Ser
945                 950                 955                 960

Thr Gln Lys Ala Ala Val Glu Thr Ser Phe Leu Asp Tyr Gly Glu Asn
                965                 970                 975

Leu Val Gln Lys Trp Gln Val Leu Ser Glu Val Leu Ser Cys Ser Glu
            980                 985                 990

Lys Leu Val Cys His Leu Gly Arg Leu Gly Ser Val Ser Glu Ala Lys
        995                1000                1005

Ala Phe Cys Leu Glu Ala Leu Lys Leu Thr Thr Lys Leu Gln Ile Pro
   1010                1015                1020

Arg Gln Cys Ala Leu Phe Leu Val Leu Lys Gly Glu Leu Glu Leu Ala
025                1030                1035                1040

Arg Asn Asp Ile Asp Leu Cys Gln Ser Asp Leu Gln Gln Val Leu Phe
               1045                1050                1055

Leu Leu Glu Ser Cys Thr Glu Phe Gly Gly Val Thr Gln His Leu Asp
           1060                1065                1070

Ser Val Lys Lys Val His Leu Gln Lys Gly Lys Gln Gln Ala Gln Val
       1075                1080                1085

Pro Cys Pro Pro Gln Leu Pro Glu Glu Glu Leu Phe Leu Arg Gly Pro
   1090                1095                1100

Ala Leu Glu Leu Val Ala Thr Val Ala Lys Glu Pro Gly Pro Ile Ala
105                1110                1115                1120
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Pro Ser Thr Asn Ser Ser Pro Val Leu Lys Thr Lys Pro Gln Pro Ile
               1125                1130                1135

Pro Asn Phe Leu Ser His Ser Pro Thr Cys Asp Cys Ser Leu Cys Ala
           1140                1145                1150

Ser Pro Val Leu Thr Ala Val Cys Leu Arg Trp Val Leu Val Thr Ala
       1155                1160                1165

Gly Val Arg Leu Ala Met Gly His Gln Ala Gln Gly Leu Asp Leu Leu
   1170                1175                1180

Gln Val Val Leu Lys Gly Cys Pro Glu Ala Ala Glu Arg Leu Thr Gln
185                1190                1195                1200

Ala Leu Gln Ala Ser Leu Asn His Lys Thr Pro Pro Ser Leu Val Pro
               1205                1210                1215

Ser Leu Leu Asp Glu Ile Leu Ala Gln Ala Tyr Thr Leu Leu Ala Leu
           1220                1225                1230

Glu Gly Leu Asn Gln Pro Ser Asn Glu Ser Leu Gln Lys Val Leu Gln
       1235                1240                1245

Ser Gly Leu Lys Phe Val Ala Ala Arg Ile Pro His Leu Glu Pro Trp
   1250                1255                1260

Arg Ala Ser Leu Leu Leu Ile Trp Ala Leu Thr Lys Leu Gly Gly Leu
265                1270                1275                1280

Ser Cys Cys Thr Thr Gln Leu Phe Ala Ser Ser Trp Gly Trp Gln Pro
               1285                1290                1295

Pro Leu Ile Lys Ser Val Pro Gly Ser Glu Pro Ser Lys Thr Gln Gly
           1300                1305                1310

Gln Lys Arg Ser Gly Arg Gly Arg Gln Lys Leu Ala Ser Ala Pro Leu
       1315                1320                1325

Ser Leu Asn Asn Thr Ser Gln Lys Gly Leu Glu Gly Arg Gly Leu Pro
   1330                1335                1340

Cys Thr Pro Lys Pro Pro Asp Arg Ile Arg Gln Ala Gly Pro His Val
345                1350                1355                1360

Pro Phe Thr Val Phe Glu Glu Val Cys Pro Thr Glu Ser Lys Pro Glu
               1365                1370                1375

Val Pro Gln Ala Pro Arg Val Gln Gln Arg Val Gln Thr Arg Leu Lys
           1380                1385                1390

Val Asn Phe Ser Asp Asp Ser Asp Leu Glu Asp Pro Val Ser Ala Glu
       1395                1400                1405

Ala Trp Leu Ala Glu Glu Pro Lys Arg Arg Gly Thr Ala Ser Arg Gly
   1410                1415                1420

Arg Gly Arg Ala Arg Lys Gly Leu Ser Leu Lys Thr Asp Ala Val Val
425                1430                1435                1440

Ala Pro Gly Ser Ala Pro Gly Asn Pro Gly Leu Asn Gly Arg Ser Arg
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```
                1445                1450                1455

Arg Ala Lys Lys Val Ala Ser Arg His Cys Glu Glu Arg Arg Pro Gln
            1460                1465                1470

Arg Ala Ser Asp Gln Ala Arg Pro Gly Pro Glu Ile Met Arg Thr Ile
        1475                1480                1485

Pro Glu Glu Glu Leu Thr Asp Asn Trp Arg Lys Met Ser Phe Glu Ile
    1490                1495                1500

Leu Arg Gly Ser Asp Gly Glu Asp Ser Ala Ser Gly Gly Lys Thr Pro
505                 1510                1515                1520

Ala Pro Gly Pro Glu Ala Ala Ser Gly Glu Trp Glu Leu Leu Arg Leu
                1525                1530                1535

Asp Ser Ser Lys Lys Lys Leu Pro Ser Pro Cys Pro Asp Lys Glu Ser
            1540                1545                1550

Asp Lys Asp Leu Gly Pro Arg Leu Gln Leu Pro Ser Ala Pro Val Ala
        1555                1560                1565

Thr Gly Leu Ser Thr Leu Asp Ser Ile Cys Asp Ser Leu Ser Val Ala
    1570                1575                1580

Phe Arg Gly Ile Ser His Cys Pro Pro Ser Gly Leu Tyr Ala His Leu
585                 1590                1595                1600

Cys Arg Phe Leu Ala Leu Cys Leu Gly His Arg Asp Pro Tyr Ala Thr
                1605                1610                1615

Ala Phe Leu Val Thr Glu Ser Val Ser Ile Thr Cys Arg His Gln Leu
            1620                1625                1630

Leu Thr His Leu His Arg Gln Leu Ser Lys Ala Gln Lys His Arg Gly
        1635                1640                1645

Ser Leu Glu Ile Ala Asp Gln Leu Gln Gly Leu Ser Leu Gln Glu Met
    1650                1655                1660

Pro Gly Asp Val Pro Leu Ala Arg Ile Gln Arg Leu Phe Ser Phe Arg
665                 1670                1675                1680

Ala Leu Glu Ser Gly His Phe Pro Gln Pro Glu Lys Glu Ser Phe Gln
                1685                1690                1695

Glu Arg Leu Ala Leu Ile Pro Ser Gly Val Thr Val Cys Val Leu Ala
            1700                1705                1710

Leu Ala Thr Leu Gln Pro Gly Thr Val Gly Asn Thr Leu Leu Leu Thr
        1715                1720                1725

Arg Leu Glu Lys Asp Ser Pro Pro Val Ser Val Gln Ile Pro Thr Gly
    1730                1735                1740

Gln Asn Lys Leu His Leu Arg Ser Val Leu Asn Glu Phe Asp Ala Ile
745                 1750                1755                1760

Gln Lys Ala Gln Lys Glu Asn Ser Ser Cys Thr Asp Lys Arg Glu Trp
                1765                1770                1775
```

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Trp Thr Gly Arg Leu Ala Leu Asp His Arg Met Glu Val Leu Ile Ala
          1780                1785                1790

Ser Leu Glu Lys Ser Val Leu Gly Cys Trp Lys Gly Leu Leu Leu Pro
       1795                1800                1805

Ser Ser Glu Glu Pro Gly Pro Ala Gln Glu Ala Ser Arg Leu Gln Glu
   1810                1815                1820

Leu Leu Gln Asp Cys Gly Trp Lys Tyr Pro Asp Arg Thr Leu Leu Lys
825                1830                1835                1840

Ile Met Leu Ser Gly Ala Gly Ala Leu Thr Pro Gln Asp Ile Gln Ala
               1845                1850                1855

Leu Ala Tyr Gly Leu Cys Pro Thr Gln Pro Glu Arg Ala Gln Glu Leu
          1860                1865                1870

Leu Asn Glu Ala Val Gly Arg Leu Gln Gly Leu Thr Val Pro Ser Asn
       1875                1880                1885

Ser His Leu Val Leu Val Leu Asp Lys Asp Leu Gln Lys Leu Pro Trp
   1890                1895                1900

Glu Ser Met Pro Ser Leu Gln Ala Leu Pro Val Thr Arg Leu Pro Ser
905                1910                1915                1920

Phe Arg Phe Leu Leu Ser Tyr Ser Ile Ile Lys Glu Tyr Gly Ala Ser
               1925                1930                1935

Pro Val Leu Ser Gln Gly Val Asp Pro Arg Ser Thr Phe Tyr Val Leu
          1940                1945                1950

Asn Pro His Asn Asn Leu Ser Ser Thr Glu Glu Gln Phe Arg Ala Asn
       1955                1960                1965

Phe Ser Ser Glu Ala Gly Trp Arg Gly Val Val Gly Glu Val Pro Arg
   1970                1975                1980

Pro Glu Gln Val Gln Glu Ala Leu Thr Lys His Asp Leu Tyr Ile Tyr
985                1990                1995                2000

Ala Gly His Gly Ala Gly Ala Arg Phe Leu Asp Gly Gln Ala Val Leu
               2005                2010                2015

Arg Leu Ser Cys Arg Ala Val Ala Leu Leu Phe Gly Cys Ser Ser Ala
          2020                2025                2030

Ala Leu Ala Val His Gly Asn Leu Glu Gly Ala Gly Ile Val Leu Lys
       2035                2040                2045

Tyr Ile Met Ala Gly Cys Pro Leu Phe Leu Gly Asn Leu Trp Asp Val
   2050                2055                2060

Thr Asp Arg Asp Ile Asp Arg Tyr Thr Glu Ala Leu Leu Gln Gly Trp
065                2070                2075                2080

Leu Gly Ala Gly Pro Gly Ala Pro Leu Leu Tyr Tyr Val Asn Gln Ala
               2085                2090                2095
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Arg Gln Ala Pro Arg Leu Lys Tyr Leu Ile Gly Ala Ala Pro Ile Ala
2100 2105 2110

Tyr Gly Leu Pro Val Ser Leu Arg
2115 2120

<210> 3
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 3
ggccaattga tatcatgagg agcttcaaaa gag 33

<210> 4
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 4
caactgtcca ctagttgggt cagg 24

<210> 5
<211> 54
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 5
gaattctaat acgactcact ataggatcca tgatccctga ggaagaactg actg 54

<210> 6
<211> 55
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 6
gaattctaat acgactcact ataggatcca tgtctgacgg ggaagactca gcctc 55

<210> 7
<211> 56
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 7
gaattctaat acgactcact ataggatcca tggattccag caagaagaag ctgccc 56

<210> 8
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 8
ttattaccgc agagagacag gcaagcc 27