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Development and validation of a fluorogenic assay to measure separase enzyme activity

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ABSTRACT

Separase, an endopeptidase, plays a pivotal role in the separation of sister chromatids at anaphase by cleaving its substrate cohesin Rad21. Recent study suggests that separase is an oncogene. Overexpression of separase induces aneuploidy and mammary tumorigenesis in mice. Separase is also overexpressed and mislocalized in a wide range of human cancers, including breast, prostate, and osteosarcoma. Currently, there is no quantitative assay to measure separase enzymatic activity. To quantify separase enzymatic activity, we have designed a fluorogenic assay in which 7-amido-4-methyl coumaric acid (AMC)-conjugated Rad21 mitotic cleavage site peptide (Ac-Asp-Arg-Glu-Ile-Nle-Arg-MCA) is used as the substrate of separase. We used this assay to quantify separase activity during cell cycle progression and in a panel of human tumor cell lines as well as leukemia patient samples.

Accurate chromosomal segregation in each cell cycle relies on a conserved protein complex called cohesin and an endopeptidase called separase. Separase is a CD clan endopeptidase (also known as Esp1 or separin in yeast) [1–3] that is bound with its inhibitory chaperone securin (also known as PTTG in human and Pds1 in yeast) prior to anaphase [1,4,5]. In metaphase, ubiquitin-mediated degradation of the securin by anaphase-promoting complex/cyclosome (APC/C)[2]–Cdc20 ubiquitin–ligase releases and activates separase. The activated separase proteolytically cleaves cohesin Rad21 at two sites, resulting in the removal of cohesin from chromosomes and separation of the sister chromatids [1,6–9].

Recent studies demonstrate that overexpression of separase induces aneuploidy (aberrant chromosome number) and mammary tumorigenesis in mice [10]. Small interfering RNA (siRNA)-mediated knockdown of separase and separase-deficient mouse embryonic fibroblasts also results in genomic instability [11–13]. Studies indicate that separase protein is overexpressed and mislocalized in a wide range of human cancers, including breast, prostate, and osteosarcoma [10,14]. Furthermore, Separase overexpression strongly correlates with relapse, metastasis, and a lower 5-year overall survival rate in breast and prostate cancer patients [14].

Fluorogenic assay has been successfully used in enzymatic assay, such as caspases [15,16] and matrix metalloproteases [17,18], and proved to be a very reliable method. Although separase activity can be assessed by immunoblot [19,20], there is currently no other method available to quantitatively estimate separase enzymatic activity. Here, we report the development of a simple, sensitive, and robust quantitative assay for separase using a fluorogenic peptide containing the separase cleavage site of Rad21 as a substrate. This assay can be used specifically to measure separase activity from cells and tumor specimens.

Materials and methods

Preparation of cytostatic factor extracts from Xenopus egg

Cytostatic factor (CSF) extracts were prepared as described previously [21]. Briefly, frogs were induced to lay eggs with an injection of 125 U of human chorionic gonadotropin (hCG) to the dorsal sac. Eggs were collected the next morning and dejellied with 2% cysteine in 1× modified Ringer's solution (MMR, pH 7.8). Eggs were packed evenly by a brief spin at 500 rpm followed by a second spin at 10,000 rpm for 10 min. Clear extract with floating membranes was collected by side puncture and collected in a fresh tube. Extracts were supplemented with CSF energy mix (40 mM phosphocreatine, 4 mM adenosine triphosphate [ATP], and 0.2 mg/ml creatine phosphokinase) and 34 nM cyclin BΔ90 and were rotated at room temperature for 15 min. Anaphase of the CSF was initiated by adding Ca^{2+} up to 0.6 mM and incubating for 20 min at room

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[2] *Abbreviations used:* APC/C, anaphase-promoting complex/cyclosome; siRNA, small interfering RNA; CSF, cytostatic factor; hCG, human chorionic gonadotropin; MMR, modified Ringer's solution; ATP, adenosine triphosphate; IgG, immunoglobulin G; cDNA, complementary DNA; mAb, monoclonal antibody; EGTA, ethyleneglycoltetraacetic acid; MCA, methyl coumaric acid; PBS, phosphate-buffered saline; AML, acute myelogenous leukemia; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AMC, 7-amido-4-methyl coumaric acid; RFU, relative fluorescence units; DAPI, 4′,6-diamidino-2-phenylindole.

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temperature. Before being used for activating separase, the activity of CSF was confirmed using securin degradation assay.

Overexpression, immunoprecipitation, and activation of separase

We obtained active separase from human cells as described previously [19,20]. Briefly, ZZ TEV_4–separase was expressed in 293T cells and immunoprecipitated from exponentially growing cells using immunoglobulin G (IgG)-conjugated Sepharose 6 (GE Healthcare, Piscataway, NJ, USA). To release active separase from its inhibitory chaperone securin, we activated separase by incubating IgG–Sepharose 6 containing ZZ TEV_4–separase with anaphase-initiated *Xenopus* CSF to degrade securin at room temperature for 1 h. Activated separase protein was eluted from the beads using TEV protease (Invitrogen, Carlsbad, CA, USA).

Separase protease assay

Separase cleavage of Rad21 was performed as described previously [19] with minor modifications. Rad21 complementary DNA (cDNA) was cloned into pFASTBac vector (Invitrogen) containing Flag tag on N terminus of Rad21 and transformed into sf9 insect cells. Flag–Rad21 was purified using Flag monoclonal antibody (mAb)-conjugated agarose beads (Sigma, St. Louis, MO, USA) and eluted with 3× Flag peptide as described previously [22]. Then 5 μl of activated separase was mixed with 1 μl of recombinant Rad21 protein (expressed and purified from sf9 cells) and incubated in 20 μl of cleavage assay buffer (30 mM Hepes–KOH [pH 7.7], 50 mM NaCl, 25 mM NaF, 25 mM KCl, 5 mM $MgCl_2$, 1.5 mM ATP, and 1 mM ethyleneglycoltetraacetic acid [EGTA]) for 1 h at 37 °C. The cleavage of Rad21 was detected by immunoblotting with Rad21 mAb.

For the fluorescence assays, the same cleavage buffer was used in a 50-μl reaction volume. First, 0.5–1 μl (~33.5 ng) of activated separase was combined with 2 μl of 10 mM Rad21–MCA (methyl coumaric acid) peptide (Ac-Asp-Arg-Glu-Ile-Nle-Arg-MCA) (Peptide International, Louisville, KY, USA) and incubated for 3 h at 37 °C. At the end of the reaction, fluorescence was measured by spectrofluorometry (Spectrafluor Plus, TECAN, Mannedorf, Switzerland) at λ_{ex} = 405 nm and λ_{em} = 465 nm.

Cell culture and synchronization

Mitotic cell lysates were prepared from HeLa cells according to Gaglio and coworkers with minor modifications [23]. Briefly, HeLa cells were synchronized with a double thymidine block and arrested at G2/M using nocodazole (40 ng/ml final concentration) 6 h after release from the thymidine block. After 4 h of nocodazole arrest, approximately 80% of the cells reached mitosis. Mitotic cells were collected by mitotic shake-off and released from nocodazole arrest. Typically, 10^5 cells were collected at 0, 60, 80, 100, 120, and 140 min after nocodazole release. Cell lysate was prepared as described by Zhang and coworkers [22].

Leukemic cell lines (HEL 92.1.7, HL60, U937, Jurkat, Molt4, RS4, and Raji, American Type Culture Collection [ATCC], Manassas, VA, USA) were cultured in RPMI 1640 supplemented with 10% bovine fetal serum (Invitrogen) to a density of 0.5–1.0 × 10^6 cells/ml. Then 10 million exponentially growing cells were pelleted and washed three times in cold phosphate-buffered saline (PBS) before making the protein lysates. Bone marrow aspirates from pediatric patients with newly diagnosed acute myelogenous leukemia (AML, $n = 6$) were collected prior to administration of chemotherapy using an institutional review board-approved protocol according to institutional guidelines. Mononuclear cells were isolated using Lymphoprep (Axis–Shield, Oslo, Norway) and washed twice with PBS, and then 1 × 10^7 cells were frozen as cell pellets at −80 °C. Lysates were prepared as described previously [22].

Results and discussion

Isolation and activation of separase enzyme from human cells

Because the development of an in vitro separase activity assay is limited by the availability of active enzyme, we used ZZ TEV_4–separase expressed in 293T cells as a source of separase enzyme. Separase was immunoprecipitated using the IgG-conjugated Sepharose 6 and eluted from the beads by cleaving the epitope using TEV protease. Because separase is inactive when bound to its inhibitory chaperone securin, the immunoprecipitated separase was incubated in *Xenopus* egg extracts (pretreated with Ca^{2+} for APC activation) to degrade securin and activating separase. We resolved the activated separase preparation on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and performed Coomassie staining (Fig. 1A) as well as immunostaining with separase mAb (Fig. 1B) to estimate the concentration of separase in the sample. According to the total immunoprecipitated protein loaded and the intensity ratio of separase bands to the total bands from the Coomassie-stained gel, the concentration of separase was estimated to be 33.5 ng/μl separase preparation. As an additional approach, the concentration of separase was also estimated by comparing the band intensities from a protein standard with known concentrations loaded alongside (see Fig. S1 in Supplementary material). Using both methods, we obtained the similar result that the separase concentration in the samples is approximately 30 ng/μl (Figs. 1 and S1). Activated separase was tested for activity in a Rad21 cleavage assay (Fig. 1C) and used as an enzyme source in developing the fluorogenic assay. Activated separase successfully cleaved full-length recombinant Rad21 protein, generating expected mitotic cleavage fragments in vitro that were detected on immunoblot using Rad21 antibody (Fig. 1C).

Designing a synthetic fluorogenic Rad21 mitotic cleavage site peptide as separase substrate

The specific mitotic separase cleavage sites of cohesin Rad21 have been mapped, and it is known that active separase cleaves Rad21 at two specific sites bearing the consensus Glu-X-X-Arg near the N-terminal and C-terminal ends of the protein, respectively

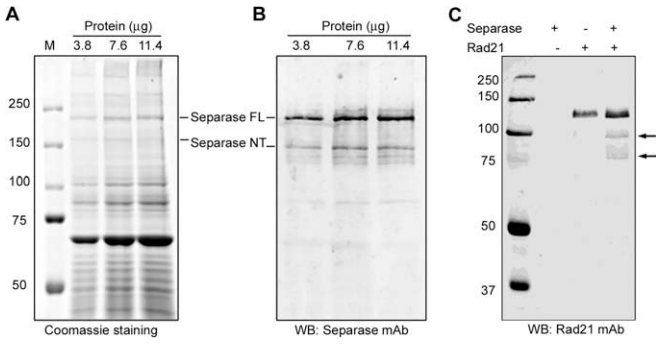

Fig. 1. Isolation and activation of separase. The separase was immunoprecipitated from 293T cells and activated with *Xenopus* egg extract. (A, B) Estimation of active separase protein concentration. Activated separase preparation (5, 10, and 15 μl, equivalent to 3.8, 7.6, and 11.4 μg of input protein, respectively) was resolved on 7% SDS–PAGE. The protein bands were visualized with Coomassie staining (A) and the separase bands were probed with anti-separase mAb on immunoblot. (B) Full-length and autocleaved separase bands versus total band intensities on Coomassie gel was quantified using ImageQuant 5.2 software. Activated separase protein concentration was calculated as a fraction of separase band optical density over the total band intensities from the Coomassie gel and was estimated to be 33.5 ng/μl. (C) Immunoblot of Rad21 cleavage assay using activated separase. The assay was performed at 37 °C for 1 h. The protein was resolved on 8% SDS–PAGE. Full-length Rad21 and cleavage fragments (arrows) were visualized with anti-Rad21 mAb. FL, Full length; NT, N-terminal; WB, Western Blot.