THE C-TERMINAL FRAGMENT OF PRESENILIN 2 TRIGGERS p53-MEDIATED STAurosporine-
INDUCED APOPTOSIS, A FUNCTION INDEPENDENT OF THE PRESENILINASE-DERIVED N-TERMINAL
COUNTERPART.

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Short Title: CTF-PS2 triggers p53-dependent apoptosis
Abstract

Mutations on presenilins are responsible for most of familial forms of Alzheimers disease. These holoproteins undergo rapid maturation by presenilinase in the early compartment of the secretory pathway, leading to the production of N- and C-terminal fragments. We show first that overexpression of the presenilinase-derived maturation product of presenilin 2 (CTF-PS2) increases Aβ recovery, the production of which is almost abolished by a caspase 3 inhibitor and increased by staurosporine. This and the observation that the apoptotic inducer staurosporine enhances CTF-PS2 degradation clearly link CTF-PS2 to apoptotic cascade effectors. This prompted us to analyze the putative ability of CTF-PS2 to modulate cell death. CTF-PS2 overexpression decreases cell viability and augments both caspase 3 activity and immunoreactivity. This is accompanied by lowered bcl2-like immunoreactivity and increased poly(ADP)-ribose polymerase cleavage and cytochrome c translocation into the cytosol. Interestingly, CTF-PS2-induced caspase 3 activation is prevented by pifithrin-α, a selective blocker of p53 transcriptional activity. On line with the latter data, CTF-PS2 drastically increases p53 immunoreactivity and transcriptional activity. Of most interest is our observation that CTF-PS2 expression also triggers increased caspase 3 activity and immunoreactivity in fibroblasts in which presenilins had been deleted. Therefore, CTF-PS2 could modulate cell death out of the NTF/CTF heterodimeric complex thought to correspond to the biologically functional entity. This is the first direct demonstration that CTF-PS2 could exhibit some of its functions in absence of the presenilin2 N-terminal fragment (NTF-PS2) counterpart derived from the presenilinase cleavage.
Introduction

Presenilins 1 and 2 (PS1 and PS2) have been the center of a huge amount of studies because most of the familial forms of Alzheimer’s disease (AD) are due to inherited mutations on genes coding for these two parent proteins (For reviews (1-4). PS1 and PS2 are highly homologous transmembrane proteins mainly located in the early compartments of the secretory pathway where they undergo various post-transcriptional modifications (5). Particularly interesting is the susceptibility of PS to an endoproteolytic cleavage giving rise to N-terminal (NTF) and C-terminal (CTF) fragments (6-9) by a yet unknown protease referred to as presenilinase. This proteolytic event appears of major importance because intact PS holoproteins are poorly detectable in the brain of affected patients. The two proteolytic counterparts appear to accumulate with a 1/1 stochiometry (7) and this complex is thought to exhibit the PS-mediated phenotypes.

Among other biological functions, PSs have been shown to control the production of the amyloid β-peptides and to modulate cell death in response to various apoptotic stimuli (10,11). Interestingly, these two functions are altered by AD-related pathogenic mutations. Thus, all mutations increase the production of amyloid β-peptides (12), and more specifically the 42-aminoacid long Aβ that appears to be more implicated in the pathology than its shorter counterpart of 40 residues. More recently, we showed that wild type PS2 and more potently, mutated PS2, trigger caspase 3 activation by a p53 dependent mechanism (13). This was accompanied by a decrease in PS1 expression, in agreement with a previous report showing that p53 depletion increases PS1 expression and delayed apoptotic cell death (14).
Interestingly, the presenilinase-derived CTF fragment undergoes phosphorylation events (15,16) that appear to affect its susceptibility to caspase-mediated proteolysis (17-19). This could suggest that at least part of the PS-associated modulation of cell death could be related to the production of the CTF or its caspase-derived CTF\textsubscript{cas}. Therefore, two main questions at least remained to be addressed. First, what are the mechanisms by which the CTF fragment could modulate the apoptotic cell death? Second, is CTF acting through the interaction of its N-terminal counterpart or is it able to trigger a phenotype in absence of NTF?

Here we establish that the overexpression of CTF-PS2 augments A\textsubscript{β}, the production of which is prevented by caspase 3 inhibitors. Furthermore, CTF-PS2 increases caspase 3 expression and activity and elevates both expression and transcriptional activity of p53. Of most interest, we show that this CTF-PS2-related phenotype was still observed in PS-deficient cells, indicating that CTF-PS2 could be biologically active \textit{per se}, in absence of any heterodimeric interaction with its N-terminal counterpart.
Materials and Methods

Cells systems.

HEK293 cells stably expressing the C-terminal fragment of PS2 (CTF-PS2) were obtained and cultured as previously described (20). Mice in which the genes of PS1 and PS2 were deleted (kindly provided by Drs. Bart De Strooper (Leuven, Belgium) and Paul Saftig (Kiel, Germany)) have been previously described (21). Mice fibroblasts were obtained and immortalized with SV40 large T antigen according to the procedure previously reported (22) and were cultured as previously reported (23). Transient transfection of fibroblasts with the cDNA encoding either for CTF-PS2 or empty vector was performed with DAC30 according to manufacturer’s recommendations (Eurogentec, France). CTF-PS2 expression in stably and transiently transfected cells was checked by western blot by means of the Ab333 antibodies (rabbit polyclonal anti-C-terminal fragment of PS2, (provided by W. Araki and T. Tabira) as previously described (24).

Flow cytometry analysis of propidium iodide (PI) incorporation

HEK 293 cells were grown in 6-well plates and incubated for 24 hours at 37°C in the presence or absence of 2µM of staurosporine. Cells were harvested, pelleted by centrifugation at 1000 x g for 10 min at 4°C, gently resuspended in 500µl of 0.1% sodium citrate buffer containing 50 µg/ml propidium iodide (PI) and incubated overnight under agitation. The PI fluorescence of individual nuclei was measured using a FACS scan flow cytometer (program CellQuest, Becton Dickinson) as described (13).
Tunel analysis

Cells were cultured in 6-well plates and then treated or not with 2 µM of staurosporine for 24 hours. After treatment cells were fixed for 30 minutes with 4% paraformaldehyde, rinsed in PBS, permeabilized overnight with 70% ethanol and then processed for the dUTP nick-end labelling TUNEL technique according to the manufacturer’s recommendations (Boehringer Kit). Staining was assessed with peroxidase-conjugated antibody and revealed with a diaminobenzidine substrate as described (25). Fragmented DNA labeling corresponds to black spots. A second labeling with erythrosine B was carried to visualize the totality of the cells.

Caspase 3 activity

HEK 293 cells were cultured in 6-well plates and then incubated for 24 hours at 37°C in the presence or absence of 2.0 µM of staurosporine (Sigma). In some cases cells were either pre-incubated overnight with 100 µM Ac-DEVD-al (caspase 3 inhibitor, Neosystem) before treatment with staurosporine or submitted to a co-incubation of 2 µM staurosporine and 10 µM of the p53 inhibitor pifithrin-α. Cells were then analyzed as extensively described (26). Fluorimetry was recorded at 360 and 460nm for excitation and emission wavelengths, respectively by means of a microtiter plate reader (Labsystems Floroskan II). Caspase-specific activity was calculated from the linear part of fluorimetry recording and expressed in units/h/mg of proteins (established by the Biorad procedure). One unit corresponds to 4 nmol of AMC released.

Western blot analyses
Plated cells were rinsed, gently scraped, pelleted by centrifugation, and then resuspended in 100µl of lysis buffer (10 mM Tris-HCl pH 7.5 containing 2% SDS and a cocktail of protease inhibitors, Roche). Equal amounts of protein (50µg) were separated on 8 and 12% SDS-PAGE gels for analysis of poly (ADP-ribose) polymerase (PARP) and active caspase 3, Bcl2, caspase 3 precursor (ProCPP32) and p53, respectively and wet transferred to Hybond C (Amersham) membranes. Membranes were then blocked with non-fat milk and incubated overnight at 4°C with the following primary antibodies: anti-PARP (Ustate Biotechnology), anti-active caspase 3 (rabbit polyclonal, R&D Systems), anti-Bcl2 (mouse monoclonal, Santa Cruz), anti-ProCCP32 (mouse monoclonal, Transduction Laboratories) and an anti-p53 (mouse monoclonal, Santa Cruz). Immunological complexes were revealed by enhanced electrochemiluminescence (Roche) with either an anti-rabbit peroxidase or with an anti-mouse peroxidase (Immunotech) antibodies depending on the host used for the production of the primary antibodies listed above.

**Cytochrome C translocation analysis**

HEK 293 cells were grown in 6-well plates and incubated for 24 hours at 37°C in the presence or absence of 2.0 µM of staurosporine. Cells were submitted to a cellular fractionating and the cytosolic /mitochondrial fractions, electrophoresed on Tris-Tricine gels and western blotted as previously described (13). In brief, 25 µg of proteins were separated on 16.5 Tris-Tricine gels, immobilized in nitrocellulose sheets and probed with an anti-cytochrome C (rabbit polyclonal, Santa Cruz Technologies) antibody. Immunological complexes were revealed with an anti-rabbit peroxidase (Immunotech) and electrochemoluminescence detection (Roche).
**p53 transcriptional activity**

The PG13-luciferase p53 gene reporter construct (kindly provided by Dr. B. Vogelstein) have been previously described (27). Cells were cultivated in 12-cell plaques till 70% confluence and then co-transfected with 1.0 µg of PG13-luciferase cDNA and 0.5 µg of a β-galactosidase transfection vector (to normalize transfection efficiency) by means of the DAC30 transfection reagent according the manufacturer conditions (Eurogentec). Forty eight hours after transfection, luciferase and β-galactosidase activities were analyzed according to manufacturers conditions (Kit Promega).

**Immunoprecipitation and detection of Aβ**

HEK293 cells stably expressing the CTF-PS2 (CPS2) were cultivated in 6-well plaques, pre-incubated with either the inhibitor of caspase 3, Ac-DEVD-al (Neosystem), for 16 hours and then submitted to a period of 7 hours of secretion in the cultivating media containing 1% instead of 10% FCS and 10µM phosphoramidon (Sigma). Media was then recovered and analyzed for total Aβ by combined immunoprecipitation FCA18 (28) and western blot with WO2 as previously described (29).
Results

The setting up and characterization of stably HEK293 cells over-expressing the C-terminal fragment (CTF) of PS2 was previously reported (24). We previously showed that overexpression of the CTF-PS2 in these cells (compare lanes without Ac-DEVD-al in Fig.1C) led to an increased recovery of total amyloid β-peptides (24). Here we confirm that CTF-PS2-expressing HEK293 cells secrete more Aβ than mock-transfected control cells in basal conditions (Fig.1A,B). Interestingly, the caspase 3 inhibitor Ac-DEVD-al drastically reduces Aβ recovery in both cell systems (Fig.1A,E) and concomitantly increases endogenous (Mock) and overexpressed (CPS2) CTF-PS2-like immunoreactivity (Fig.1C,F). Conversely, CTF-PS2 immunoreactivity was drastically lowered by the treatment of transfected cells with the apoptotic stimulus staurosporine (Fig.1D), in an Ac-DEVD-al-sensitive manner (not shown). This was accompanied by a staurosporine-stimulated increase of Aβ recovery in both mock- and CTF-PS2- transfected cells (Fig.1B). This modulation of the CTF-PS2-mediated control of βAPP maturation by apoptotic effector and inhibitor led us to deeply examine the putative role of CTF-PS2 in the control of cell death.

First, we examined the capacity of CTF-PS2 to alter cell viability by means of Tunel and DNA fragmentation analyses. Tunel labeling of apoptotic cells shows that over-expression of CTF-PS2 drastically increases the number of stained nuclei in both basal and apoptotic conditions when compared to Mock-transfected cells (table 1). FACS analysis of propidium iodide nuclei incorporation also showed increased DNA fragmentation in basal and staurosporine-stimulated cells (Table 1). These two CTF-PS2-mediated phenotypes clearly illustrate a pro-apoptotic phenotype
underlying associated decrease in cell viability.

Another signature of such an apoptotic process is the drastic increase of the Ac-DEVD-al sensitive caspase 3-like activity triggered by CTF-PS2 in both basal and staurosporine-induced conditions (Fig.2A). In agreement, Fig. 2B shows a drastic reduction of the immunoreactivity of the pro-caspase 3 precursor in cells over-expressing CTF-PS2, reflecting an increased maturation of the inactive precursor into its bioactive counterpart in CTF-PS2 transfected cells. We further examined caspase 3 modulation through the monitoring of the cleavage of one of its favored substrate, the poly-(ADP-ribose polymerase (PARP). It is well documented that PARP occurs as an inactive precursor that is proteolytically converted into its active counterpart by caspase 3. Thus the ratio between PARP precursor over its product is generally an index of caspase 3 activity. In Mock transfected cells, as expected the apoptotic stimulus staurosporine increases PARP product (Fig.3A) and therefore, reduces the above-described ratio (Fig.3B). Hereagain cells expressing CTF-PS2 are associated with increased production of PARP product in both basal and staurosporine-stimulated conditions (Fig.3A), resulting in a drastically lower ratio when compared to Mock-transfected cells that indirectly again reflects increased caspase 3 activity (Fig.3B).

In order to delineate the mechanisms by which CTF-PS2 could exert its caspase 3-mediated pro-apoptotic phenotype, we examined the putative contribution of the mitochondrial pathway. Staurosporine increases the translocation of cytochrome C from the mitochondrial fraction to the cytosol in Mock transfected cells (Fig.4A,B). Over-expression of CTF-PS2 triggers a significant increase of the translocation of cytochrome C (Fig.4A,B) associated with reduced immunoreactivity
of the antiapoptotic oncogene Bcl2 (Fig.5A,B) while Bax-like immunoreactivity remained unaffected (not shown). This likely contributed to the perturbation of the mitochondrial membrane permeability resulting in cytochrome C translocation. It should be noted that in agreement with these data, CTF-PS2-expressing cells show a 250% and 175% increase in PI incorporation in basal and stimulated conditions, respectively after cell treatment with etoposide, an apoptotic effector targeting the mitochondrial compartment (not shown).

We examined whether CTF-PS2 expression could elicit its pro-apoptotic phenotype by a p53-dependent mechanism. As shown in Fig.6A, stably transfected CPS2 cells show an increased level of p53 immunoreactivity that is virtually doubled in transfected cells (Fig.6B). This increased immunoreactivity is accompanied by a drastic potentiation of p53 transcriptional activity (Fig.6C).

We took advantage of the recent description of pifithrin-α (PFT-α), a selective blocker of p53 transcriptional activity to show a direct relationship between CTF-PS2-mediated p53 and caspase 3 activations. Fig.7 shows little if any effect of PTFα in Mock transfected cells examined in basal conditions while about 50% of the caspase 3 activity was inhibited in CTF-PS2-expressing cells (compare black and white bars in Fig.7), indicating that the higher caspase3 activity observed in basal conditions in the latter cell system was related to p53 activity. An identical blockade of caspase 3 activation was observed in staurosporine-stimulated conditions (Fig.7) indicating that both basal and staurosporine induced caspase activation triggered by CTF-PS2 expression was blockable by PTFα. This demonstrates the direct modulation of caspase 3 activity by p53 as the main mechanism underlying CTF-mediated proapoptotic phenotype.
Finally, to examine whether the CTF-PS2 was able to elicit its pro-apoptotic function in absence of its presenilinase-derived N-terminal counterpart, we transiently transfected fibroblasts in which the genes coding for PS1 and PS2 were invalidated with CTF-PS2 cDNA. Analyses of caspase 3-like activity in basal and STS-induced apoptosis clearly shows that the CTF-PS2 remains proapoptotic in PS^-/- fibroblasts (Fig.8C). It is noteworthy that the effect of CTF-PS2 was significant only in stimulated conditions (8C). This could be explained by the relatively low transfection efficiency observed (Fig.8A). Accordingly, the immunoreactivity of active caspase 3 (Fig.8B) was enhanced in staurosporine-treated CTF-PS2-transfected PS^-/- fibroblasts.

Additionally, these results show that CTF-PS2 triggers the same phenotype in cells types of different origin, namely, HEK 293 human cells (Fig.2-7), Mouse Embryonic fibroblasts (Fig.8), and telencephalon specific murine (TSM1) neurons (data not shown) indicating that its pro-apoptotic function is not cell specific.

Discussion

PS are multipotent proteins likely involved in distinct functions including among others, control of βAPP processing, Notch and Wnt pathways, and adhesion (5). PS1 and PS2 also appear involved in the modulation of cell death although the two parent proteins seem to behave differently in this paradigm. Thus, PS2 was clearly characterized by several groups as a proapoptotic effector (13, 30-33) while PS1 decreases the susceptibility of neurons to apoptotic stimuli (34). It is interesting to note that while the two proteins elicit opposite phenotypes, they both appear to be
linked to the tumor suppressor p53 activity. Thus, it was established that p53 down-regulated PS1 expression thereby leading to delayed apoptotic response (14). By contrast, we recently showed that PS2 and its pathogenic mutant N141I-PS2 drastically increased p53 expression and transcriptional activity in various cell systems (13). Interestingly, this was accompanied by a reduction of PS1 expression, suggesting a cross-talk between the two congener proteins (13).

PS are rapidly processed in the endoplasmic reticulum, leading to N-terminal (NTF-PS) and C-terminal (CTF-PS) fragments that appear to associate with a 1/1 stochiometry (7). This heterodimer is thought to correspond to the biologically active complex responsible for PS-associated functions. Whether the maturated fragments could per se modulate PS-related functions remained a matter of speculation. However, a pivotal work by Vito and colleagues indicated that ALG3, the C-terminal 103 amino-acids of mouse PS2 was able to rescue a T cell hybridoma from Fas-induced apoptosis (35). Furthermore, the same group showed that a C-terminal fragment of PS2 that can be physiologically generated by proteolysis and alternative transcription was able to rescue HeLa cells from Fas- and TNF-induced apoptosis (36). These studies could be envisioned as clues of functions specifically associated with PS-derived maturated fragments although it could be argued that overexpression of these products displace their endogenous counterparts from the biological heteromeric complex. This possibility would not explain how the sole expression of one fragment could trigger a phenotype if this just results in a substitution limited stochiometrically by the other endogenous counterpart.

We previously showed that CTF-PS1 and CTF-PS2 overexpressions led to an up regulation of βAPP processing, yielding increased amounts of both α- and β/γ-
derived products APPα and Aβ, respectively (24). We confirm that Aβ production is increased upon overexpression of CTF-PS2 in the present study. This phenotype appears blocked by caspase 3-like inhibitors and stimulated by staurosporine with accompanying increase or lowering of CTF-PS2-like immunoreactivity, respectively. This suggests that increased Aβ production is related to the caspase-derived cleavage product of CTF-PS2. The fact that CTF-PS2 undergoes breakdown upon staurosporine stimulus also argues for the possibility that CTF-PS2 behaves as the precursor of a caspase-derived fragment that could control cell death. This would well agree with the study carried out by Walter et al. that showed that phosphorylation of PS2 slowed down caspase-mediated cleavage and retarded apoptosis in HeLa cells (32).

The mechanisms by which CTF-PS2 could trigger cell death remained to be established. Here we show for the first time that CTF-PS2 overexpression led to increased caspase 3 activity and immunoreactivity, enhanced cleavage of one of the favorite caspase 3 substrate, polyADP-ribose polymerase, and decreased expression of the antiapoptotic oncogene bcl-2. Whether the physical interaction between CTF-PS2 with bcl-2 described by Passer and colleagues (37) could explain our observed increased translocation of cytochrome c in the cytosol remains to be established. We established that the CTF-PS2-associated cell death was a p53-dependent phenomenon. Thus, CTF-PS2 increases both p53 expression and transcriptional activity (see Fig. 7). That CTF-PS2 associated p53 modulation and caspase activation were directly linked was evidenced by the fact that pifithrin-α, a selective blocker of p53 fully prevents the CTF-PS2-induced caspase activation (see fig. 8). The mechanisms by which CTF-PS2 triggers cell death appears similar to the one we
described for PS2 itself. Therefore, the possibility that CTF-PS2-associated phenotype could account entirely for that observed with PS2 remains a possibility.

Interestingly, we demonstrate that CTF-PS2 also triggers caspase 3 activation in cells devoid of presenilins. Thus, PS-/- cells respond to transient transfection with CTF-PS2 by an augmentation of both caspase 3 immunoreactivity and activity. This means that unlike it appears to be the case for APP processing, the presence of the NTF counterpart is not an absolute requirement for CTF-PS2 proapoptotic phenotype. It is interesting to note that CTF-PS2-like immunoreactivity could be detected in the liver of adult mice in which PS genes had been disrupted (35,38). This observation, combined with the present work, could suggest possible functions specifically associated with CTF-PS2 in absence of its presenilinase-derived N-terminal counterpart.

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Table 1: CTF-PS2 triggers DNA fragmentation and increases the number of tunel-positive cells. Apoptosis and DNA fragmentation were monitored by Tunel and FACS analyses as described in the methods in Mock- or CTF-PS2-

| TUNEL labeling (% of control) | MOCK | CTF-PS2 | t-test, p |
|-------------------------------|------|---------|-----------|
| Basal                         | 3.3 +/- 0.5 | 27 +/- 4 | p<0.005   |
| Staurosporine                 | 5.2 +/- 0.2 | 40 +/- 3 | p<0.00005 |

| FACS analysis (% of control) | MOCK | CTF-PS2 | t-test, p |
|-------------------------------|------|---------|-----------|
| Basal                         | 3 +/- 0.7  | 10 +/- 1.7 | p<0.05    |
| Staurosporine                 | 13 +/- 4.9 | 21 +/- 5.2 | p<0.01    |
expressing cells, in absence (basal) or in the presence of staurosporine. Means +/- S.E.M correspond to 3 independent experiments carried out in duplicates.

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Legends

Fig.1. CTF-PS2 increases Aβ recovery: effect of caspase 3 inhibitor (Ac-DEVD-al) and staurosporine. Indicated cells were pre-treated overnight with (+) or without (-) 100 µM Ac-DEVD-al (A) then total secreted Aβ was analysed by immunoprecipitation and western blot as depicted in Methods and quantitated by densitometry (E). CTF-PS2-like immunoreactivity in both cell lysates was analysed (C) and quantitated in CPS2 cells (F) by densitometry. Bars in E and F are the means +/-SEM of 5 independent experiments (n=5). In B, the indicated cells were
incubated for the indicated time periods with 2µM of staurosporine (STS) and analyzed for their Aβ production. CTF-PS2-like immunoreactivity in D corresponds to a 24 h treatment with 2µM staurosporine.

**Fig.2. CTF-PS2 increases caspase 3-like activity.** (A) Basal (black bars) and STS-stimulated (2 µM STS, 24 h, white bars) caspase 3-like activity was fluorimetrically recorded in the indicated cell lines as described in the Methods. Bars represent the Ac-DEVD-al sensitive caspase 3-like activity and are the means +/- SEM of 8 determinations carried out in duplicates. (B) Determination of Pro-CPP32-like immunoreactivity (insert) and quantitative densitometric analysis in basal conditions in the indicated cell lines. The histograms represent the quantitative analysis of 5 independent determinations expressed in % (means +/- SEM) of control mock-transfected cells taken as 100.

**Fig. 3. CTF-PS2 over-expression increases PARP cleavage in HEK 293 cells.** (A) A representative gel of the PARP precursor (Prec) cleavage into its product (Prod) performed in basal (CT) and stimulated (STS) conditions (2 µM STS, 24 h) in Mock- or CTF-PS2-expressing cells. The histograms in B represent the quantitative analysis of 5 independent determinations expressed as the ratio precursor/product densitometric analysis reflecting PARP inactivation.

**Fig.4. CTF-PS2 increases cytochrome C translocation to the cytosol.** (A) The indicated cell lines were treated (STS) or not (C) with staurosporine (2µM, 24 h) and analysed for their cytochorome C content in cytosolic (c) and mitochondrial (m)
fractions obtained as detailed in Methods. Bars in B correspond to the means +/- SEM of 5 independent experiments and represent the ratios of cytochrome C immunoreactivities in cytosol versus mitochondrial fractions.

**Fig.5. CTF-PS2 overexpression decreases Bcl2-like immunoreactivity.** (A) Determination of Bcl2 expression in the indicated cell lines as detailed in Methods and their quantitative densitometric analysis in (B). Bars represent of 5 independent determinations (means +/- SEM) expressed in percent of control Mock-transfected cells taken as 100.

**Fig.6. CTF-PS2 increases p53 expression and transcriptional activity.** (A) Determination of p53 immunoreactivity in basal (CT) and staurosporine (STS) stimulated conditions (2 μM STS, 24 h) in the indicated cell lines. Bars in B represent the densitometric analysis of p53-like immunoreactivity and are means +/- SEM of 3 independent determinations. (C) Determination of p53 transcriptional activity (see Methods) measured with a p53 gene reporter (PG13-luciferase). Bars represent the means +/- SEM of 3 independent experiments made in duplicate.

**Fig.7. Effect of pifithrin-α on CTF-PS2-induced activation of caspase 3.** The indicated cell lines were treated for 24 h in the presence or absence of 10 μM pifithrin-α in basal and staurosporine stimulated conditions (2 μM, STS). Bars represent the means +/- SEM of 3 independent fluorimetric analysis (duplicates) of caspase 3 activity expressed in percent of control (taken as 100) corresponding to untreated cells.
Fig.8. CTF-PS2 increases caspase 3 activity in PS1−/−PS2−/− mouse embryonic fibroblasts. (A) CTF-PS2-like immunoreactivity after its cDNA transient transfection. B) Two independent determinations of active caspase 3 immunoreactivity by western blot analysis in basal (Ct) and staurosporine (STS)-stimulated conditions (1 µM, 2h) in PS−/− deficient cells transiently transfected either with empty vector (pcDNA3) or CTF-PS2 cDNA as detailed in Methods. Bars in C correspond to caspase 3 fluorimetric activity and are the means of 3 independent experiments performed in duplicate in basal (CT) and stimulated (STS) conditions (STS, 1 µM, 2h). Data were normalized for CTF-PS2 transfection efficiency assessed by western blot as described in the Methods.
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