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A primate-specific POTE-actin fusion protein plays a role in apoptosis

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Abstract

The primate-specific gene family, *POTE*, is expressed in many cancers but only in a limited number of normal tissues (testis, ovary, prostate). The 13 *POTE* paralogs are dispersed among 8 human chromosomes. They evolved by gene duplication and remodeling from an ancestral gene, *Ankrd26*, recently implicated in controlling body size and obesity. In addition, several *POTE* paralogs are fused to an actin retrogene producing POTE-actin fusion proteins. The biological function of the *POTE* genes is unknown, but their high expression in primary spermatocytes, some of which are undergoing apoptosis, suggests a role in inducing programmed cell death. We have chosen HeLa cells as a model to study POTE function in human cancer, and have identified *POTE-2 α -actin* as the major transcript and the protein it encodes in HeLa cells. Transfection experiments show that both POTE-2 α -actin and POTE-2 γ C are localized to actin filaments close to the inner plasma membrane. Transient expression of POTE-2 α -actin or POTE-2 γ C induces apoptosis in HeLa cells. Using wild-type and mutant mouse embryo cells, we find apoptosis induced by over-expression of POTE-2 γ C is decreased in *Bak*^{-/-} or *Bak*^{-/-} *Bax*^{-/-} cells indicating POTE is acting through a mitochondrial pathway. Endogenous POTE-actin protein levels but not RNA levels increased in a time dependent manner by stimulation of death receptors with their cognate ligands. Our data indicates that the *POTE* gene family encodes a new family of proapoptotic proteins.

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Conflict of interest statement The authors declare that they have no conflict of interest.

Keywords

POTE paralogs; Ankrd26; Bax/Bak; Death receptor

Introduction

Apoptosis is a basic biological process that plays an essential role in all multicellular organisms and particularly in cancer biology [1, 2]. Apoptosis can be induced by intrinsic or extrinsic pathways [3–5]. In the intrinsic pathway stimuli such as cellular stress, DNA damage and chemotherapeutic drugs act on mitochondria, which release a group of proapoptotic factors such as cytochrome C and trigger activation of caspases and cell death [6]. The extrinsic pathway can be activated through the TNF receptor super-family with their cognate ligands, including Fas ligand and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Binding of TRAIL or Fas ligand results in cross-linking and activation of death receptors, which in turn activate caspase-8 and caspase-3 in a mitochondrial dependent and independent manner [1, 5, 7, 8].

POTE is a primate specific gene family that is expressed in many human cancers (prostate, colon, lung, ovary, pancreas and breast) and cancer cell lines, but only in a limited number of normal organs, including prostate, ovary, testis and placenta [9, 10]. In testis the POTE protein has been detected by immunohistochemistry in primary spermatocytes [11].

The *POTE* genes entered the primate genome about 30 million years ago and rapidly evolved into 13 closely related paralogs, which are located on 8 different chromosomes. All POTE proteins are made up of three distinct regions: N-terminal cystine rich domains, followed by 3–6 ankyrin repeats with spectrin-like helices at the C-terminus [12]. Several *POTE* paralogs are fused with an actin cDNA, producing POTE-actin fusion proteins [13]. *POTE* paralogs are named according to the chromosome they are located on; for example, *POTE-2* and *POTE-22* are located on chromosomes 2 and 22, respectively. There are 4 paralogs on chromosome 2: *POTE-2α*, *2β*, *2γ*, and *2δ* [14]. Each *POTE* paralog has several spliced variants, an example is *POTE-2γ* which has at least four different splice forms, *POTE-2γA*, *POTE-2γB*, *POTE-2γC* and *POTE-2γD*. Paralogs on chromosome 2 and 22 are the types predominantly expressed in normal tissues; *POTE-2α* and *POTE-2γ* are abundantly expressed in many cancers and cancer cell lines [14].

Despite information about the expression pattern and structure of the POTE paralogs, the biological function of the *POTE* genes is unknown. Recently a *POTE* ancestral gene, *Ankrd26*, was identified in humans and mice. Mice with a mutation inactivating *Ankrd26* were found to be obese and to have increased body size, indicating that *Ankrd26* has a role in controlling body size and growth [15]. In this study, we used Hela cells as a model cell line to study the function of POTE. We found that *POTE-2α-actin* is the major paralog expressed in Hela cells and that over-expression of a *POTE-2α-actin* cDNA by transfection induces apoptosis. In addition, endogenous *POTE-2α-actin* expression is increased by Fas ligand, antibodies to Fas and by TRAIL.

Materials and methods

Cell culture and transient transfection

Hela cells was purchased from ATCC (Manassas, VA). The four MEF cell lines, wild-type, *Bax*^{-/-}, *Bak*^{-/-} and dko (*Bax*^{-/-} *Bak*^{-/-} double knock-out) were the gift of Dr. Richard Youle (NIH). Hela cells or MEF cells were grown in DMEM media containing 10% fetal bovine serum, penicillin and streptomycin in a humidified chamber under 5% CO₂ at 37°C. Hela and MEF cell lines were transfected with Lipofectamine 2000 (Invitrogen Life Technology, Carlsbad, CA) following the manufacture's instructions.

Expression vectors, antibodies and reagents

pEGFP-N1 was purchased from Clontech/BD Biosciences (Bedford, MA). pPOTE-2 α -actin-EGFP and pPOTE-2 γ C-EGFP were described previously [16]. The apoptosis staining kit (Caspase GLOW Red Multi-Caspase Staining Kit) was purchased from Biovision (Mountview, CA). Phalloidin-TRITC, was from Sigma (St. Louis, MO). Anti-Fas (clone CH11) was purchased from Upstate (Temecula, CA); anti-cleaved caspase-3 was from Cell Signaling (Danvers, MA).

RT-PCR and real-time PCR analysis

RNA was reverse transcribed using the First-Strand cDNA synthesis kit and random hexamer priming according to the manufacture's instructions (Amersham Pharmacia Biosciences, Piscataway, NJ). PCR primers T444 and T445 were used to amplify *POTE* genes as reported [14], except 25 PCR cycles were used instead of 35 cycles. For real-time PCR analysis, RNA was digested with DNAase before cDNA synthesis. Real-time PCR was performed using Quantifast SYBR green master mix (Qiagen, Valencia, CA) with primer T444 and primer Rev T860 (GCATGGCCTCACACCACTGTTAC).

Caspase assay

Cells grown on cover slips were transfected with pPOTE-2 α -actin-EGFP or pPOTE-2 γ C-EGFP or EGFP vector. After 48 or 72 h of transfection, RED-VAD-FMK (1:300 dilutions from stock) was added to the culture dishes, and incubated 1 h at 37°C with 5% CO₂. After washing with PBS, cells were fixed in 2% PFA for 3 min, and mounted on cover slips for visualization by confocal microscopy. Images were obtained using a Zeiss LSM 510 confocal microscope.

RNA interference

Double strand siRNA oligo #1 ACAACGAAAGUGAAGAGUA was synthesized by Dharmacon (Lafayette, CO). Hela cells (2×10^5) were plated on 35 mm dishes, cultured overnight and transfected with 8 μ l of 20 μ M siRNA oligo and 10 μ l of Oligofectamine (Invitrogen). Forty-eight hours post-transfection, RNA was made using Trizol (Invitrogen) and protein lysate was prepared as described below.

Membrane and soluble protein fractionation

Hela cells were washed with PBS, scraped from dishes in PBS, and centrifuged at low speed to collect cells. The cell pellet was resuspended in hypotonic buffer containing 10 mM NaCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl, pH 7.5. The swollen cells were broken in a Dounce homogenizer followed by low speed centrifugation to remove the nuclei and unbroken cells. The low-speed supernatant was then centrifuged at 100,000g for 1 h. The pellets were resuspended in lysis buffer to solubilize the membrane fraction. The pellet was taken up in the same volume as the supernatant and equal amounts were resolved by SDS-PAGE and used for western blot analysis.

Western blots

Hela cells grown in six well dishes were washed once with PBS and then disrupted in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.5% Triton X-100 and 0.5% NP-40, 10 µg/ml leupeptin, 2 µg/ml aprotinin and 20 µg/ml PMSF). Total cellular lysate was subjected to high speed centrifugation and supernatants were used for western blotting after SDS-PAGE and transferred to PVDF membranes. Blots were incubated with 5% blocking agent (Amersham Pharmacia Biosciences) for 30 min in Tris-buffered saline plus 0.05% Tween 20, and then incubated with anti-POTE, PG5 (1 µg/ml), anti-cleaved caspase-3 (1:500) or anti-Fas (1:400) antibodies for 2 h or overnight, followed by secondary antibody for 1 h. After washing 4 times with TBST (Tris-Buffered Saline with 0.1% Tween 20), signals were detected by ECL or ECL plus reagents (Amersham Pharmacia Biosciences).

Results

POTE-2 α -actin is the major paralog in Hela cells

Because there are many *POTE* gene family members and splicing variants [14], we determined which paralogs exist in Hela cells by RT-PCR and sequence analysis using a primer set (T444/T445) that detected all *POTE* paralogs (Fig. 1). A total of 41 cloned RT-PCR products were sequenced. Analysis of these sequences showed 29/41 clones are from *POTE-2 α* , 11/41 clones are from *POTE-2 γ* and 1 clone is from *POTE-2 δ* , indicating that *POTE-2 α* and *POTE-2 γ* are the major *POTE* paralogs expressed in Hela cells. We then used specific primers that span from the 5' UTR to actin in all the *POTE-2* paralogs (Fig. 1, primer set T1 and T07) to identify the spliced forms of *POTE* present in Hela cells. A total of 12 clones were sequenced of which 7 clones were from the *POTE-2 α* locus and contained an in-frame actin insertion. The remaining clones were from *POTE-2 γ* (4 clones) and *POTE-2 δ* (1 clone). The actin insert in these clones is not in-frame with the *POTE* open reading frame. These results indicate that *POTE-2 α -actin* is the major transcript expressed in Hela cells.

To identify which POTE proteins could be detected in Hela cells, we carried out western blots with MAb PG5, previously shown to be able to detect most of the *POTE* paralogs [11]. The anti-POTE antibody detected two major bands, one at 120 kDa, the predicted size of a POTE-actin fusion protein and a smaller band at 35 kd, which does not correspond to any identified *POTE* transcript. To confirm that the 120 kDa protein is POTE-2 α -actin we used siRNA to specifically knock down *POTE-2 α -actin* RNA and protein. As shown in Fig. 1b,

transfection of siRNA oligo1, which should reduce the RNA levels of all the *POTE* paralogs expressed in Hela cells specifically reduced the amount of protein at the molecular weight of 120 kd, indicating that this band is POTE-2 α -actin (Fig. 1b). The 35 kd protein recognized by PG5 antibody did not change, indicating it is not a member of the POTE family. The level of *POTE* mRNA was also remarkably decreased by the siRNA (Fig. 1c). This result indicates that the 120 kd protein detected by PG5 is the endogenous POTE-2 α -actin protein.

POTE-2 α -actin localization in the cell

Because the endogenous POTE protein can not be detected with the PG5 monoclonal antibody by immunofluorescence in Hela cells (and many other cell lines, unpublished observation), we transiently transfected *POTE-2 α -actin-EGFP* into cells to determine the location and possible function of POTE. Only about 10% of the transfected cells express the fusion protein indicating that the transfection efficiency of this large protein is quite low, in contrast to cells transfected with EGFP alone, where over 80% of cells expressed EGFP (data not shown). As shown in the confocal photographs in Fig. 2a, POTE-2 α -actin-EGFP co-localizes with actin fibers close to the plasma membrane, whereas EGFP alone is found throughout the cell. POTE-2 α -actin-EGFP is also detected at the spreading edge of the cell in membrane ruffles where actin is also enriched (Fig. 2a, arrows). We stained transfected cells with antibodies to the focal adhesion proteins vinculin and paxillin, and found that POTE-2 α -actin did not localize with these proteins (data not shown), indicating POTE-2 α -actin is not localized to areas of focal adhesion.

To determine if the localization of POTE-2 α -actin near the plasma membrane is dependent on intact actin fibers, we treated cells with the depolymerization agent Latrunculin A (Lat A) to disrupt actin fibers. Hela cells transfected with *POTE-2 α -actin-EGFP* or *EGFP* plasmids were incubated with Lat A for 30 min in culture before fixation. After treatment with Lat A, the prominent actin filaments disappeared and were replaced by a very faint diffuse fluorescence throughout the cell, indicating most actin filaments were disrupted (Fig. 2b compared with Fig. 2a). Concomitantly POTE-2 α -actin-EGFP became dispersed through the cell. This result indicates that POTE localization near the plasma membrane is dependent on intact actin fibers and raises the possibility that the proteins are associated with each other in some manner.

To confirm that endogenous POTE is in the membrane fraction we subjected the cells lysate to centrifugation at 100,000g as described in “Materials and methods” and western blotting with antibodies to actin and POTE. We detected actin in both the membrane fraction and the soluble fraction, suggesting some of the actin had depolymerized in the fractionation procedure. We also found POTE-2 α -actin in both the membrane fraction and soluble fraction (Fig. 2c). As proof that we had successfully separated the membrane from the soluble fraction. Fas was only detected in the membrane fraction and GAPDH in the soluble fraction. These experiments together with the microscopic localization results, indicate that POTE-2 α -actin is closely associated with actin filaments near the plasma membrane.

We previously found that we could detect *POTE-2 γ CRNA* and protein in two breast cancer cell lines [13, 14]. In the current study we were able to detect *POTE-2 γ CRNA* in Hela cells, but could not detect the protein, presumably because the levels are very low. To determine

the location of POTE-2 γ C protein, we transfected HeLa cells with POTE-2 γ C-EGFP and determined its location by confocal microscopy. As shown in Fig. 2a, POTE-2 γ C protein is distributed within the cell in a pattern similar to POTE-2 α -actin in close association with actin filaments. We also performed an experiment to disrupt actin fibers by Lat A as was done for POTE-2 α -actin, and found that POTE-2 γ C was also dependent on intact actin fibers for localization to the plasma membrane (data not shown).

Over-expression of POTE induces apoptosis

To determine the function of POTE-2 α -actin or POTE-2 γ C we attempted to produce stable cell lines expressing large amounts of POTE but were not successful. We concluded that high expression of *POTE* genes might be toxic to cells and set out to investigate this in more detail. To determine if the POTE-2 α -actin-EGFP transfected cells were dying due to apoptosis, we used a co-localization approach in which we identified transfected cells based on the green fluorescence of EGFP and determined if they were undergoing apoptosis by staining them with a pan-caspase family inhibitor VAD-FMK labeled with rhodamine (Biovision). Using this approach we found that 60% of POTE-2 α -actin expressing cells were undergoing apoptosis 48 h after transfection, and 94% of POTE-2 α -actin expressing cells were undergoing apoptosis at 72 h (Fig. 3a, b). Control cells transfected with EGFP alone were healthy and did not stain red for apoptosis even after 72 h of transfection.

To investigate if over-expression of another POTE paralog known to be expressed in cancer cells could induce apoptosis, we transfected HeLa cells with *POTE-2 γ C* which has a higher transfection efficiency than POTE-2 α -actin and found that 32% of the *POTE-2 γ C-EGFP* transfected cells were undergoing apoptosis 48 h after transfection and 65% after 72 h (Fig. 3a, b). These studies show that expression of high levels of both POTE paralogs (2 α -actin and 2 γ C) in transfected cells results in programmed cell death and prevent the establishment of permanent cell lines.

POTE-2 γ C induced apoptosis is dependent on BAK

BAK and BAX are major regulators of apoptosis [4, 17]. To investigate if POTE induced apoptosis is dependent upon either of these pro-apoptotic proteins, we utilized MEF cells that were either *Bak*^{-/-}, *Bax*^{-/-} or mutant in both genes *Bak*^{-/-}/*Bax*^{-/-} (dko) [1, 5, 18]. We chose to transfect the MEF cells with *POTE-2 γ C* rather than *POTE-2 α -actin* due to the higher transfection efficiency of *POTE-2 γ C*. After 2 or 3 days of transfection, apoptotic cells were detected by staining with VAD-FMK-rhodamine. As shown in Fig. 4a, control MEFs and *Bax*^{-/-} cells expressing POTE-2 γ C-EGFP underwent apoptosis (red), but *Bak*^{-/-} and dko cells were resistant to apoptosis (Fig. 4). We quantified the apoptotic response by VAD-FMK-rhodamine staining, and found that expression of POTE-2 γ C-EGFP caused about 50% of wild-type or *Bax*^{-/-} cells to undergo apoptosis in 2 days and 70% of the cells by 3 days. In contrast, less than 2% of POTE-2 γ C-EGFP transfected *Bak*^{-/-} MEF cells underwent apoptosis (Fig. 4b). None of the cells expressing EGFP by itself became apoptotic. These findings indicate that POTE induced apoptosis is dependent on Bak, but not Bax expression.

Endogenous POTE protein expression is stimulated by FasL/TRAIL

Because POTE protein is closely associated with the plasma membrane, we investigated whether or not activation of the extrinsic apoptosis pathway would affect POTE protein levels. We found that antibodies to Fas (Fig. 5a), and TRAIL (Fig. 5b) both increased POTE-2 α -actin protein levels in a time dependent manner with a peak around 4–7 h and a subsequent fall at 18 h. The activation of caspase-3 was detected after the rise in POTE protein beginning at the 7 h time point. Once caspase-3 was fully activated, POTE-actin levels decreased. We also found that increasing the amount of Fas antibody further increased POTE-actin levels (Fig. 5a) and that Fas ligand also increased POTE-actin protein levels in these cells (data not shown).

To determine if the increase in POTE-actin is due to the induction of mRNA for *POTE*, we performed real-time RT-PCR analysis of RNA from cells that were treated with Fas antibody for 3 and 5 h. As shown in Fig. 5c, *POTE* mRNA is not increased in treated cells indicating that the regulation of POTE-actin is not at the transcriptional level, but rather at the translational or protein stability level.

To determine if POTE-actin expression is required for apoptosis induced by TRAIL, we carried out knock down experiments in HeLa cells. The cells were transfected with siRNA specific for POTE-actin as described in the “Materials and methods” and 24 h later the cells were treated with TRAIL at 400 ng/ml to induce apoptosis. We found that POTE-actin levels were decreased by about 70% but there was no decrease in the levels of cleaved caspase-3. These data suggest that POTE-actin is not essential for caspase-3 activation in TRAIL induced apoptosis.

Discussion

In the current study we have shown that HeLa cells mainly express the *POTE-2 α -actin* isoform of *POTE* and that the levels of POTE-2 α -actin protein are elevated in cells treated with pro-apoptotic factors Fas ligand, antibodies to Fas or TRAIL. We also demonstrate that over-expression of POTE-2 α -actin or POTE-2 γ C induces apoptosis, preventing the establishment of permanent cell lines making large amounts of POTE protein. We investigated possible pathways for POTE involvement in cell death and found that a cell line lacking Bak, or Bak and Bax, but not Bax alone, was resistant to POTE induced apoptosis. Although both Bak and Bax are required for mitochondria fragmentation and the subsequent release of apoptotic factors for activation of the apoptotic cascade [18]. Bak and Bax exist in different locations and bind to different partners [6, 19, 20]. POTE contains ankyrin and spectrin repeats and is located in close association with actin near the plasma membrane, making it a good candidate for a scaffolding protein. In some cell lines, but not in HeLa, preventing cellular attachment to substrate activates a cell death pathway called anoikis. It is possible that POTE as a signaling transducing scaffold protein, has a role in this anoikis. In addition, over-expression of POTE may sequester activators or inhibitor proteins required for interaction with Bak. Imbalance of those factors by the sequestration of POTE could activate Bak and induce subsequent apoptosis.

Although *POTE* mRNA has been detected in many cancers, its expression in normal tissues is restricted to testis, ovary and prostate, with the highest expression being detected in testis. Immunohistochemical studies with anti-POTE antibodies have shown that POTE protein is located within spermatocytes, some of which are undergoing apoptosis. This raises the possibility that POTE has a role in sperm maturation in humans. Fas ligand, which raises POTE levels in HeLa cells, is also expressed at high levels in spermatocytes [21] suggesting that Fas ligand is associated with and may control POTE levels in spermatocytes. In addition, *POTE* RNA levels have been reported to be decreased in teratozoospermia, a condition in which less than 4% of sperm cells are morphologically normal (<http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2697>). We are currently examining POTE and apoptosis protein expression in various types of testicular abnormalities to gain further information about the possible role of POTE in spermatogenesis.

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Fig. 1.

a Schematics showing exons and predicted protein domains of POTE-2 α -actin. Primer binding sites are shown by *arrows*. The position of Si RNA oligos are shown by the red bar.

b Western blot analysis of POTE expression after 48 h of siRNA treatment. *Arrow* shows the expected 120 kD POTE-2 α -actin protein. GAPDH is the loading control.

c RT-PCR analysis of POTE expression after 48 h of siRNA treatment: 30 cycles for POTE and 25 cycles for actin. *CRDs* Cysteine Rich Domain, *ANKs* Ankyrin repeats

Fig. 2.

POTE-2 α -actin and POTE-2 γ C are associated with actin protein in HeLa cells. **a** HeLa cells were transfected with pPOTE-2 α -actin-EGFP, pPOTE-2 γ C-EGFP or pEGFP vector. After 24 h of transfection, cells were fixed with 4% PFA and stained with phalloidin-TRITC (*red*) and DAPI (*blue*). *Yellow* shows merged image of co-localization of endogenous actin and POTE-2 α -actin-EGFP or POTE-2 γ C-EGFP. **b** POTE localization is dependent on intact actin filaments. HeLa cells transfected with POTE-2 α -actin-EGFP were treated Lat A for 30 min before PFA fixation. Cells were then stained with phalloidin-TRITC and DAPI for 5

min and analyzed by confocal microscopy. **c** HeLa cells were lysed with hypotonic buffer as described in “Experimental procedures”. Equivalent volume of soluble and membrane fractions were analyzed by western blot with anti-POTE (PG5), anti-Fas (membrane marker), anti-actin or anti-GAPDH (soluble fraction marker) antibodies

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Fig. 3.

POTE over-expression induces apoptosis in HeLa cells. **a** HeLa cells transfected with pEGFP, pPOTE-2 α -actin-EGFP or pPOTE-2 γ C-EGFP. At 2 or 3 days post-transfection, cells were incubated with RED-VAD-FMK for 1 h before fixation with 2% PFA. Cells were visualized with confocal microscopy. **b** Quantitative analysis of POTE induced apoptosis. Transfected cells were stained with RED-VAD-FMK for 1 h and red-staining cells and total transfected cells were counted under microscopy. Three separate experiments were repeated and each time about 100 transfected cells were counted

Fig. 4.

POTE induced apoptosis is dependent on pro-apoptotic protein Bak. **a** & **b** MEF cells, wild-type (wt), *Bax*^{-/-}, *Bak*^{-/-} and dko *Bax*^{-/-} *Bak*^{-/-} (double knock-out) were transfected with pEGFP or pPOTE-2 γ C-EGFP. At 2 or 3 days post-transfection, cells were incubated with RED-VAD-FMK for 1 h, and then fixed with 2% PFA for 3 min. Red labeled cells and total transfected cells were analyzed by confocal microscopy (**a**) or counted (**b**). Three separate experiments were performed and each time more than 300 cells were analyzed

Fig. 5.

Endogenous POTE-2 α -actin expression is regulated by death receptor activation. **a** & **b** HeLa cells at about 60% confluence were treated with anti-Fas antibody (**a**) or with 30 ng/ml of TRAIL (**b**) at the indicated concentrations and times before western blot analysis. Equal amount of cell lysate was analyzed by western blot. POTE-2 α -actin was detected by PG5; GAPDH as a loading control. **c** HeLa cells were treated with 400 ng/ml anti-Fas antibody at the indicated times. RNA was prepared and real-time PCR was performed. The relative expression was quantified using actin RNA levels as the control