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Phosphorylation-dependent cleavage of p130cas in apoptotic rat-1 cells☆

Do Hoon Kim,[a] Soo Jeon Choi,[a] Seunghyi Kook,[b] Wook Kim,[a] and Woo Keun Song[a,*]

[a] *Department of Life Science, Kwangju Institute of Science and Technology, 1 Oryong-dong, Puk-gu, Kwangju 500-712, South Korea*
[b] *Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0295, USA*

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Abstract

We previously demonstrated caspase-mediated cleavage of p130cas during apoptosis and identified two caspase-3 cleavage sites [1]. In this study, we investigated the phosphorylation-dependent cleavage of p130cas in apoptotic Rat-1 fibroblast cells. Lysophosphatidic acid and fibronectin induced p130cas phosphorylation, which in turn resulted in resistance to caspase-mediated cleavage. Alternatively, dephosphorylation by calf intestinal alkaline phosphatase, PP1, and LAR stimulated cleavage of p130cas by caspase-3, generating a 31-kDa fragment. During apoptosis, p130cas dephosphorylation seems to precede its cleavage. The phosphorylation of tyrosine and serine residues immediately adjacent to the two cleavage sites ($DVPD^{416}$ and $DSPD^{748}$) strongly affected p130cas cleavage by caspase-3, both in vitro and in vivo. Furthermore, the generation of the 31-kDa cleavage fragment was strongly regulated by phosphorylation of a tyrosine residue at position 751 ($DSPD^{748}$ and GQY^{751}). Our results collectively suggest that degradation of p130cas during apoptosis is modulated in a phosphorylation-dependent manner.

Keywords: Phosphorylation-dependent cleavage; Focal adhesion complex degradation; P130cas degradation during apoptosis; Phosphorylation mimic mutation

Apoptosis, or programmed cell death, is critical to many biological processes, including the development of organisms and maintenance of tissue homeostasis [2]. An important aspect in the biochemical mechanism of apoptosis is the role of caspases. Caspases comprise a family of highly conserved aspartate-specific proteases that are responsible for numerous biological and morphological changes associated with apoptosis [3]. Protein cleavage by caspases is not only specific, but also highly efficient. The preferred tetrapeptide recognition motif differs significantly among caspases and leads to the diversity of their biological functions [4]. Not all proteins containing the optimal tetrapeptide sequence are cleaved, implying that tertiary structural elements influence substrate recognition and cleavage. The substrates for caspases include a growing number of cytoskeletal and structural proteins such as gelsolin [5,6], lamin A [7,8], and actin [9,10].

During apoptosis, adhesive cells change from a flat shape to a round shape, detach from the substratum, and lose connection with neighboring cells or the extracellular matrix (ECM). Maintenance of cell–matrix contact is an important cell survival factor [11–13] and the loss of cell–matrix and cell–cell contact or 'rounding up' is a hallmark of apoptosis. Proteolysis of cytoskeleton-associated proteins is possibly involved in the morphological alterations observed during apoptosis in vitro and in vivo, i.e., membrane blebbing and rounding up of cells. Therefore, focal adhesion proteins linking the cytoskeleton and ECM via integrins may be critical substrates of caspase. The degradation of focal adhesion kinase (FAK) has been demonstrated in several types of cells undergoing apoptosis [14–16]. In addition, we previously reported that p130cas, an important focal adhesion protein, is cleaved by caspase-3 in apoptotic Rat-1 cells [1].

☆ *Abbreviations:* p130cas, Crk-associated substrate 130 kDa; LPA, lysophosphatidic acid; LAR, leukocyte common antigen-related tyrosine phosphatase; PP1, protein phosphatase 1; ECM, extracellular matrix; FAK, focal adhesion kinase; PLC, phospholipase C.

* Corresponding author. Fax: +82-62-970-2484.
E-mail address: wksong@kjist.ac.kr (W.K. Song).

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Protein phosphorylation is a fundamental mechanism of cellular signal transduction. Extracellular stimuli, such as cytokines, growth factors, cell–cell, and cell–substratum adhesive interactions activate a number of tyrosine kinases which in turn send signals downstream by phosphorylating various proteins to regulate cell morphology, migration, differentiation, proliferation, and survival. The overexpression of LAR, transmembrane receptor-like protein tyrosine phosphatase, induces caspase-dependent apoptosis by reducing tyrosine phosphorylation and protein stability of p130cas [17,18]. Moreover, phosphorylated proteins, such as IκB-α, presenilin-2, and PLC-γ1, are resistant to cleavage by caspase-3 [19–21].

p130cas was initially identified as a prominent tyrosine-phosphorylated substrate of the oncoproteins v-Crk and v-Src [22–24]. Tyrosine phosphorylation of p130cas is stimulated by a variety of factors, including ECM proteins [25,26], the protein kinase inhibitor, k252a, and bioactive lipid LPA [27–29]. Since p130cas has numerous phosphorylation sites, specifically, Ser^{411}, Ser^{743}, and Tyr^{751}, adjacent to the cleavage sites, Asp^{416} and Asp^{748}, it is hypothesized that phosphorylation at these residues affects p130cas cleavage by caspase-3. In this study, we investigate the phosphorylation-dependent cleavage of p130cas in apoptotic Rat-1 cells. Our data demonstrate that p130cas phosphorylation attenuates cleavage by caspase-3 during etoposide-induced apoptosis.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic, and trypsin-EDTA were purchased from Gibco-BRL (Grand island, NY). Etoposide was obtained from Sigma Chemical (St. Louis, MO) and enhanced chemiluminescence (ECL) reagent was from Amersham (Buckinghamshire, England). Monoclonal antibody (mAb) against p130cas (Cas mAb) was purchased from Transduction Laboratories (Lexington, KY). Anti-Cas polyclonal antibody (Cas-2 Ab; 38]) and rat Cas cDNA were provided by Dr. Hismaru Hirai (University of Tokyo, Japan). Anti-phosphotyrosine monoclonal antibody (4G10) was obtained from Upstate Biotechnologies (Lake Placid, NY). Horseradish peroxidase (HRP)-labeled anti-mouse and anti-rabbit immunoglobulin (IgG) were acquired from Jackson ImmunoResearch Labs (West Grove, PA). We employed the Quik Change Site-Directed Mutagenesis System from Stratagene (La Jolla, CA). K252a and lysophosphatidic acid (LPA) were obtained from Calbiochem (San Diego, CA). PP1 and LAR were purchased from New England BioLabs (Beverly, MA) and the Fugene 6 reagent was from Roche (Mannheim, Germany).

Cell culture. Rat-1 cells were grown in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 incubator. After 36 h, cells were placed in DMEM and exposed to 2 μM LPA for selected periods of time to induce phosphorylation of p130cas. To trigger apoptosis, cells were exposed to 40 μM etoposide for selected time-periods. Apoptotic cells were harvested by centrifugation at 1000*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 subjected to immunoblotting.

Electrophoresis and immunoblot analysis. For immunoblot analyses, cells treated as described above were lysed in lysis buffer [1% SDS, 1 mM sodium orthovanadate, 10 mM Tris–HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 1.5 μM pepstatin, and 10 μg/ml aprotinin], collected, boiled, and centrifuged for 5 min to remove insoluble material. Protein concentrations were measured using the BCA method (Pierce, Rockford, IL). Proteins in lysate aliquots were separated by 10–12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h at room temperature in buffer containing 5% non-fat dried milk in 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 (TBST). Next, membranes were incubated with primary antibody, followed by TBST containing HRP-conjugated anti-mouse or anti-rabbit immunoglobulin. Bands were detected using ECL according to manufacturer's protocol (Amersham). In some cases, blots were stripped by heating 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. The band intensity p130cas and the 31-kDa fragment was measured using GS-700 Imaging Densitometer (BioRad, CA).

Immunoprecipitation and cleavage by caspases. Cells were lysed for 1 h at 4 °C in 800 μl 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 orthovanadate, 10 μM leupeptin, 1.5 μM pepstatin, and 10 μg/ml aprotinin]. Lysates were clarified by centrifugation at 10,000*g* for 10 min at 4 °C, incubated overnight at 4 °C with anti-Cas-2 Ab raised against residues 456–690 of rat Cas, and 30 μl protein A–Sepharose 4B Fast Flow beads (Pharmacia Biotech, Uppsala, Sweden) for an additional 4 h at 4 °C. Next, beads were pelleted by brief centrifugation at 3000*g* for 5 min and washed twice with Triton X-100 buffer to remove non-specific binding proteins and once with caspase reaction buffer [200 mM Hepes (pH 7.4), 100 mM NaCl, 0.05% NP-40, and 200 mM DTT]. Immunoprecipitated Cas proteins were incubated for 30 min at 30 °C with 1 μl bacterial cell lysates containing the respective recombinant caspase-3. In other experiments, immunoprecipitates were treated with LAR or PP1 (protein phosphatase 1) for 30 min at 30 °C and incubated with recombinant caspase-3. Proteins were eluted from the beads by boiling samples for 5 min in SDS–PAGE loading buffer containing β-mercaptoethanol and separated by 10% SDS–PAGE, followed by Western blotting.

Site-directed mutagenesis. Cas mutants were generated from pcDNA3.0-Cas using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). Serine (S) residues at positions 411 and 743 and a tyrosine (Y) residue at position 751 of p130cas were replaced with either glutamate (E) or alanine (A). Three sets of primers, specifically, 5′-GTTCC TCCTTCTGTGGAGAAGGATGTGCCTGATGG-3′ and 5′-CCAT CAGGCACATCCTTCTCCACAGAAGGAGGAAC-3′ (for the S411E mutation), 5′-CCTCCAAAGTTCACCGAGCAGGACTCTCCGGA TG-3′ and 5′-CATCCGGAGAGTCCTGCTCGGTGAACTTTGGA GG-3′ (for the S743E mutation), and 5′-CTCTCCGGATGGCCAGGA GGAGAACAGTG AAGGGG-3′ and 5′CCCCTTCACTGTTCTCCT CCTGGCCATCCGGAGAG-3′ (for the Y751E mutation) were used to construct the glutamate mutants. Another three sets of primers, specifically, 5′-GTTCCTCCTTCTGTGGCCAAGGATGTGCCTGATG G-3′ and 5′-CCATCAGGCACATCCTTGGCCACAGAAGGAGGA AC-3′ (for the S411A mutation), 5′-CCTCCAAAGTTCACCGCCC AGGACTCTCCGGATG-3′ and 5′-CATCCGGAGAGTCCTGGGC GGTGAACTTTGGAGG-3′ (for the S743A mutation), and 5′-CTC TCCGGATGGCCAGGCTGAGAACAGTGAAGGGG-3′ and 5′-C CCCTTCACTGTTCTCAGCCTGG CCATCCGGAGAG-3′ (for the Y751A mutation) were used to generate the alanine mutants. Mutations were confirmed by DNA sequencing analyses.

Construction of expression plasmids and transient transfections. Full-length wild-type and mutant Cas cDNAs were amplified from pcDNA3.0-Cas using the forward and reverse primers 5′-TGTGCTG GAATTCGGCGGCCG-3′ and 5′-ACCGGATCCGGGCGGCAGC CAGCTGG-3′, respectively. The PCR was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) digested with *Bam*HI and *Eco*RI, and cloned into pFLAG-CMV-5c (Eastman Kodak, New Haven, CT) to generate pFLAG-CMV-Cas. Rat-1 cells were main-

tained in DMEM with 10% FBS. To generate cells transiently expressing either wild-type or mutant Cas, Rat-1 cells were transfected with the appropriate constructs using Fugene 6 reagent. Protein expression was analyzed by Western blotting, using Flag M2 mAb (Sigma, St. Louis, MO).

Results

Loss of p130cas phosphorylation during etoposide-induced apoptosis in Rat-1 cells

Cell adhesion results in the phosphorylation of focal adhesion proteins, including FAK and p130cas [30], which is critical in the transmission of signals to downstream effector molecules for cell survival. We previously demonstrated the degradation of p130cas during etoposide-induced apoptosis in rat-1 cells [1]. To elucidate the relationship between p130cas phosphorylation and degradation during apoptosis, Rat-1 cells were exposed to 40 μM etoposide for the indicated time-periods and cell lysates were blotted with anti-phosphotyrosine (4G10) and Cas mAb (Fig. 1A). A caspase-mediated cleavage product (31-kDa) of p130cas was initially observed at 12 h following etoposide treatment and increased in concentration up to 36 h as apoptosis progressed. Reprobing the blot with anti-phosphotyrosine Ab (4G10) revealed significant dephosphorylation of p130cas. Moreover, no phosphorylated product was detected after 18 h (Fig. 1B). These results strongly indicate that dephosphorylation of p130cas accompanies apoptosis.

Effect of p130cas phosphorylation on caspase-3-mediated cleavage

To determine whether phosphorylation of p130cas affects caspase-mediated cleavage of the protein during apoptosis, cells were treated for the indicated time-periods with 2 μM LPA, which stimulates tyrosine phosphorylation of focal adhesion proteins, such as p130cas and FAK [31]. As shown in Fig. 2A, tyrosine phosphorylation of p130cas (detected with 4G10 Ab) was significantly increased by LPA in a time-dependent manner. Next, cells were pre-treated with LPA for 20 min in serum-free media and exposed to 40 μM etoposide for 24 h. The p130cas protein was rapidly cleaved in untreated cells, generating a 31-kDa fragment. In contrast, significantly lower amounts of the 31-kDa cleavage fragment were detected in LPA-treated cells (Fig. 2B). To determine the susceptibility of phosphorylated p130cas to caspase-3, LPA-treated cells were immunoprecipitated with Cas-2 polyclonal antibody and treated with caspase-3 in vitro. To some extent, p130cas phosphorylated by LPA was resistant to caspase-3-mediated cleavage (Fig. 2C).

Since cell adhesion to fibronectin induces p130cas phosphorylation [25], we compared the 31-kDa cleavage products in cells grown on different ECMs, including fibronectin, laminin, and poly-L-lysine. As expected, cell adhesion to fibronectin resulted in elevated tyrosine phosphorylation of p130cas (Fig. 2D) and the 31-kDa product was significantly reduced in cells grown on fibronectin, compared with those grown on uncoated or poly-L-lysine coated dishes (Fig. 2E). These data further confirm the fact that phosphorylation of p130cas attenuates caspase-3-mediated cleavage.

Effect of p130cas dephosphorylation on caspase-3-mediated cleavage

To examine the susceptibility of dephosphorylated p130cas to caspase-mediated cleavage, p130cas was initially immunoprecipitated with Cas-2 polyclonal antibody followed by pre-treatment with calf intestinal phosphatase (CIP) for 30 min to eliminate the phosphorylation of serine, threonine, and tyrosine residues.

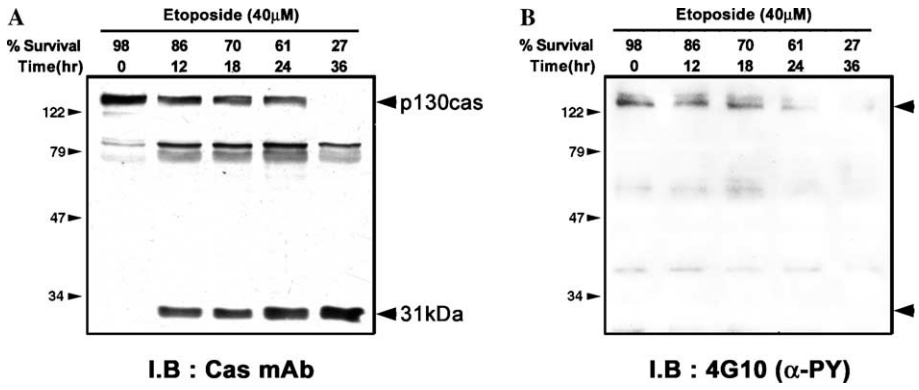

Fig. 1. Dephosphorylation of p130cas during etoposide-induced apoptosis. (A) Rat-1 cells were incubated with 40 μM etoposide for the indicated times and degradation of p130cas was detected with Cas mAb. Cell viability was assessed by trypan blue exclusion. The 31-kDa cleavage fragment was clearly detected at 12 h after etoposide treatment. (B) The same blot was reprobed with 4G10 Ab (α-PY) to examine tyrosine phosphorylation of p130cas. Tyrosine phosphorylation was decreased upon etoposide treatment and not detected after 24 h.

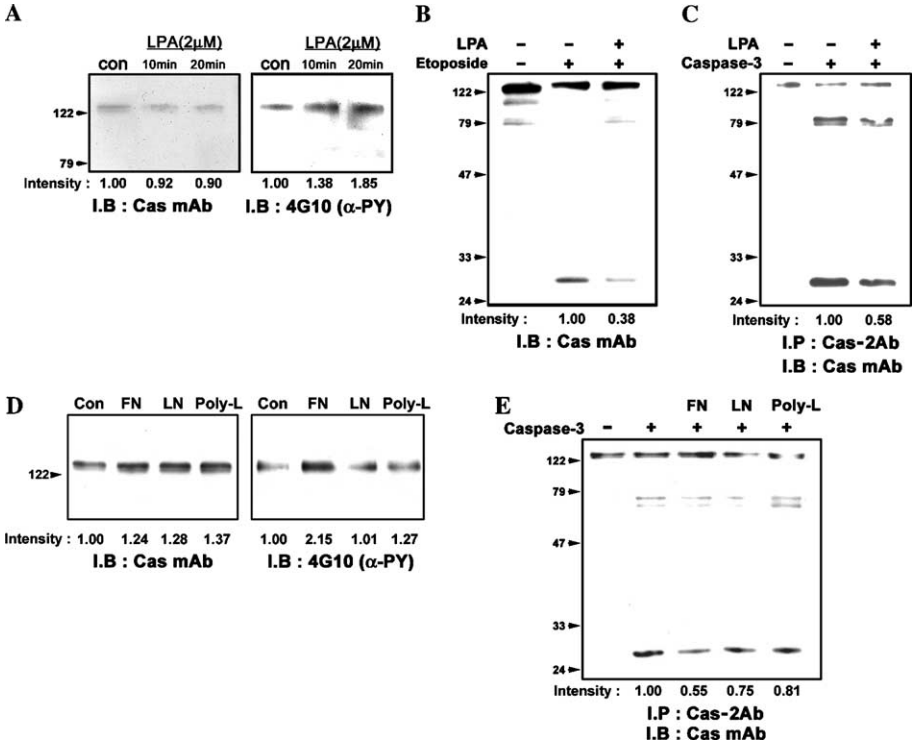

Fig. 2. Effect of p130cas phosphorylation on caspase-mediated cleavage. (A) To induce phosphorylation of 130cas, cells were treated with LPA (2 μM) in a time-dependent manner and immunoblotted with Cas mAb. Increased phosphorylation of a 130-kDa protein (p130cas) was detected following LPA treatment. The membrane was reprobed with 4G10 (α-PY) Ab. (B) Cells pretreated with LPA for 20 min were exposed to etoposide (40 μM) for 12 h and immunoblotted with Cas mAb. (C) Cells were pretreated with LPA for 20 min and immunoprecipitated with Cas-2 polyclonal antibody. Treatment of each immunoprecipitate with caspase-3 revealed that phosphorylated p130cas was resistant to cleavage. (D) Rat-1 cells were grown on dishes coated with fibronectin (FN), laminin (LN), and poly-L-lysine (Poly-L). The cells were lysed, immunoprecipitated with Cas-2 polyclonal antibody, and immunoblotted with Cas mAb or 4G10 (α-PY). (E) Also, the cell lysates were immunoprecipitated with Cas-2 polyclonal antibody and treated with caspase-3. Immunoprecipitated p130cas from cells grown on FN was more resistant to cleavage by caspase-3. The band intensity of p130cas (A and D) and the 31-kDa fragment (B, C, and E) was measured and presented, compared to the control (the band intensity, 1.00).

As shown in Fig. 3A, CIP treatment resulted in complete tyrosine dephosphorylation of p130cas, as detected with the 4G10 antibody. Dephosphorylated p130cas was easily cleaved by caspase-3, thus generating larger amounts of the 31-kDa cleavage fragment (Fig. 3B). These results confirm the theory that dephosphorylated p130cas is more susceptible to cleavage by caspase-3.

LAR, a transmembrane receptor-like protein tyrosine phosphatase, dephosphorylates p130cas [18], while PP1 is a general protein serine/threonine phosphatase. When immunoprecipitates of p130cas (using Cas-2 Ab) were treated with phosphatase, PP1, or LAR and subsequently exposed to caspase-3, p130cas treated with PP1 or LAR was more effectively cleaved and generated larger amounts of the 31-kDa cleavage fragment (Fig. 3C). To further determine whether dephosphorylation is critical for cleavage by caspase-3, immunoprecipitates were preincubated with sodium orthovanadate (Na_3VO_4) to inhibit the phosphatase activity of PP1 and LAR. Na_3VO_4 treatment effectively inhibited p130cas cleavage and to some extent, intact p130cas was recovered from PP1/LAR-treated samples (Fig. 3C). These results additionally support the theory that the caspase-mediated cleavage of p130cas during apoptosis is phosphorylation-dependent.

In vivo/in vitro cleavage of phosphorylation mimic mutants of p130cas

We previously identified two cleavage sites ($DVPD^{416}G$ and $DSPD^{748}G$) of caspase-3 in p130cas and showed that the cleavage of $DVPD^{416}G$ generates a 74-kDa fragment. Further cleavage at the $DSPD^{748}G$ site generates a 31-kDa fragment (Fig. 4A). Here, we demonstrate the phosphorylation-dependent cleavage of p130cas by caspase-3. To confirm that phosphorylation at Ser and Tyr residues adjacent to the caspase-3 cleavage sites affects p130cas cleavage, these residues were substituted with either Ala to generate non-phosphorylated protein or Glu to mimic phosphorylated forms. In single mutants of both glutamate and alanine ($S^{411}E/A$, $S^{743}E/A$, and $Y^{751}E/A$), similar amounts of the 31-kDa cleavage fragment were generated (Fig. 4B). In contrast, the phosphorylation mimic double Glu

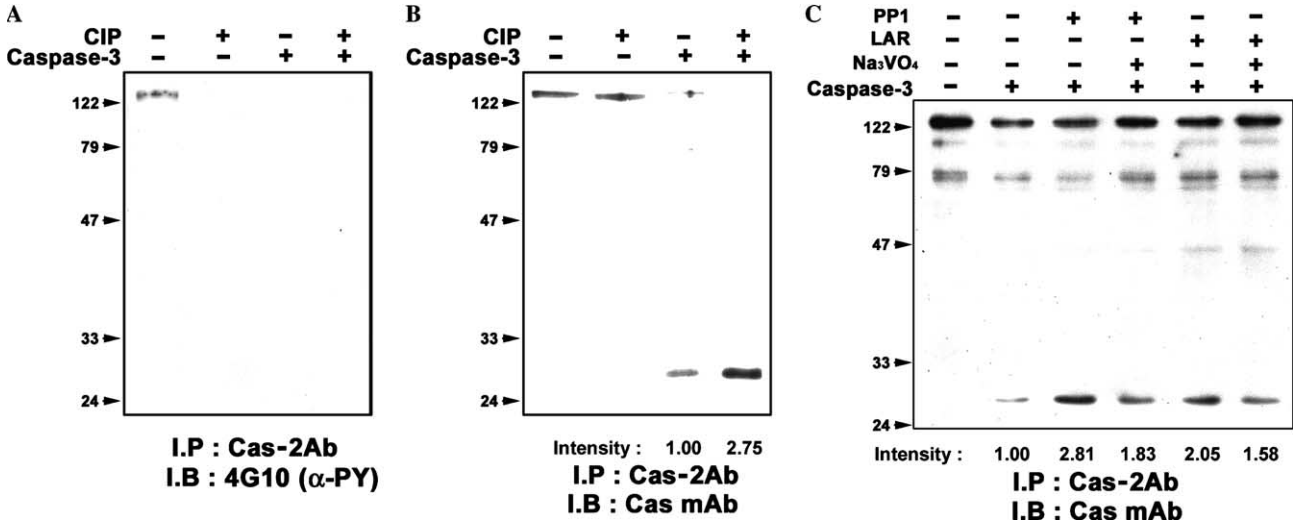

Fig. 3. Effect of p130cas dephosphorylation on caspase-mediated cleavage. (A) Cells grown for 48 h were lysed and immunoprecipitated with Cas-2Ab. For in vitro dephosphorylation of p130cas, immunoprecipitates were treated with calf intestinal phosphatase (CIP, 5U) for 30 min at 37 °C, followed by exposure to caspase-3. The p130cas protein was completely dephosphorylated by CIP and tyrosine phosphorylation in cleavage fragment of p130cas was not detected. (B) The blot was reprobed with Cas mAb. Increased concentrations of the 31-kDa cleavage fragment were observed in CIP-treated cell lysates. (C) p130cas immunoprecipitated with Cas-2 Ab was incubated with transmembrane tyrosine phosphatase, LAR (10 U) and common Ser/Thr phosphatase, PP1 (1 U) for 30 min at 30 °C. Sodium orthovanadate (Na_3VO_4), an inhibitor of phosphatase, was employed to inhibit phosphatase activity. The relative intensity of the 31-kDa band was presented, compared to the control (the band intensity, 1.00).

mutants effectively attenuated cleavage by caspase-3, compared to the double Ala mutants. The double $S^{743}E/Y^{751}E$ mutant displayed the greatest resistance to caspase-mediated cleavage (Fig. 4C). Our results indicate that phosphorylation of Ser^{743} and Tyr^{751} located immediately adjacent to the caspase recognition site affects the production of the 31-kDa cleavage fragment. To confirm this in vivo, cells were transiently transfected with pFLAG-CMV-5c vectors containing double or single mutant p130cas DNA and exposed to etoposide for 18 h. In wild-type and single mutant p130cas-transfected cells, no differences in caspase-mediated cleavage of exogenous p130cas (immunoprecipitated by FLAG-Ab) were noted during apoptosis. However, the double mutants were effectively resistant to caspase-mediated cleavage and generated significantly lower amounts of the 31-kDa fragment (Fig. 5A). When the same blot was reprobed with Cas mAb, endogenous p130cas in all double mutant cas-transfected cells was cleaved and revealed a similar cleavage pattern. These results are consistent with in vitro data, thus confirming that cleavage of p130cas is modulated by phosphorylation.

Discussion

We previously reported etoposide-induced proteolytic cleavage of p130cas in Rat-1 cells during apoptosis and identified two proteolytic cleavage sites for caspase-3 [1]. Proteolysis of p130cas contributes to the morphological characteristics of apoptotic cells that reflect the disassembly of focal adhesion complexes and interruption of survival signals from ECM. In this study, we demonstrate that caspase-mediated cleavage of p130cas is highly dependent on the phosphorylation state of the protein. Several results indicate that phosphorylated p130cas is resistant to cleavage by caspase-3 and that p130cas dephosphorylation precedes cleavage by caspase-3 during apoptosis. First, loss of p130cas phosphorylation was observed during etoposide-induced apoptosis. Second, p130cas phosphorylated by LPA was resistant to cleavage by caspase-3, both in vivo and in vitro. Third, p130cas dephosphorylated by CIP, LAR, and PP1 was effectively cleaved by caspase-3 in vitro and in vivo. These results are in accordance with previous reports that FAK dephosphorylation by DCVC precedes the activation of apoptotic machinery, such as caspases, as well as the proteolysis of FAK [32]. Modification of susceptibility to caspase-mediated cleavage by phosphorylation has been demonstrated in previous studies: phosphorylation of I-κB [19] and presenilin-2 [20] at serine residues adjacent to the caspase recognition site inhibits cleavage by caspase. In addition, phosphorylation-dependent cleavage during apoptosis has been reported for Akt [33] and α-adducin [34].

The optimal target tetrapeptide cleavage sequence of caspase-3, DXXD, is highly dependent on the primary amino acid sequence preceding the essential aspartate residue [35]. In a previous report, we identified two putative cleavage sites of p130cas, specifically at $DVPD^{416}$ and $DSPD^{748}$ [1]. Since p130cas contains numerous phosphorylation sites, specifically, Ser^{411}, Ser^{743}, and Tyr^{751}, which are adjacent to the cleavage sites (Asp^{416} and Asp^{748}), it is possible that phosphorylation

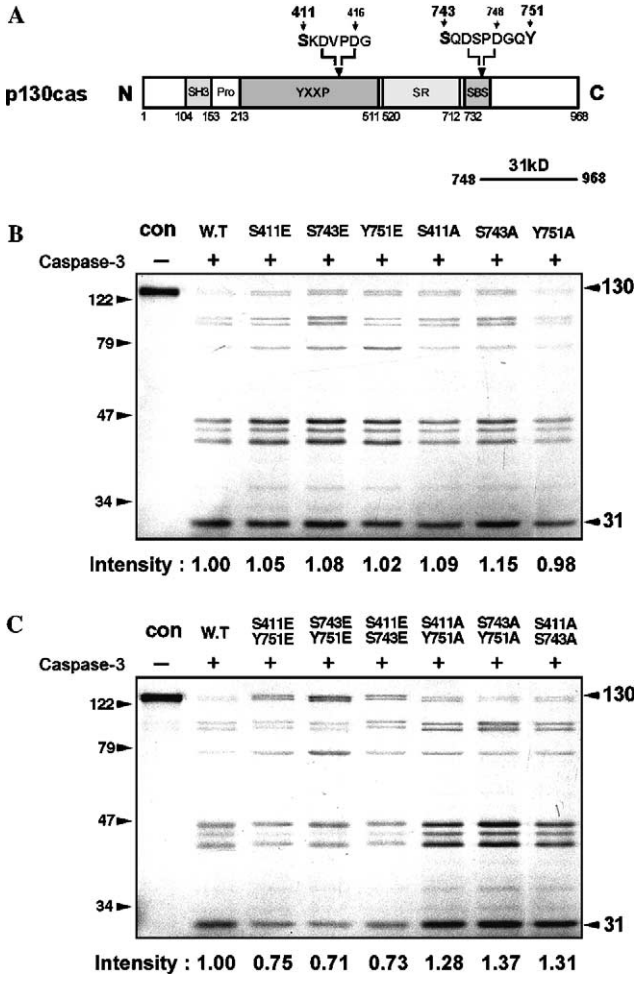

Fig. 4. In vitro cleavage of p130cas mutants. (A) Schematic diagram of the structure of p130cas with two identified cleavage sites and functional domains. The following regions are indicated: SH3 domain (SH3), proline-rich sequence (Pro), substrate domain (YXXP, tyrosine phosphorylation sites), serine-rich domain (SR), and Src binding site (SBS). The 31-kDa cleavage fragment is specified and the putative phosphorylation sites (S411, S743, and Y751) adjacent to the cleavage sites are indicated. (B) The phosphorylation sites were substituted with either Ala (S411A, S743A, and Y751A) or Glu (S411E, S743E, and Y751E). Single-point mutants of p130cas were translated in vitro and cleaved by caspase-3. (C) Double point Glu mutants (S411E/Y751E, S743E/Y751E, and S411E/S743E) or Ala mutants (S411A/Y751A, S743A/Y751A, and S411A/S743A) were translated in vitro and subjected to caspase assays. The relative intensity of the 31-kDa band was presented, compared to the control (the band intensity, 1.00).

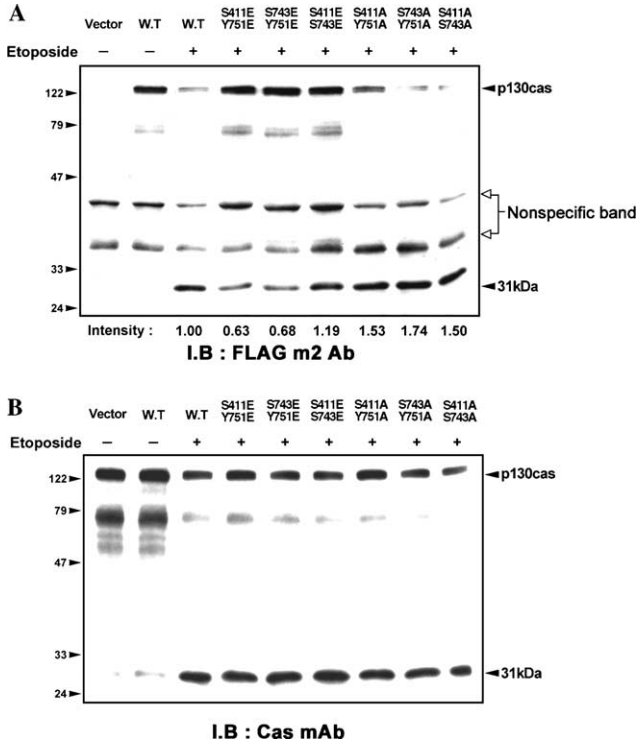

Fig. 5. In vivo cleavage of p130cas mutants. (A) Cells transfected with FLAG-tagged p130cas double mutants were cultured for 48 h and exposed to 40 μM etoposide for 18 h. Cell lysates were immunoblotted with FLAG mAb. Double Glu mutants were more resistant to cleavage than Ala mutants. (B) The same samples were immunoblotted with Cas mAb. The relative intensity of the 31-kDa band was presented compared to the control (the band intensity, 1.00).

at these sites affects protein cleavage by caspase-3. Our findings indicate that phosphorylation of p130cas inhibits caspase-mediated cleavage in vitro and in vivo. Single substitution of the phosphorylation sites of p130cas with an unphosphorylatable Ala residue and mimicking phosphorylated residues using Glu does not inhibit cleavage by caspase-3. However, a double substitution with glutamate appears to attenuate caspase-mediated cleavage. Moreover, tyrosine phosphorylation (Y751) adjacent to the cleavage site (D748) renders the protein more resistant to caspase-mediated cleavage, both in vitro and in vivo. These results support data showing that tyrosine phosphorylation of p130cas by LPA results in significantly reduced amounts of the 31-kDa fragment, while treatment of the protein with LAR generates increased quantities of the cleavage fragment.

p130cas is a substrate of several kinases including FAK [36,37], p60Src [38,39], and p60Src family tyrosine kinases [26] and is additionally a target of several other protein tyrosine phosphatases (PTPs), such as PTP-PEST [40] and PTP-1B [41]. Moreover, since p130cas contains multiple tyrosine phosphorylation sites, different PTPs may preferentially dephosphorylate distinct sites. Therefore, p130cas phosphorylation/dephosphorylation may be modulated by kinase/phosphatases during apoptosis. A number of results support this theory. For example, FAK that phosphorylates p130cas is cleaved by caspases and its degradation contributes to the loss of paxillin from focal adhesions [32]. The degradation of p130cas also correlates temporally with the onset of apoptosis. Moreover, initiation of p130cas cleavage blocks paxillin binding to the protein and the resultant localization of paxillin within focal adhesion sites [1]. Loss of FAK activity may contribute to the loss of p130cas phosphorylation during apoptosis. Alternatively, LAR, a tyrosine phosphatase, preferentially

dephosphorylates p130cas, which is critical for its stability. Moreover, overexpression of LAR leads to instability and downregulation of p130cas during LAR-induced apoptosis [17,18]. It is possible that degradation of p130cas contributes to multiple signaling pathways that are essential for normal cell growth, survival, and cell death. Interestingly, the two Src binding motifs located at the sequence RPLPSPP (733–739) and Tyr762 near the C-terminus of p130cas are separated by caspase-3 cleavage at the D748 site, and phosphorylation of Tyr751 adjacent to the caspase recognition site attenuates p130cas cleavage. Since dephosphorylation of p130cas occurs prior to protein degradation, the Tyr751 residue may be a primary target for phosphatases during apoptosis.

In summary, we demonstrate that caspase-mediated cleavage of p130cas is highly dependent on the phosphorylation status of p130cas and that phosphorylation at Ser or Tyr residues adjacent to the caspase recognition site in p130cas modulates protein cleavage during apoptosis.

Acknowledgments

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