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Caspase-mediated Cleavage of p130cas in Etoposide-induced Apoptotic Rat-1 Cells

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Apoptosis causes characteristic morphological changes in cells, including membrane blebbing, cell detachment from the extracellular matrix, and loss of cell–cell contacts. We investigated the changes in focal adhesion proteins during etoposide-induced apoptosis in Rat-1 cells and found that during apoptosis, p130cas (Crk-associated substrate [Cas]) is cleaved by caspase-3. Sequence analysis showed that Cas contains 10 DXXD consensus sites preferred by caspase-3. We identified two of these sites ($DVPD^{416}G$ and $DSPD^{748}G$) in vitro, and point mutations substituting the Asp of $DVPD^{416}G$ and $DSPD^{748}G$ with Glu blocked caspase-3-mediated cleavage. Cleavage at $DVPD^{416}G$ generated a 74-kDa fragment, which was in turn cleaved at $DSPD^{748}G$, yielding 47- and 31-kDa fragments. Immunofluorescence microscopy revealed well-developed focal adhesion sites in control cells that dramatically declined in number in etoposide-treated cells. Cas cleavage correlated temporally with the onset of apoptosis and coincided with the loss of p125FAK (focal adhesion kinase [FAK]) from focal adhesion sites and the attenuation of Cas–paxillin interactions. Considering that Cas associates with FAK, paxillin, and other molecules involved in the integrin signaling pathway, these results suggest that caspase-mediated cleavage of Cas contributes to the disassembly of focal adhesion complexes and interrupts survival signals from the extracellular matrix.

INTRODUCTION

Apoptotic cell death is a fundamental biological process crucial for proper organism development and for maintenance of tissue homeostasis (Raff, 1992). Apoptosis causes characteristic morphological changes that include membrane blebbing, decreased adhesion and intercellular contacts, chromatin condensation, nuclear fragmentation, and the packing of the nuclear fragments into membrane-enclosed apoptotic bodies (Wyllie *et al.*, 1980; Darzynkiewicz *et al.*, 1994). Caspases are a family of highly conserved aspartate-specific cysteine-proteases related to mammalian interleukin-1β-converting enzyme, which are involved in the final execution phase of apoptosis (Nicholson, 1996; Nicholson and Thornberry, 1997). The substrates for caspases are now known to include a growing number of molecules involved in cytoskeletal regulation and signaling, including α-fodrin, gelsolin, growth arrest–specific gene (Gas 2), MEKK-1, and PKC (Brancolini *et al.*, 1995; Emoto *et al.*, 1995; Martin *et al.*, 1995; Ghayur *et al.*, 1996; Cardone *et al.*, 1997; Kothakota *et al.*, 1997; Janicke *et al.*, 1998). Recent studies have shown that attachment to the extracellular matrix (ECM) actively regulates cell survival, and that cells undergoing apoptosis lose their connections to neighboring cells and to the ECM. For example, epithelial and endothelial cells undergo apoptosis ("anoikis") when displaced from the ECM (Frisch and Francis, 1994; Re *et al.*, 1994), whereas cell adhesion to ECM suppresses apoptosis in mammary epithelial cells (Frisch and Francis, 1994; Boudreau *et al.*, 1995; Aharoni *et al.*, 1996).

Cell attachment to the ECM is mediated by the association of integrins with specific ECM components. The binding of integrin receptors to the ECM results in the clustering of integrins and the recruitment of a wide variety of proteins, leading to the formation of focal adhesions, polyprotein structures linking the ECM to the actin cytoskeleton and perhaps providing a structural basis for cell viability.

Focal adhesion complexes are made up of such components as focal adhesion kinase (FAK), Crk-associated substrate (Cas), paxillin, pp60src, vinculin, tensin, talin, and α-actinin. Among these, FAK, Cas, and paxillin are closely associated with changes in the cytoskeleton; thus reorganization of the actin cytoskeleton is accompanied by striking changes in the level of tyrosine phosphorylation of these

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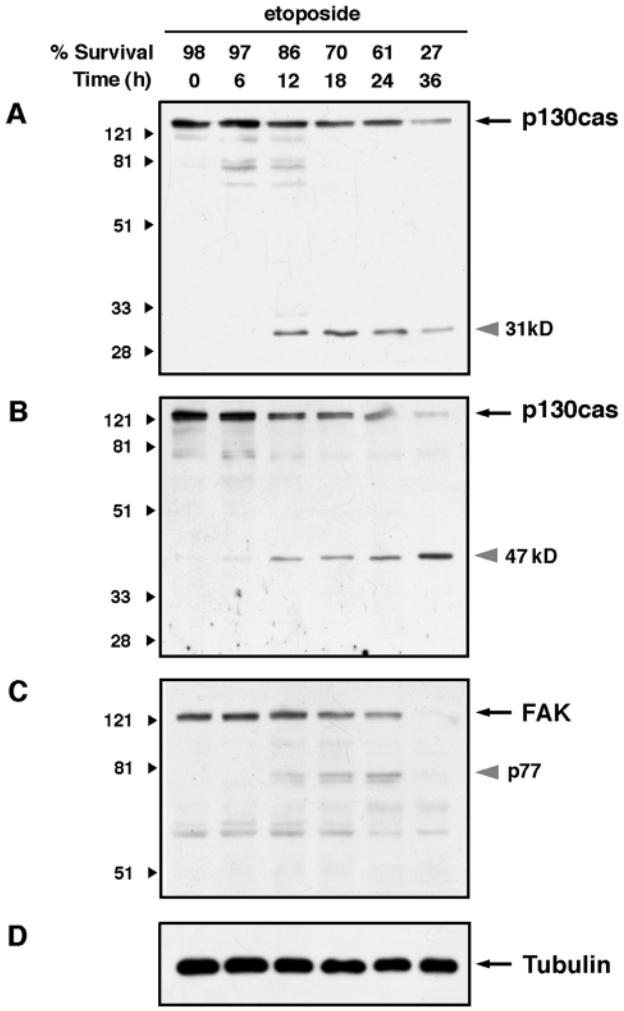

Figure 1. Proteolytic cleavage of Cas during etoposide-induced apoptosis in Rat-1 cells. Rat-1 cells were exposed to 40 μM etoposide for the indicated periods. Cell lysates were then subjected to immunoblot analysis using Cas mAb (A) and Cas-2 (B). Intact Cas migrates at a molecular mass of 130-kDa. Cleavage products of Cas are shown (large arrowheads on the right; 31 and 47 kDa). The blot was then reprobed with FAK mAb (C), after which, to verify equal loading of protein, the blot was stripped once again and reprobed with tubulin mAb (D). Molecular mass standards are indicated by the small arrowheads on the left.

proteins (Rozengurt, 1995; Rozengurt and Rodriguez-Fernandez, 1997; Tanaka *et al.*, 1997; Zhu *et al.*, 1998). For instance, tyrosine dephosphorylation of focal adhesion proteins is correlated with decreases in the length and number of actin stress fibers (Rankin and Rozengurt, 1994; Casamassima and Rozengurt, 1997).

In particular, several lines of evidence suggest that Cas is closely associated with changes in the cytoskeleton: Cas is colocalized with F-actin in peripheral regions of osteoclast-like cells where its phosphorylation is involved in actin ring formation (Nakamura *et al.*, 1998); actin stress fiber formation is severely impaired in Cas-deficient fibroblasts; and Cas-deficient embryos have poorly developed hearts in which myofibrils are disorganized and Z-disks are disrupted (Honda *et al.*, 1998). In addition, the transition from flat to round cell morphology, which is a characteristic feature in cells undergoing apoptosis, is accompanied by cytoskeletal rearrangement and changes in focal adhesion proteins. Indeed, proteolytic cleavage of FAK by caspase during apoptosis further suggests the important role played by the association of focal adhesion proteins, the cytoskeleton, and the ECM in the maintenance of cell morphology and viability.

In addition to its structural functions, Cas is activated and phosphorylated in response to a number of mitogens and growth factors and influences a variety of cellular functions, including growth, migration, and differentiation. Cas contains a Src homology 3 (SH3) domain, proline-rich regions, and a cluster of 15 putative SH2-binding motifs (Sakai *et al.*, 1994), characteristics that identify Cas as an adaptor molecule capable of transmitting cellular signals in association with other intracellular proteins containing SH2 and SH3 domains. Such proteins include Src, Crk, FAK, paxillin, and tensin (Sakai *et al.*, 1994; Turner and Miller, 1994; Miyamoto *et al.*, 1995; Schaller and Parsons, 1995; Harte *et al.*, 1996; Nakamoto *et al.*, 1996; Salgia *et al.*, 1996). Thus, Cas may play essential roles in the integrin-mediated signaling pathway.

In this study, we examined changes in focal adhesion proteins during apoptosis and observed that Cas is cleaved by caspase-3 in apoptotic cells. The degradation of Cas likely results in its loss from focal adhesions, disrupting cell adhesion to the ECM and/or integrin-mediated cell survival signaling.

MATERIALS AND METHODS

Materials

Culture media and supplements were purchased from Life Technologies (Grand Island, NY). Etoposide was from Sigma (St. Louis, MO), and the ECL reagent was from Amersham (Buckinghamshire, England). Monoclonal antibodies (mAbs) against p130Cas (Cas mAb), FAK, and paxillin were from Transduction Laboratories (Lexington, KY). Anti-α-tubulin mAb, anti-Talin mAb, and anti-FLAG M2 mAb were from Sigma. Polyclonal paxillin antibody was kindly provided by Dr. Man Sik Kang (Seoul National University. Seoul, Korea). Anti-Cas polyclonal antibody (Cas-2 Ab; Sakai *et al.*, 1994) and rat Cas cDNA were generously provided by Dr. Hismaru Hirai (University of Tokyo, Tokyo, Japan), and Cas mAb (4F4) was provided by Dr. J.T. Parsons (University of Virginia, Charlottesville, VA). HRP-labeled anti-mouse immunoglobulin (Ig) and FITC- and TRITC-conjugated goat anti-mouse and anti-rabbit IgG or IgM were from Jackson ImmunoResearch (West Grove, PA). The Quik Change site-directed mutagenesis system was from Stratagene (La Jolla, CA). The TNT- coupled reticulocyte lysate system was from Promega (Madison, WI). The tetrapeptide caspase inhibitors ZVAD-fluoromethylketone (fmk) and DEVD-chloromethylketone (cmk) were from Bachem (Torrance, CA).

Cell Culture and Cell Viability Assays

Rat-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To induce apoptosis, cells were exposed to 40 μM etoposide for selected periods. Apoptotic cells, which appeared as "floaters" in the culture medium, were harvested by centrifugation at 1000 $\times$ *g* for 5 min. Adherent, viable cells remaining on the culture dish and control cells (cultured in normal growth medium) were scraped from the dishes, collected by centrifugation, and subsequently lysed. When used, ZVAD-fmk

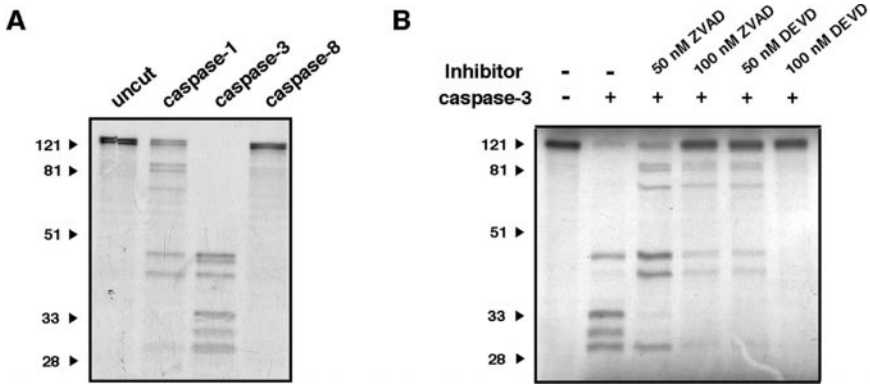

Figure 2. In vitro cleavage of Cas by recombinant caspases. (A) ^{35}S-Labeled rat Cas was generated by in vitro translation and incubated with bacterial cell lysates (20 μg) containing overexpressed recombinant caspases (caspase-1, -3, and -8). Cas was cleaved by caspase-3 and partially cleaved by caspase-1 but was unaffected by caspase-8. (B) Incubation of the in vitro–translated products with either DEVD-cmk or ZVAD-fmk, two membrane-permeant caspase antagonists, inhibited Cas cleavage in a concentration-dependent manner. Molecular mass standards are shown on the left.

or DEVE-cmk was added to the cells for 3 h before the induction of apoptosis. Cell viability was assessed by trypan blue exclusion.

Electrophoresis and Immunoblot Analysis

For immunoblot analysis, cells treated as described above were lysed in a lysis buffer (1% SDS, 1 mM sodium ortho-vanadate, 10 mM Tris, pH 7.4, 1 mM PMSF, 10 μM leupeptin, 1.5 μM pepstatin, and 10 μg/ml aprotinin) and then collected, boiled, and centrifuged for 5 min to remove insoluble material. Protein concentrations in the resultant lysate were measured using the BCA method (Pierce, Rockford, IL). The proteins present in aliquots of lysate were separated by 10–12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked for 1 h at room temperature in buffer containing 5% nonfat dry milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20 (TBST). The membranes were incubated first with primary antibody and then incubated in TBST containing an HRP-conjugated anti-mouse or anti-rabbit immunoglobulin (Jackson ImmunoResearch). Bands were detected using ECL according to the manufacturer's protocol. In some cases, blots were stripped by heating them to 65°C for 30 min in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) and reprobed.

Immunoprecipitation

Cells were lysed for 1 h at 4°C in 1 ml of Triton X-100 buffer (10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 1 mM sodium ortho-vanadate, 10 μM leupeptin, 1.5 μM pepstatin, and 10 μg/ml aprotinin). The lysates were clarified by centrifugation at 20,000 $\times$ *g* for 10 min at 4°C, after which aliquots (1 mg) were incubated overnight at 4°C with anti-Cas-2 Ab raised against amino acid residues 456–690 of rat Cas and then incubated for an additional 4 h at 4°C with protein A-agarose beads (Pharmacia Biotech, Uppsala, Sweden). The beads were then washed three times with Triton X-100 buffer to remove nonspecific binding proteins. Immune complexes were treated with SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol, and 0.006% bromophenol blue) and subjected to SDS-PAGE.

In Vitro Caspase Cleavage Assays

Wild-type and mutant p130cas cDNAs were constructed in pcDNA3.0 and used as templates for coupled transcription–translation using a T7 kit (Promega) with [^{35}S]methionine (1175Ci/mmol; New England Nuclear, Boston, MA). Recombinant caspases were prepared from bacterial cells transformed with caspase-expressing plasmids. The p30 domains of mouse caspase-1, human caspase-8, and the full-length human caspase-3 were amplified by PCR using the following oligonucleotide primers: 5′-CGCGG ATCCT GGCAC ATTTC CAGGA C-3′ and 5′-CGCGG ATCCT AAGGA AGTAT TGGC-3′ (p30 caspase-1); 5′-CGCGG ATCCG GAGAA CACTG AAAAC TC-3′ and 5′-CGCGG ATCCT ACCAT CTTCT CACTT GG-3′ (caspase-3); and 5′-CGGGA TCCTA CCATC TTCTC ACTTG G-3′ and 5′-CCGCA AGCTT ATCAG AAGGG AGACA AG-3′ (p30 caspase-8). The PCR products were subcloned into the *Bam*HI site (caspase-1 and -3) or the *Bam*HI–*Hin*dIII sites (caspase-8) of pET-15b (Novagen, Madison, WI). Exponential growth of bacterial cells was induced for 2 h with 0.2 mM isopropyl-1-thio-α-D-galactopyranoside, after which the cells were harvested and lysed by sonication in buffer containing 0.05% NP-40, 20 mM HEPES, pH 7.4, and 100 mM NaCl. The lysates were cleared by centrifugation, and the protein concentrations were determined using the BCA method (Pierce). The bacterial cell lysates were then used as sources of recombinant caspases.

To assay caspase-catalyzed cleavage, in vitro–translated Cas was incubated for selected times at 30°C with bacterial cell lysates (20 μg) containing the respective recombinant caspases. The reactions were terminated by the addition of SDS-PAGE loading buffer and heating to 100°C for 5 min. Samples were then subjected to 10–12% SDS-PAGE, fixed with destaining solution for 30 min at room temperature, dried, and exposed to x-ray film. When endogenous Cas in cell lysates was the substrate, cells were first lysed on ice in 10 mM HEPES/KOH, pH 7.4, 2 mM EDTA, 5 mM dithiothreitol, 1% NP-40, 10 μM leupeptin, and 10 μg/ml aprotinin. The lysates were then cleared by centrifugation at 27,000 $\times$ *g* for 5 min, and 40-μg aliquots were incubated for selected times at 30°C with recombinant caspase-3 (20 μg of bacterial cell lysates).

Site-directed Mutagenesis

Cas mutants were generated from pcDNA3.0-Cas using the Quik Change site-directed mutagenesis kit (Stratagene). Asp residues at positions 416 (DVPD^{416}) and 748 (DSPD^{748}) of rat p130cas were replaced with Glu. Two sets of primers, 5′-GCAAG GATGT GCCTG AAGGC CCACT GCTGC G-3′ and 5′-CGCAG CAGTG GGCCT TCAGG CACAT CCTTG C-3′ for the D^{416} mutation and 5′-CCCAG GACTC TCCGG AAGGC CAGTA TGAGA ACAG-3′and 5′-CTGTT CTCAT ACTGG CCTTC CGGAG AGTCC TGGG-3′ for the D^{748} mutation, were used for construction of the mutants. The sequences of the mutants were then confirmed by DNA sequence analysis.

Contruction of Expression Plasmids and Transient Tranfection

The full-length cDNA of wild-type and mutant p130cas were PCR amplified from pcDNA3.0-cas using the forward and reverse primers 5′-TGTGCTGGAATTCGGCGGCCG-3′ and 5′-ACCGGATC-CGGGCGGCAGCCAGCTGG-3′. The PCR reaction was carried out using pfu DNA polymerase (Stratagene), digested with *Bam*HI and

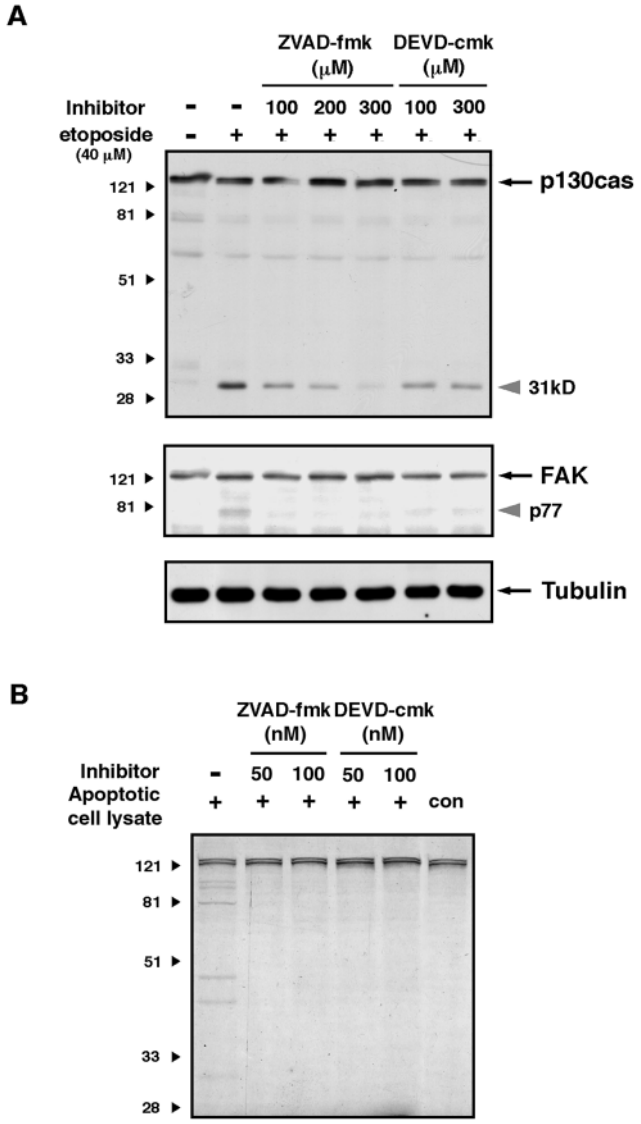

Figure 3. Inhibition of Cas proteolysis in vivo by ZAVD-fmk and DEVD-cmk. (A) Rat-1 cells were pretreated for 3 h with the indicated concentrations of ZVAD-fmk or DEVD-cmk and then exposed to 40 μM etoposide for an additional 12 h. Cas proteolysis was analyzed by immunoblot analysis using Cas mAb as a probe. The same membrane was then stripped and reprobed with FAK mAb. Both antagonists effectively inhibited Cas and FAK cleavage. Molecular mass standards are indicated by the small arrowheads on the left. Original proteins (arrows) and cleavage fragments (large arrowheads) are shown on the right. (B) Apoptotic cell lysates (30 μg) were obtained from etoposide-treated cells for 36 h and incubated with in vitro–translated Cas in the presence of either ZVAD-fmk or DEVD-cmk. Both caspase inhibitors completely inhibited the cleavage of in vitro–translated Cas even at 50 nM.

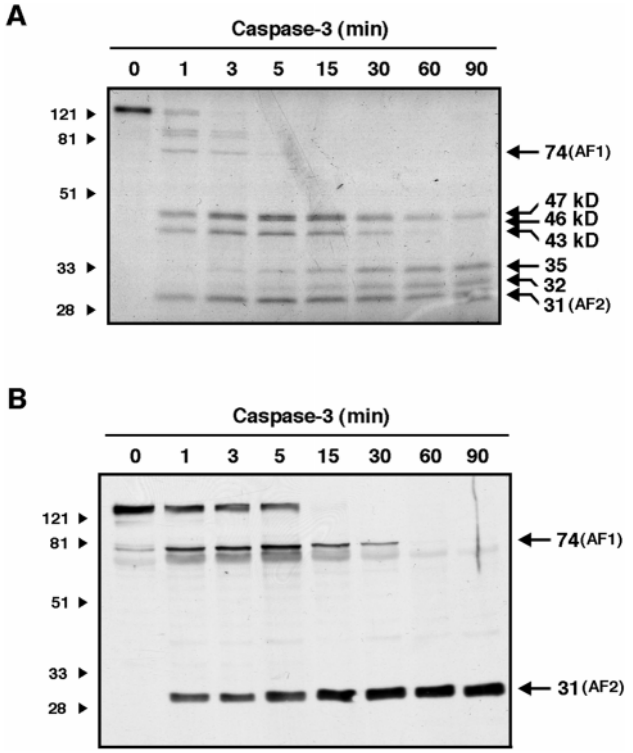

Figure 4. Time course of the in vitro proteolysis of Cas by caspase-3. (A) In vitro–translated Cas was incubated with caspase-3 for the indicated times. Samples were subjected to SDS-PAGE, and Cas cleavage fragments were detected by autoradiography. Seven fragments with molecular masses of 74, 47, 46, 43, 35, 32, and 31 kDa were observed. (B) Cell lysates from control cells were used as a source of endogenous Cas and incubated with caspase-3 for the indicated times. Cas cleavage fragments with molecular masses of 74 and 31 kDa were detected on immunoblots probed with Cas mAb. Molecular mass standards are shown by the arrowheads on the left. Molecular masses of the cleavage products are shown by the arrows on the right.

*Eco*RI, and cloned into pFLAG-cytomegalovirus (CMV)-5c (Eastman Kodak, New Haven, CT) to generate pFLAG-CMV-Cas. Rat-1 cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS (Life Technologies, Gaithersburg, MD). To generate cells transiently expressing either the wild-type Cas or each mutant, Rat-1 cells were transfected with the constructed DNAs using LipofectAMINE following the manufacturer's instructions (Life Technologies). The expression of the constructed DNAs was analyzed by Western blotting using Flag M2 mAb (Sigma).

Immunofluorescence

Rat-1 cells grown on 0.1% gelatin-coated coverslips were fixed with 1.5% paraformaldehyde for 10 min and washed in PBS. They were then permeabilized with 0.5% Triton X-100 in PBS for 5 min. The cells were then incubated for 1 h in a humidified chamber at 37°C with appropriate combinations of the primary Ab in PBS containing 1% BSA, washed four times with 0.1% Triton X-100 in PBS, and incubated for 1 h at room temperature with FITC-conjugated donkey anti-IgG/IgM in PBS containing 1% BSA. The coverslips were then washed with 0.1% Triton X-100 in PBS and mounted with 90% glycerol and 0.1% *o*-phenylenediamine in PBS. Immunofluorescence was analyzed under a Leica (Nussloch, Germany) DMRBE microscope equipped with a 100× objective lens and filters for epifluorescence. Fluorescence micrographs were taken on T-max P3200 film (Kodak).

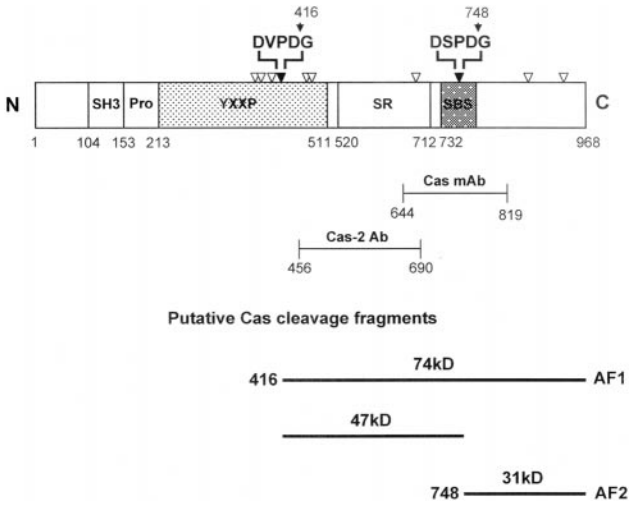

Figure 5. Schematic representation of the structure of Cas with the predicted cleavage sites and the functional domains. The following regions within Cas are indicated: the SH3 domain (SH3), the proline-rich sequence (Pro), the substrate domain (YXXP, tyrosine phosphorylation sites), and the serine-rich domain (SR), which includes multiple serine phosphorylation consensus motifs, the Src binding site (SBS), a proline-rich sequence that associates with the SH3 domain, and a tyrosine residue that upon phosphorylation binds to the SH2 domain. The immunogens for Cas mAb and Cas-2 Ab are illustrated. Ten DXXD sequences conserved in rat and mouse p130 Cas are indicated (209). The putative fragments (AF1 and AF2) generated by caspase-3-catalyzed cleavage during etoposide-induced apoptosis are indicated (τ, putative cleavage site).

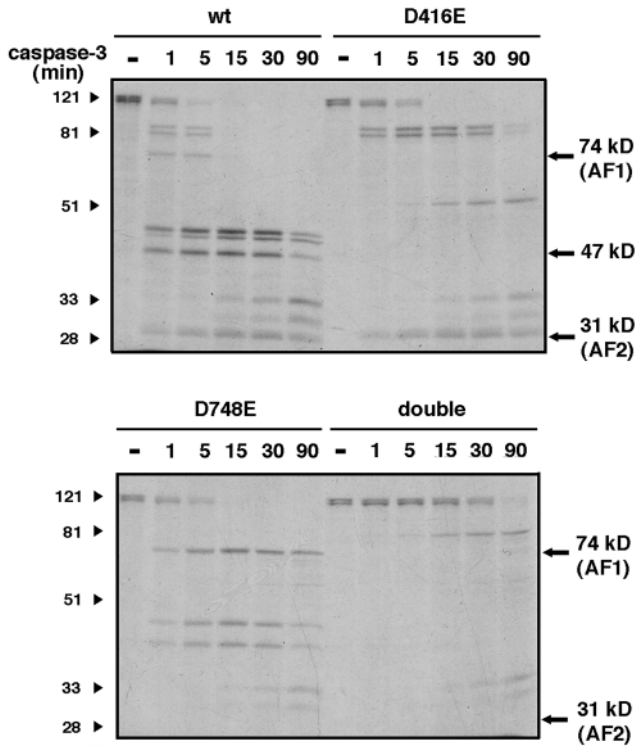

Figure 6. Determination of the caspase-3 cleavage sites in Cas using site-directed mutagenesis. (A) Cas mutants containing point mutations replacing Asp ($DVPD^{416}$ and/or $DSPD^{748}$) with Glu were translated in vitro and incubated with extracts (20 μg) prepared from bacterial cells overexpressing recombinant caspase-3. Mutations at D416 and/or D748 are able to block Cas cleavage at those sites. Molecular mass standards are shown by the arrowheads on the left. Molecular masses of the cleavage products are shown by the arrows on the right.

RESULTS

Cleavage of Cas during Etoposide-induced Apoptosis in Rat-1 Cells

Exposing cells to etoposide induces apoptosis and gross changes in cell shape. The transition from flat to round cell morphology likely results from detachment from the ECM and leads to the appearance of apoptotic cells floating in the medium. Because cell detachment from the ECM is well known to result from loss of focal adhesion sites, we examined in vivo changes in focal adhesion proteins during apoptosis.

Rat-1 cells were incubated with 40 μM etoposide for selected periods, lysed, and subjected to immunoblot analysis using Cas mAb and Cas-2 Ab as probes. When blots were probed with Cas mAb, it was found that Cas had undergone proteolytic cleavage during etoposide-induced apoptosis (Figure 1A). A 31-kDa proteolytic fragment was first detected at 12 h, markedly increased for up to 24 h in the continued presence of etoposide, and then gradually declined until, after 48h, it was no longer detectable. In contrast, the 31-kDa fragment was barely detected in untreated control cells (Figure 1A**).** The appearance of the 31-kDa proteolytic fragment is in accord with the previous report by Bannerman *et al.* (1998). In similar manner, a 47-kDa cleavage fragment was detected when blots were probed with Cas-2 Ab (Figure 1B).

The appearance of Cas degradation products correlated well with the reduction of FAK, which time dependently declined in the presence of etoposide and was undetectable within 36 h (Figure 1C). A ~77-kDa cleavage product was faintly detected at 12 h and became intense within 18–24 h after treatment. This suggests proteolytic cleavage of FAK, which is consistent with several lines of evidence indicating that caspases also degrade FAK in cells undergoing apoptosis (Wen *et al.*, 1997; Levkau *et al.*, 1998).

In Vitro Cleavage of Cas by Caspase-3

Analysis of its amino acid sequence showed that Cas has several probable caspase cleavage sites: 10 sites with the DXXD consensus sequence preferentially cleaved by caspase-3-like caspases and 5 sites with the sequence (IV-L)XXD preferentially cleaved by caspase-6-like caspases (Talanian *et al.*, 1997). We therefore investigated whether Cas is cleaved by caspases in vitro. The cDNA encoding rat Cas was transcribed and translated in vitro and then incubated with recombinant caspases-1, -3, and -8. The specific activities of recombinant caspases-1 and -3 were previously confirmed by cleaving GDP dissociation inhibitor type D4, and the activity of caspase-8 was confirmed by cleaving poly-(ADP-ribose) polymerase (our unpublished data).

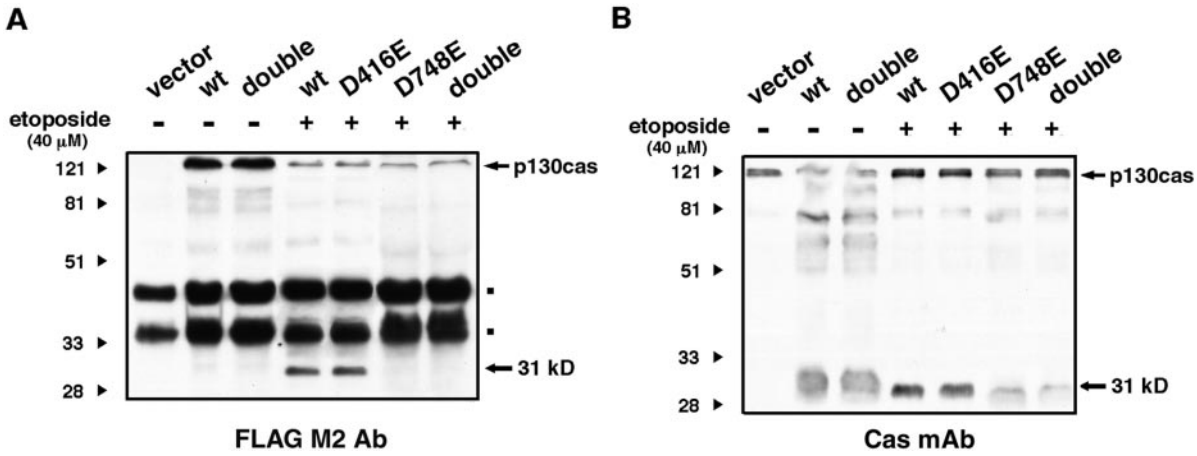

Figure 7. In vivo cleavage of mutant Cas. Rat-1 cells transiently transfected with pFLAG-CMV-5c containing each mutant Cas DNA such as vector only (lane 1), wild-type Cas (wt, lanes 2 and 4), single-mutant Cas ($D^{416}E$, lane 5; and $D^{748}E$, lane 6), and double-mutant Cas ($D^{416}E$ and $D^{748}E$, lanes 3 and 7) were cultured with (lanes 4- 7) or without (lanes 1–3) etoposide for 24 h. The cell lysates were immunoblotted with anti-FLAG M2 mAb (A), and the same membrane was then stripped and reprobed with Cas mAb (B). Note that the overexpresed mutant Cas were resistant to in vivo cleavage. Molecular mass standards are shown by the arrowheads on the left. Molecular masses of the cleavage products are shown by the arrows on the right. Nonspecific binding of antibodies is indicated by closed squares on the right.

As shown in Figure 2A, caspase-3 completely cleaved in vitro–translated Cas, generating six cleavage products with molecular masses of ~ 47, 46, 43, 35, 32, and 31 kDa, respectively. The 31-kDa band appeared identical to the band detected by Cas mAb in etoposide-treated cells. Most likely because of differences in substrate specificity, Cas was cleaved to a lesser extent by caspase-1 and was not cleaved at all by caspase-8.

To confirm that the Cas proteolysis was catalyzed by caspase, recombinant caspases were preincubated with membrane-permeant caspase antagonists ZVAD-fmk and DEVD-cmk before incubation with in vitro–translated Cas. DEVD-cmk (100 nM), a specific caspase-3 inhibitor, completely blocked cleavage of Cas (Figure 2B), whereas ZVAD-fmk (100 nM), which is a less specific inhibitor, was a less effective in the inhibition of Cas cleavage. Thus, cleavage of Cas appears to be catalyzed primarily by caspase-3.

When Rat-1 cells were preincubated with either ZVAD-fmk or DEVD-cmk for 3 h and then exposed to etoposide for an additional 12 h, ZVAD-fmk suppressed the appearance of the 31-kDa cleavage product in a concentration-dependent manner, whereas DEVD-cmk did so only partially even at 300 μM (Figure 3A). DEVD-cmk was less effective at inhibiting FAK cleavage as well (Figure 3A). However, when apoptotic cell lysates obtained from the cells exposed to etoposide for 36 h were incubated with in vitro–translated Cas in the presence of either ZVAD-fmk or DEVD-cmk, cleavage of in vitro–translated Cas was blocked by both caspase inhibitors even at 50 nM (Figure 3B). Thus, although other caspases may be involved in Cas cleavage during apoptosis, caspase-3 appears to be the primary mediator of Cas cleavage. In addition, the observed effects of the caspase inhibitors from in vivo and in vitro cleavage experiments reflect relative differences in cell membrane permeability.

Determination of the Sites of Cas Cleavage by Site-directed Mutagenesis

To define the sites at which caspase-3 sequentially cleaves Cas, both in vitro–translated and endogenous Cas were digested with caspase-3, and the time courses of the proteolysis were analyzed. In vitro–translated Cas generated a total of nine different cleavage products (Figure 4A). Within 1 min after exposure to caspase-3, 100-, 90-, 74-, 47-, 46-, 43-, and 31-kDa fragments were detected; levels of the 47-, 46-, and 43-kDa fragments increased up to 15 min, and 35- and 32-kDa fragments first appeared after 15 min. The cleavage pattern of endogenous Cas was then probed using Cas mAb raised against the amino acid sequence (644–819) of rat Cas. Incubating recombinant caspase-3 with cell lysates generated the 74-kDa (AF1) and 31-kDa (AF2) fragments (Figure 4B), which appear to correspond to the 74- and 31-kDa fragments detected when in vitro–translated Cas was digested by caspase-3. Immunoblot analysis using Cas-2 Ab and Cas mAb showed that the 74-kDa fragment generates two cleavage products with apparent molecular masses of 47 and 31 kDa, the former being detected by Cas-2 Ab (Figure 1B) and the latter by Cas mAb (Figure 4B). In immunoblot analysis, the 31- and 47-kDa fragments shown in cell lysates are matched with the cleavage fragments of in vitro–translated wild type (our unpublished data).

To define the cleavage sites generating the 74- and 31-kDa fragments, we diagrammed the amino acid sequence of rat Cas (Figure 5) and determined that there were two probable cleavage sites at $DVPD^{416}G$ and $DSPD^{748}G$. That these sites were cleaved by caspase-3 was confirmed by inserting point mutations replacing Asp at the sites with Glu and then exposing the translated products to caspase-3. As shown in Figure 6, the 74-kDa fragment completely disappeared after mutation at $DVPD^{416}G$, whereas the 31-kDa fragment disappeared after mutation at $DSPD^{748}G$. The 47-kDa fragment

was still detected after mutation at DSPD^{748}G, but it was not the same 47-kDa fragment reactive with Cas-2 Ab (our unpublished data). The disappearance of the 74- and 31-kDa fragments was more apparent in a double mutant (D^{416}E and D^{748}E) with which only a single, 90-kDa cleavage fragment appeared. To further confirm in vivo cleavage of Cas, mutant Cas DNAs were cloned into pFLAG-CMV-5c vectors and transiently transfected into Rat-1 cells. After 24 h of exposure to 40 μM etoposide, cell lysates were immunoblotted with anti-FLAG M2 mAb (Figure 7A), and the same membrane was reprobed with Cas mAb (Figure 7B). Without etoposide treatment, full-length Cas (130-kDa) was well detected in wild-type Cas-transfected cells by anti-FLAG M2 mAb and Cas mAbs. After treatment with etoposide for 24 h, a 31-kDa fragment was apparently detected in wild-type Cas- and single-mutant Cas-transfected cells (D^{416}E) by anti-FLAG M2 mAb. However, the 31-kDa fragment was not detected in single-mutant (D^{748}E) and double-mutant Cas-transfected cells, respectively. As shown in Figure 7B, Cas mAb was able to detect a 31-kDa fragment in etoposide-treated cells, which is a cleavage product of endogenous Cas. These data further confirm that DVPD^{416}G and DSPD^{748}G are cleavage sites of Cas.

Cleavage of Cas Alters Its Association with Paxillin

Our findings thus far indicate that in etoposide-treated cells, the decrease in Cas is most likely due to caspase-mediated proteolysis during apoptosis. We, therefore, examined how this cleavage affects the interaction between Cas and another focal adhesion protein, paxillin. In this experiment, cells were grouped into adherent and apoptotic cells, the former being cells adhering to the bottom of culture dish and the latter being apoptotic cells floating in the medium after 36 h of exposure to etoposide. Although paxillin was unchanged in control and adherent cell lysates, Cas was dramatically decreased in adherent cells and was not even detected in apoptotic cells. Furthermore, the cleavage fragment (31-kDa) was also detected in both adherent and apoptotic cells (Figure 8A). In a parallel experiment, Cas was coprecipitated with paxillin in control adherent cells, whereas full-length Cas (130-kDa) associated with paxillin dramatically decreased, and a 100-kDa truncated Cas additionally appeared in adherent cells after treatment with etoposide for 12 h. In adherent cells exposed with etoposide for 36 h, a 100-kDa truncated Cas but not full-length Cas was only coprecipitated with paxillin (Figure 8B). This reflects the decline in the availability of Cas by apoptotic degradation.

In addition, Cas was detected as a 100-kDa truncated form in adherent cells rather than its full-length, 130-kDa form (Figure 8B), which suggests that initiation of Cas degradation inhibits the binding of Cas to paxillin, rendering Cas incapable of functioning as a docking molecule. It is interesting that, if already bound, the partially cleaved Cas remained bound to paxillin in adherent cells. This likely reflects the essential role played by Cas in the assembly of focal adhesion complexes.

To test whether the molecular changes attenuating the interaction of Cas and paxillin correlate with changes in the cellular architecture of focal adhesions, we used a Cas mAb (4F4), which recognizes the tyrosine-phosphorylated form of Cas, to probe the cellular distribution of Cas in cells exposed to etoposide for 24 h. In control cells, Cas was well localized

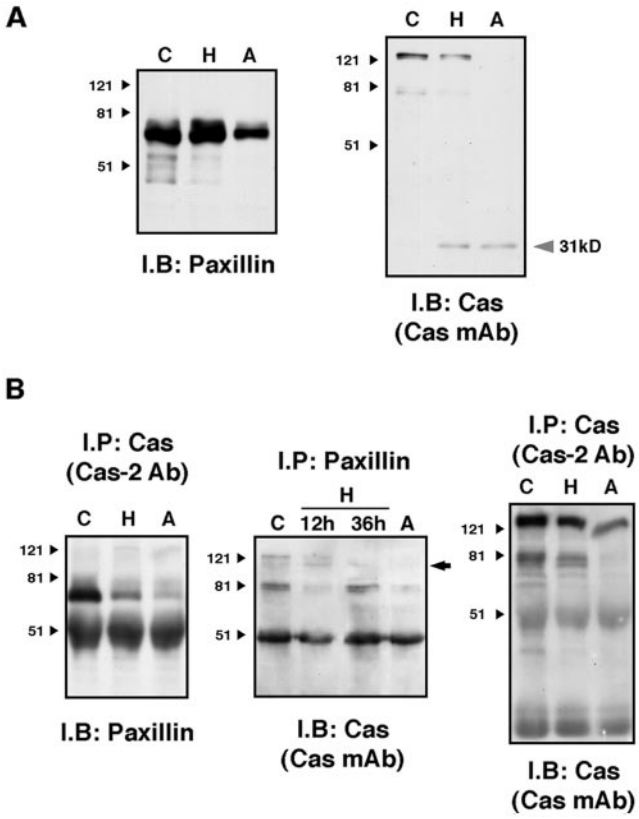

Figure 8. Interaction between Cas and paxillin in apoptotic cells. (A) Cell lysates were immunoblotted with Cas mAb or paxillin mAb. (B) Cas and paxillin were immunoprecipitated from control (C), adherent (H), or apoptotic (A) cells by Cas-2 Ab or paxillin mAb, and the interaction between Cas and paxillin was detected on immunoblots probed with Cas mAb or paxillin mAb. The partial cleaved Cas (>100-kDa band) binding to paxillin is indicated by the arrow on the right. The interaction between Cas and paxillin was dramatically decreased in adherent cells and absent in apoptotic cells. Molecular mass standards are shown on the left (arrowheads).

in focal adhesion sites (Figure 9A). But in etoposide-treated cells, Cas was lost from focal adhesions at the bottom of cells and was instead found in cell peripheries to occur membrane retraction during the process of cell detachment from the substratum (Figure 9B). Paxillin and talin underwent the same change in cellular localization as well (Figure 9, C–F). In addition, actin stress fibers in control cells formed well-arranged straight lines in the cytoplasm and in peripheral regions (Figure 9G), whereas most of the cytoplasmic stress fibers had disappeared in etoposide-treated cells, and only discontinuous, truncated fibers were seen in the cell periphery (Figure 9H). This finding is consistent with changes in the actin cytoskeleton observed in endothelial cells undergoing apoptosis (Palladini *et al.*, 1996) and suggests that degradation of Cas during apoptosis leads to relocalization of focal adhesions into the periphery of the cell and the rearrangement of the cytoskeleton and consequently results in disassembly of focal adhesions.

To examine further the role of Cas in the assembly of focal adhesions, cells were transiently transfected pFLAG-

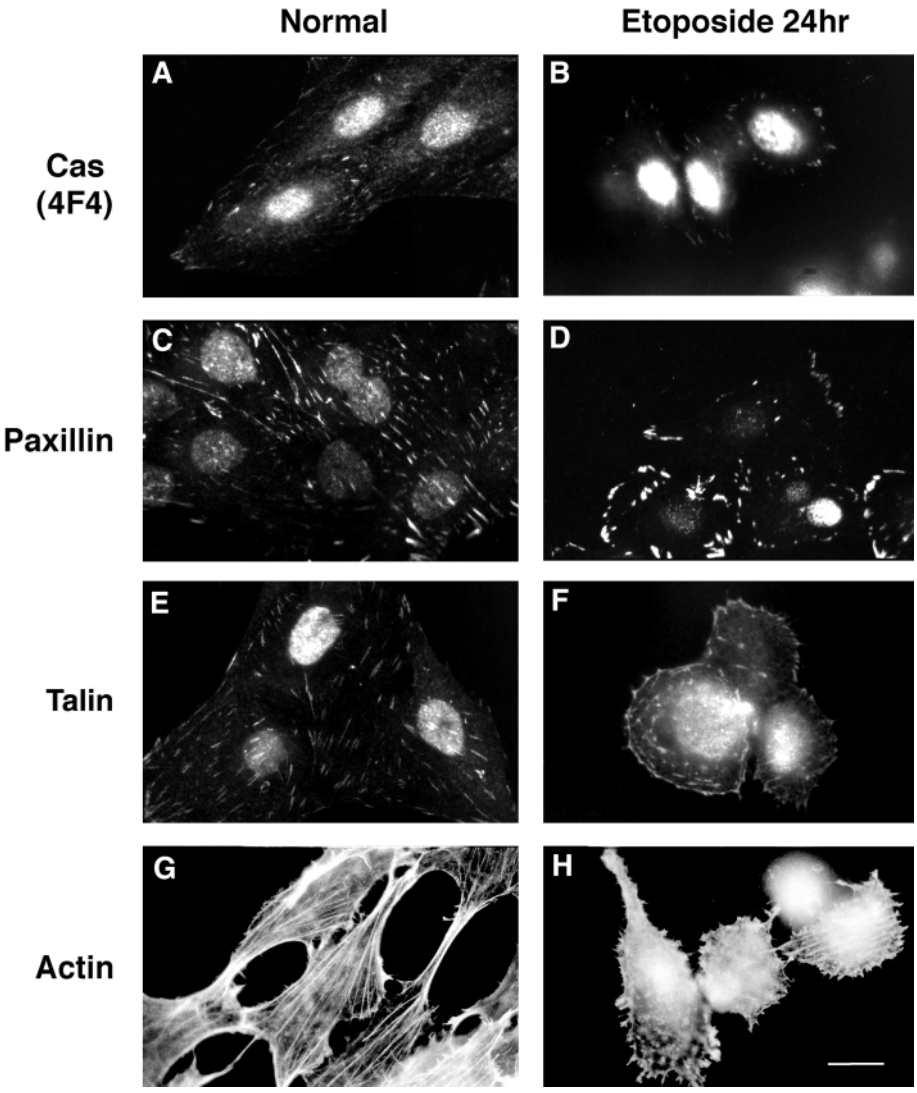

Figure 9. Fluorescent images depicting the changes in the cellular localization of Cas, paxillin, talin, and actin in apoptotic cells. The distribution of Cas (4F4, A and B), paxillin (C and D), and talin (E and F) within control cells or etoposide-treated cells reveals that Cas, paxillin, and talin are localized in focal adhesion sites in control cells (A, C, and E) but is lost from focal adhesions during apoptosis and redistributes into the periphery of cells (B, D, and F). Actin labeling using TRITC-phalloidin reveals that the actin stress fibers seen in control cells (G) are virtually absent from the cytoplasm of apoptotic cells, although some truncated fibers are seen at the cell margins (H). Bar, 10 μm.

CMV-5c vectors containing double-mutant Cas or wild-type DNAs and double immunostained with anti-FLAG M2 mAb and paxillin Ab (Figure 10). Cells expressing double-mutant Cas are indicated by positive staining with anti-FLAG M2 mAb. Immunofluorescence analysis using paxillin Ab in double-mutant Cas-transfected cells demonstrated that focal adhesions still remained in the bottom of cells after treatment with etoposide for 18 h, whereas focal adhesions in untransfected cells and wild-type Cas-transfected cells were completely lost from the bottom of cells and instead relocalized into the pheripery of cells (Figure 10, A–D). After treatment with etoposide for 24 h, both transfected and untransfected cells displayed the relocalization of focal adhesions into the pheripery of cells **(**Figure 10, E and F**)**. These data suggest that mutation in cleavage sites may attenuate Cas degradation at the beginning of apoptosis and consequently protect, at least in part, the disassembly of focal adhesions in the bottom of cells. However, the prolonged treatment with etoposide seems to induce the cleavage of mutant Cas and results in the loss of focal adhesions from the bottom of cells.

DISCUSSION

In the present study, we identified the focal adhesion molecule Cas as a substrate for apoptotic caspases. Previously, another focal adhesion molecule, FAK, was also identified as a substrate for cleavage by caspases, a proteolytic event that may be key to the execution of the suicide pathway (Crouch *et al.*, 1996; Wen *et al.*, 1997; Gervais *et al.*, 1998). Our data show that etoposide induces proteolytic cleavage of Cas in Rat-1 cells undergoing apoptosis. Cleavage of Cas has also been observed when apoptosis was induced by staurosporine or nocodazole, a microtubule-depolymerizing agent (Kook *et al*., 2000), which suggests that Cas degradation is a general phenomenon associated with apoptosis.

The cleavage patterns of in vitro–translated and native Cas were similar and interesting in several respects. The

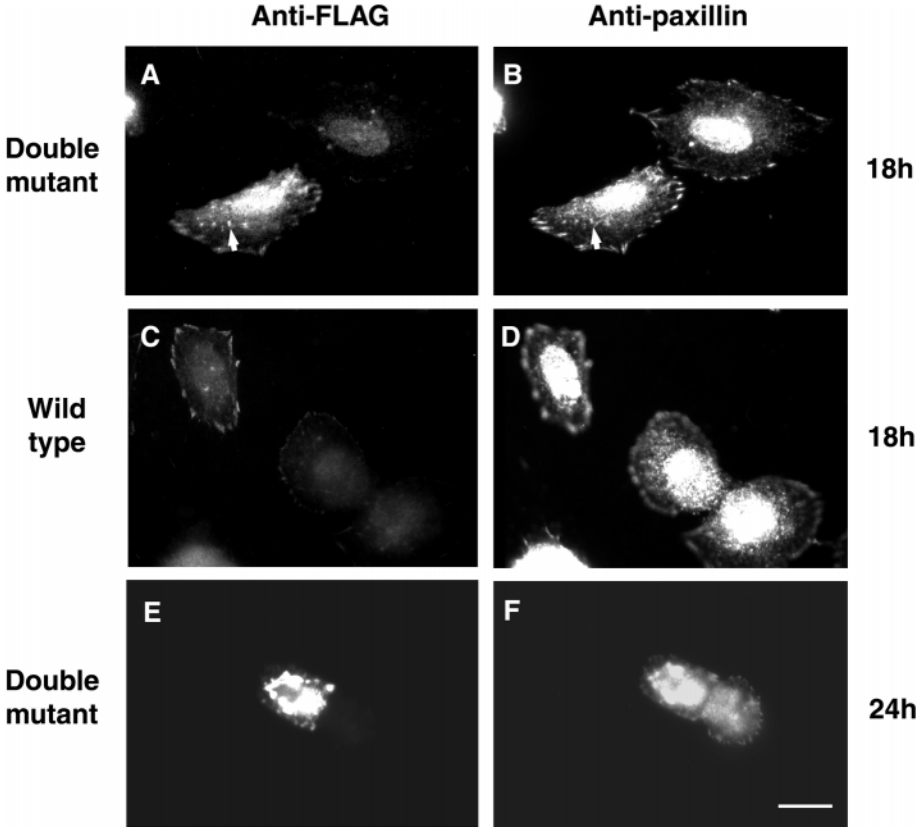

Figure 10. Changes of cellular localization of Cas in double-mutant Cas-transfected Rat-1 cells. Rat-1 cells were transiently transfected with pFLAG-CMV-5c containing double-mutant ($D^{416}E$ and $D^{748}E$) or wild-type Cas DNAs and incubated with 40 μM etoposide for 18 h (A– D) or 24 h (E and F). Cells were double immunostained with anti-FLAG M2 mAb (A, C, and E) and paxillin Ab (B, D, and F). Positive staining with anti-FLAG M2 mAb indicates cells expressing double-mutant or wild-type Cas. The images in A, C, and E depict alternate staining of the same cells shown in B, D, and F, respectively. Focal adhesions at the cell bottom are indicated by white arrows. Note that double-mutant Cas could attenuate the Cas degradation and consequently partially blocked the relocalization of focal adhesion proteins into the periphery of cells. Bar, 10 μm.

time course of these cleavages suggests that there is a hierarchy in the proteolytic cascade, perhaps in part because not all potential cleavage sites are simultaneously exposed. For example, a 100-kDa band appeared at early times (Figure 4), which was likely generated by cleavage of the C terminus, because the N terminus lacks a putative cleavage site that would yield a fragment of a corresponding size. Considering the potential sites for Cas proteolysis, it is reasonable to predict that the $DAVD^{860}A$ sequence in the C terminus would be preferentially cleaved. The C-terminal cleavage fragment would not be detected in our experiment, because it would lack a methionine residue. Cleavage near the C terminus may induce a conformational change that in turn causes other potential cleavage sites to be exposed. Using Cas mAb, we were able to detect 74- and 31-kDa fragments.

An increasing number of caspase-3 substrates sharing the DXXD consensus sequence for caspase-3 are being identified (Harvey *et al.*, 1998; Wang *et al.*, 1998). Ten potential cleavage sites found in Cas fit with the preferred consensus sequence for caspase-3, and using site-directed mutagenesis, we confirmed that two of them, $DVPD^{416}G$ and $DSPD^{748}G$, were cleaved by caspase-3. Cleavage of $DVPD^{416}G$ generates a 74-kDa fragment, further cleavage of which at the $DSPD^{748}G$ site generates the 31- and 47-kDa fragments. Substituting D416 or D748 with Glu resulted in the complete disappearance of the 74- and 31-kDa fragments, respectively, confirming that these two sites are in fact cleaved by caspase-3. Other potential cleavage sites could not be investigated, because the specificity of our antibodies is limited.

Cas degradation correlated temporally with the onset of apoptosis. Initiation of Cas cleavage blocks the binding of paxillin to Cas and the resultant localization of paxillin within focal adhesion sites. Consistent with that finding, Cas-deficient embryos exhibit disorganized myofibrils, disrupted Z-disks, and impaired actin stress fiber formation (Honda *et al.*, 1998). We also found that Cas was concentrated in the periphery of cells where membrane retraction is ongoing during apoptosis, and that actin fibers were truncated, no longer forming continuous stress fibers. In addition, we demonstrated that the double-mutant Cas ($D^{416}E$ and $D^{748}E$) could attenuate the Cas degradation and consequently partially blocked the relocalization of focal adhesion proteins into the periphery of cells. Because focal adhesions provide a structural link between the actin cytoskeleton and ECM, these data suggest that Cas degradation during apoptosis disrupts the architecture of focal adhesions, disrupts molecular interactions within focal adhesions, and, consequently, disrupts the cytoskeleton. Thus, Cas can be added to the list of proteins associated with regulation of the cytoskeleton and cell attachment that undergo proteolytic cleavage during apoptosis.

Cas was initially identified as a prominent tyrosine-phosphorylated substrate of the oncoproteins v-Crk and v-Src (Reynolds *et al.*, 1989; Mayer and Hanafusa, 1990; Kanner *et al.*, 1991). It was also reported that Cas was phosphorylated when cells adhere to the ECM and β1-integrin is cross-linked (Nojima *et al.*, 1995; Petch *et al.*, 1995). This phosphorylation is apparently involved in recruiting signaling mole-

cules into focal adhesions. Moreover, Cas is an adaptor protein with an SH3 domain, a proline-rich domain, a substrate domain containing nine Tyr-Asp-X-Pro motifs, and a C-terminal domain containing the consensus SH3 and SH2 binding sites for Src (Sakai *et al*., 1994). The association of Cas with these molecules is likely to be functionally important in the formation and maintenance of focal adhesion complexes, and SH2- and SH3-containing molecules associated with Cas may transduce a common set of signals that regulate cell growth, differentiation, and survival (Chan *et al*., 1999). Degradation of Cas during apoptosis may thus have downstream effects interrupting survival signals from the ECM and reducing the likelihood of cell survival.

The sequence RPLPSPP (733–739) and Tyr^{762} near the C terminus of Cas are involved in the association of Cas with Src; mutation of either the sequence or Tyr^{762} significantly reduced Cas binding to Src (Nakamura *et al*., 1998). It is of interest, therefore, that one of the Cas cleavage sites identified in this study is located at $DSPD^{748}G$, because cleavage at that site would be expected to separate the two Src-binding motifs causing Cas to dissociate from Src, which would in turn cause disassembly of focal adhesion complexes. Confirmation of this scenario, however, will require further study.

In summary, we have demonstrated that early caspase-catalyzed proteolysis of Cas during etoposide-induced apoptosis contributes to the morphological characteristics of apoptotic cells that reflect disassembly of focal adhesion complexes and the interruption of survival signals from the ECM.

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