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Cleavage of p130^{Cas} in Anoikis

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Abstract p130^{Cas} is a multifunctional signaling adaptor protein. It integrates and relays signals generated from a variety of extracellular stimuli and regulates a number of cellular activities including cell death. In this study, we analyzed the regulation and function of p130^{Cas} in anoikis, a type of apoptosis caused by disruption of cell-matrix interactions. We found that p130^{Cas} was specifically cleaved during anoikis in anoikis-sensitive epithelial cells, but not in anoikis-resistant tumor cells. There is a close correlation between p130^{Cas} cleavage and anoikis. Furthermore, we found that the cleavage of p130^{Cas}, as well as another focal adhesion component FAK, is different from that of caspase substrate PARP and spectrin. Although caspases and calpain were found to be involved in the cleavage of p130^{Cas}, there appear to be other unidentified proteases that are mainly responsible for the cleavage of p130^{Cas}, particularly at the early stage of anoikis. Overexpression of the p130^{Cas} cleavage product induced apoptosis. Taken together, these data suggest that there are novel proteases involved in the cleavage of p130^{Cas} during anoikis, which may be functionally involved in the onset of anoikis. p130^{Cas} may have a dual role in the regulation of anoikis. On one hand, it mediates a survival signal from cell-matrix interactions when cells are attached to the extracellular matrix. On the other hand, it participates in executing cell death when cell-matrix interactions are disrupted. These observations provide new insights into the understanding of the function of p130^{Cas} and the molecular mechanism of anoikis. J. Cell. Biochem. 91: 325–335, 2004. © 2003 Wiley-Liss, Inc.

Key words: p130^{Cas}; caspase; calpain; anoikis

Extracellular matrix (ECM) plays a critical role in regulating morphology, growth, migration, and differentiation of mammalian cells [Fisher, 1994; Cooray et al., 1996]. Disruption of cell–matrix interactions results in apoptosis of many types of cells, particularly epithelial and endothelial cells [Fisher, 1994]. This type of apoptosis is termed anoikis [Fisher, 1994]. The molecular mechanism of anoikis is poorly understood. Understanding of the molecular nature of anoikis is important to the study of tumor cell survival and growth because more than 80% of malignant tumor cells are derived from epithelial cells and are generally anoikis-resistant [Wei et al., 2001].

The response of cells to matrix proteins is mediated primarily through the integrin family of adhesion receptors, which are in complexes with intracellular signaling proteins at focal adhesions. Disruption of cell–matrix interactions causes changes in cell morphology, organization of cytoskeleton, and in modification and interaction of focal adhesion proteins. Changes in focal adhesion complexes upon cell detachment are likely involved in the disruption of cell adhesion-mediated cell survival signals and the initiation of anoikis. Focal adhesion complexes are composed of intracellular signaling proteins such as focal adhesion kinase (FAK), paxillin, pp60Src, p130^{Cas}, vinculin, tensin, talin, and α-actinin. Among them, FAK, p130^{Cas}, and paxillin are closely associated with changes in the cytoskeleton. Reorganization of the actin and cytoskeleton is accompanied by changes in the modifications and activities of these proteins [Tanaka et al., 1997; Zhu et al., 1998; Kook et al., 2000].

p130^{Cas} is an signaling adaptor protein, which contains multiple functional domains including a SH3 domain, several proline-rich regions, and a cluster of tyrosine phosphorylation sites that are SH2 binding motifs [Sakai

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et al., 1994]. This unique molecular structure enables p130^{Cas} to interact with many signaling proteins, such as FAK, Src, Crk, Grb2, PI 3K, Nck, and PLC-γ [Sakai et al., 1994; Burnham et al., 1996]. These multiple interactions allow p130^{Cas} function as a signal assembly protein to integrate and coordinate signals to control cellular activities, including cell transformation, cell adhesion, actin organization, and cell migration [Nojima et al., 1996; Nakamoto et al., 1997; Klemke et al., 1998; Nakamura et al., 1998]. Recently, a number of experiments suggest that p130^{Cas} is also involved in the regulation of cell death [Chan et al., 1999; Weng et al., 1999; Weyant et al., 2000; Harrington et al., 2001; Lesay et al., 2001; Shim et al., 2001; Wang et al., 2001]. Overexpression of wild-type p130^{Cas} protects cells from apoptosis [Weng et al., 1999; Cho and Klemke, 2000], whereas overexpression of dominant-negative forms of p130^{Cas} induces cell death [Chan et al., 1999; Almeida et al., 2000]. The finding that p130^{Cas} is cleaved during etoposide-induced apoptosis of Rat-1 cells also supports a role of p130^{Cas} in apoptosis [Kook et al., 2000].

In this study, we examined changes in focal adhesion proteins during anoikis and found that p130^{Cas} was cleaved early during anoikis and that there is a close correlation between the cleavage of p130^{Cas} and the initiation of anoikis. Furthermore, we found evidences to suggest that novel proteases, in addition to caspases and calpains, were also involved in the cleavage of p130^{Cas}. These observations provide new insights into the understanding of the function of p130^{Cas} and the molecular mechanism of anoikis.

MATERIALS AND METHODS

Cell Culture and Anoikis Assay

Immortalized human bronchial epithelial cell line HBE4-E6/E7 and Madin–Darby canine kidney epithelial cell line MDCK and lung adenocarcinoma cell lines A549, H1792, SK-LU-1, and H23 were purchased from the American Type Culture Collection (Manassas, VA). The cells, except HBE4-E6/E7, were maintained in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum. HBE4-E6/E7 was cultured in Keratinocyte-SFM supplemented with brain pituitary extracts and human recombinant EGF as suggested by the manufacturer (GIBCO-BRL, Grand Island, NY).

The anoikis assay was essentially performed as described by Frisch and Francis [1994]. Briefly, cells were grown to confluence in 100-mm tissue culture dishes. Cells were then trypsinized, and 10^6 cells were plated into 60-mm polyHEMA-coated Petri dishes. The polyHEMA- coated dishes were prepared by applying 2-ml polyHEMA solution (10 mg/ml polyhydroxyethylmethacrylate, Aldrich Chemical Co., Milwaukee, WI, in ethanol) onto the dish, dried in tissue culture hood, repeated once, followed by extensive wash with PBS (>3 times). Cells cultured in the polyHEMA dishes were collected by pipetting; cells cultured in regular tissue culture dishes were collected by scraping. Cytosolic nucleic acids, which contain fragmented genomic DNA, were extracted with 0.6-ml cell lysis buffer containing 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris (pH 7.4). The cell lysates were extracted with phenochloroform three times, precipitated with ethanol, and analyzed by electrophoresis using a 1.5% agarose gel. The gel was incubated in a RNase A-containing solution (5 μg/ml) to digest away RNAs before photographing.

Determination of Apoptotic Cells by Flow Cytometry

Cells were fixed in 70% ethanol for 10 min at room temperature, resuspended in 0.5 ml solution of 20 μg/ml propidium iodide and 100 μg/ml DNase-free Rnase in PBS, and incubated for 30 min at room temperature. For flow cytometric analysis, at least 10,000 cells were evaluated using a FACSCalibur™. Cell cycle distribution and pre-G_1 fraction were determined and quantitated using the Cell QUEST™ program. Cells in Sub-G_1 fraction were defined as apoptotic cells.

Antibodies and Immunoblotting

Mouse monoclonal antibodies against p130^{Cas}, FAK, and paxillin were purchased from Transduction Laboratory (San Diego, CA). Mouse monoclonal antibody to Src and rabbit polyclonal antibody to p130^{cas} were purchased from Santa Cruze Biotech, Inc. (Santa Cruze, CA). Rabbit polyclonal antibodies to cleaved caspase 3, phospho-Akt, Akt, phospho-MAPK, and MAPK were purchased from New England BioLabs (Beverly, MA). Mouse monoclonal antibody to PARP was from Pharmagen (San Diego, CA). All commercially purchased antibodies were used as recommended by the manufactures.

For immunoblotting, cells were lysed in a modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), aprotinin (5 μg/ml), and 1 mM Na_3VO_4]. Cell nuclei were removed from lysates by centrifugation for 10 min. Protein concentration was determined with the Bradford reagent (Bio-Rad). Proteins were resolved by SDS–PAGE (7.5%) and transferred to a nitrocellulose membrane. For immunoblotting, membranes were pre-incubated for 1 h at room temperature in a solution containing 5% fat-free milk, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% (v/v) Tween-20. After the pre-incubation, the membranes were incubated with primary antibodies at 4°C overnight. The membranes were then washed three times with the blocking solution and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison), followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ).

Plasmid Constructs

A construct of p130^{Cas} encoding the C-terminal amino acids from residue 665 (a putative caspase cleavage site) to 881 resembling the cleavage product of p130^{Cas} was subcloned in frame into an EGFP c2 expression vector (Clontech, Palo Alto, CA) to generate green fluorescent protein (GFP) fusion protein. The construct was verified by restriction enzyme digestion mapping. The GFP–p130cas fusion protein produced from the construct was analyzed by transfection and immunoblotting.

Transfection and Apoptosis Assay

Cells (293 or A549) were plated onto 30-mm cell culture dishes at 60∼75% confluence in DMEM supplemented with 10% FBS. Cells were transfected using the Lipofectamine Plus transfection kit (GIBCO-BRL). Plasmid DNA (1 μg) was used per transfection. Protein expression of the transfected constructs was analyzed by visual inspection of the green fluorescence produced by the GFP fusion proteins and by immunoblotting of lysates of the transfected cells 24 h after transfection. For apoptosis analysis, three separate transfections were performed for the construct. After expression of the fusion protein, cells were incubated in suspension for 24 h and stained with DAPI and analyzed under a Nikon-inverted fluorescence microscope equipped with dual FITC/DAPI filters. Total GFP positive and GFP positive with fragmented nuclei apoptotic cells were counted. The apoptotic index represents the percentage of GFP-positive cells that were scored as apoptotic after DAPI staining.

Treatment of Cells With Caspases and Calpain Inhibitors

The specific caspases inhibitors (ZVAD, ZAEVD, ZVDVAD, and ZWEHD) and calpain inhibitors (ALLM, ALLN, Calpeptin, and EST) were purchased from Calbiochem (La Jolla, CA) and dissolved in DMSO. In experiments that cells were treated with the inhibitors, the same volumes of DMSO were added to the controls.

RESULTS

p130^{Cas} Is Cleaved During Anoikis in Anoikis-Sensitive Epithelial Cells, but not in Anoikis-Resistant Tumor Cells

Immortalized epithelial cells (HBE4-E6/E7, MDCK) and human lung adenocarcinoma cells (A549, H1792, SK-LU-1, and H23) were analyzed and compared for their sensitivity to anoikis by culturing them in suspension on polyhydroxyethylmethacrylation (poly-HEMA)-coated petri-dishes. FACS analysis demonstrated that the percentages of cell death of the control epithelial cells MDCK and HBE4-E6/E7 were more than 30% when they were cultured in suspension for 24 h, whereas that of most of the lung tumor cells was less than 10% (Fig. 1A). The MDCK control cells were completely dead after 72 h incubation, but the tumor cells survived and continued to grow in suspension (data not shown). These observations are consistent with our previous report [Wei et al., 2001], confirming that normal epithelial cells are anoikis-sensitive (anchorage-dependent), whereas most of the tumor cells are relatively anoikis-resistant (anchorage-independent).

Since p130^{Cas} was found to be cleaved during etoposide-induced apoptosis of Rat-1 cells [Kook et al., 2000], we looked for possible cleavage of p130^{Cas} during anoikis in normal versus tumor cells. We found that p130^{Cas} was indeed cleaved during anoikis in anoikis-sensitive HBE4-E6/E7 and MDCK cells, but not in the anoikis-resistant tumor cells or in the attached growing cells (Fig. 1B). The identity of the cleavage product was further confirmed by using independent antibodies that specifically recognize

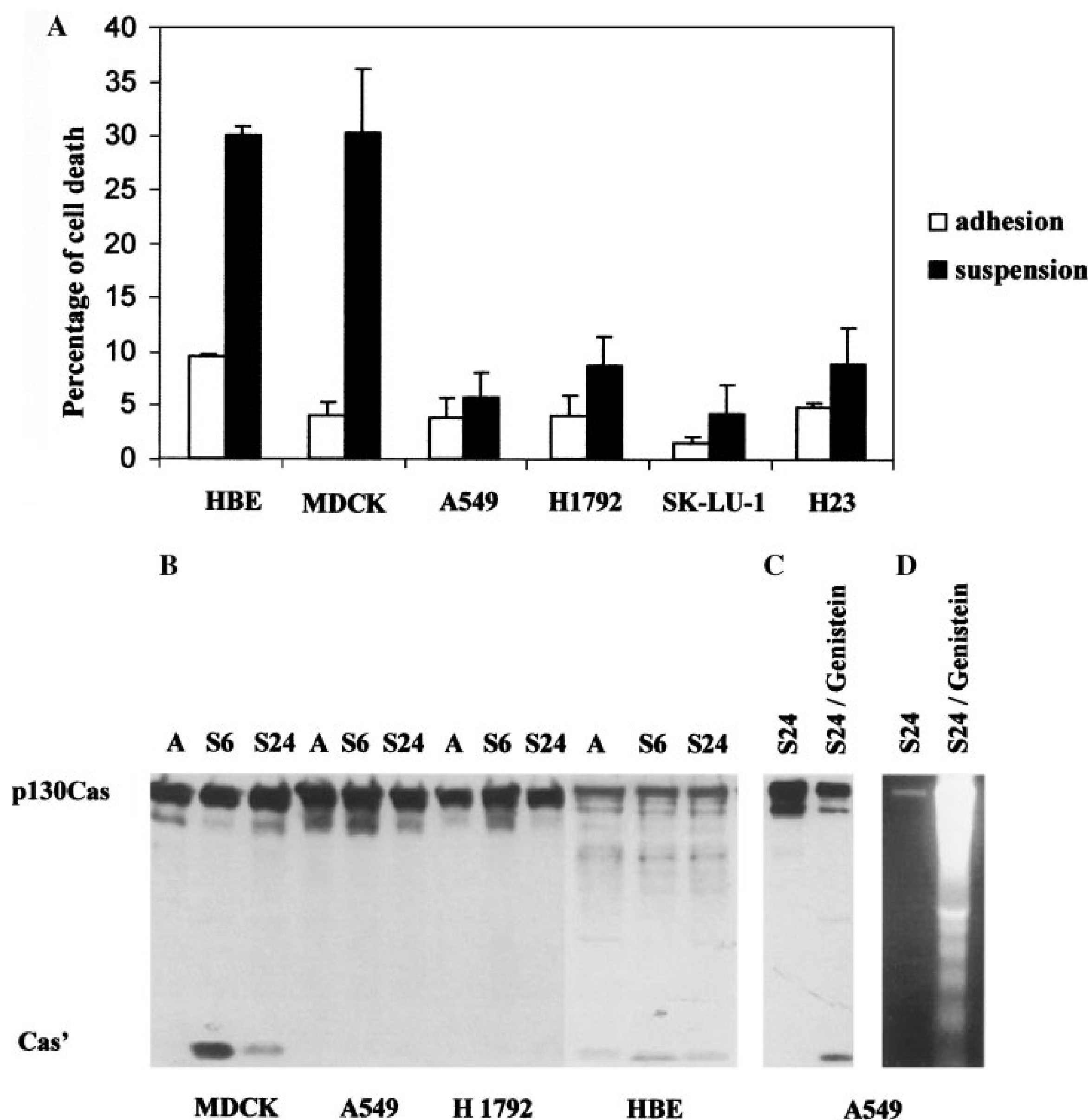

Fig. 1. Cleavage of p130^{Cas} in anoikis. **A**: Percentage of cell death caused by culturing in suspension. Normal (MDCK and HBE) and human lung adenocarcinoma cells (A549, H1792, SK-LU-1, and H23) were cultured in suspension on polyHEMA-coated Petri dishes for 24 h. Meanwhile the same amount of the cells was cultured on the regular tissue culture dishes for 24 h as the control. The apoptosis was measured with propidium iodide staining by flow cytometry. The data presented are the mean from three independent experiments. **B**: Proteolytic cleavage of p130^{Cas} in anoikis-sensitive cells (MDCK and HBE) and anoikis-resistant cells (A549 and H1792). Cells were plated on regular tissue culture dishes or poly-HEMA-coated dishes for the indicated periods of time and were lysed with RIPA buffer. The total cell lysates were resolved by 7.5% SDS–PAGE and analyzed by Western blotting using a mAb to p130^{Cas}. A: attached cells. S6: cells cultured in suspension for 6 h. S24: cells cultured in suspension for 24 h. **C** and **D**: Anoikis-resistant lung tumor cells A549 was induced to undergo anoikis with genistein. A549 cells were cultured in suspension (S) with or without 0.2 mM genistein for 24 h. Half of the cells were lysed with RIPA buffer and the total cell lysates were resolved by 7.5% SDS–PAGE and immunoblotted with antibody to p130^{Cas} (C). The other half of the cells were used for the isolation of the low-molecular weight DNA and analyzed by agorose (1.5%) gel electrophoresis (D).

p130^{Cas} (data not shown). The cleavage of p130^{Cas} occurred rather rapidly, within 1 h after the cells were plated on poly-HEMA-coated dishes (Fig. 2). These data suggest that p130^{Cas} is cleaved during the early stages of anoikis and that this cleavage is prevented in the anoikis-resistant tumor cells. When the anoikis-resistant lung tumor cells were induced to die by

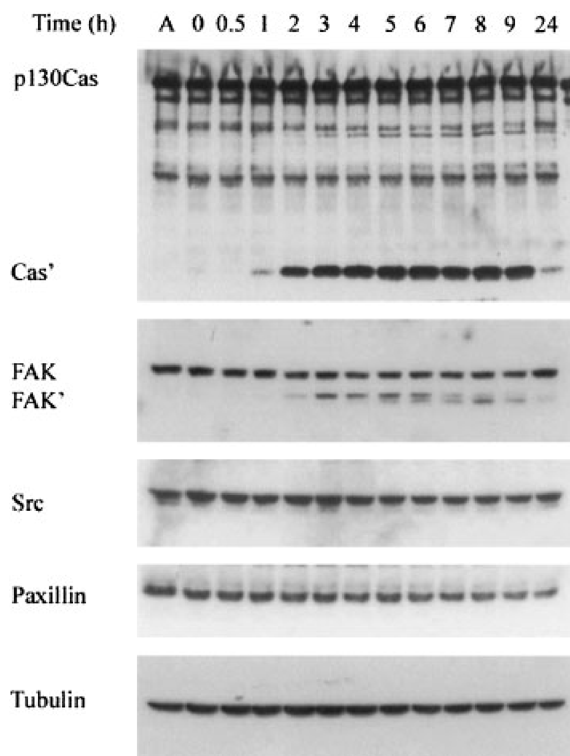

Fig. 2. Time-course of the cleavage of focal adhesion proteins during anoikis in MDCK cells. MDCK cells were cultured on regular tissue culture dishes (**A**) or in suspension for the indicated time periods and lysed with RIPA buffer. The total cell lysates were resolved by 7.5% SDS–PAGE and analyzed by Western blotting using mAbs to p130^{Cas}, Src, FAK, paxillin, and tubulin sequentially.

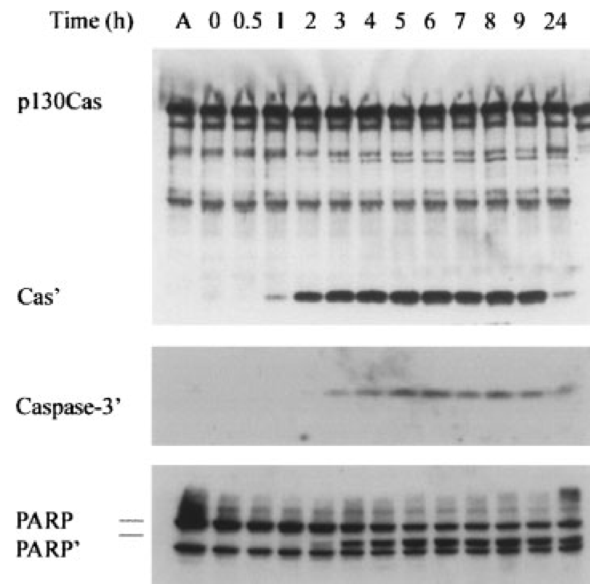

Fig. 3. Time-course of cleavage of p130^{Cas}, caspase 3, and PARP during anoikis in MDCK cells. MDCK cells were cultured on regular tissue culture dishes (**A**) or in suspension for the indicated time periods and lysed with RIPA buffer. Total cell lysates were resolved by 7.5% SDS–PAGE and analyzed by Western blotting using mAbs to p130^{Cas}, PARP, and tubulin, and a rabbit Ab to cleaved caspase-3.

genistein, a protein tyrosine kinase inhibitor, the cleavage of p130cas occurred in these tumor cells (Fig. 1C). There appears to be a close correlation between p130^{Cas} cleavage and the onset of anoikis.

We then examined possible cleavage of other components of the focal adhesion complexes during anoikis. We found that in addition to p130^{Cas}, FAK, but not Src and paxillin, was also cleaved during anoikis with similar kinetics (Fig. 2), suggesting that the cleavages of p130^{Cas} and FAK are specific.

Caspases and Calpain Are Involved in the Cleavage of p130^{Cas} in Anoikis

Caspases are responsible for initiation and executing apoptotic cell death by cleaving a number of critical homeostatic, repair, and structural proteins in the dying cells [Nicholson and Thornberry, 1997]. p130^{Cas} contains putative caspase cleavage sites and has been reported to be cleaved by caspase-3 in etoposide-induced apoptotic rat-1 cells [Kook et al., 2000]. To find out whether caspase-3 is also responsible for the cleavage of p130^{Cas} in anoikis, we analyzed the cleavage of p130^{Cas} and compared with that of caspase-3 and its substrate PARP (Fig. 3). Cleavage products of p130^{Cas} were detected as early as 1 h after MDCK cells were plated into suspension, reached its maximum within 4 h, whereas the cleavage of caspase-3 occurred 3 h after the cells were incubated in suspension. Similarly, the cleavage of PARP also began 3 h after the cell detachment, which correlated with that of caspase-3 (Fig. 3). These data suggest that cleavage of p130^{Cas} occur before caspase-3 activation.

To further investigate the involvement of caspases in the cleavage of p130^{Cas} during anoikis, ZVAD (inhibitor of caspases 1, 3, 4, 7), ZAEVD (inhibitor of caspases 6, 8, 9, 10), ZVDVAD (inhibitor of caspase 2), and ZWEHD (inhibitor of caspase 5, 1, 4) were added into MDCK cells cultured on poly-HEMA-coated dishes. Cleavage of PARP and spectrin were completely blocked by ZVAD and ZAEVD, whereas the cleavage of p130^{Cas} was only partially blocked (Fig. 4A and data not shown). These data suggest that although caspases do play a role in the cleavage of p130^{Cas} during

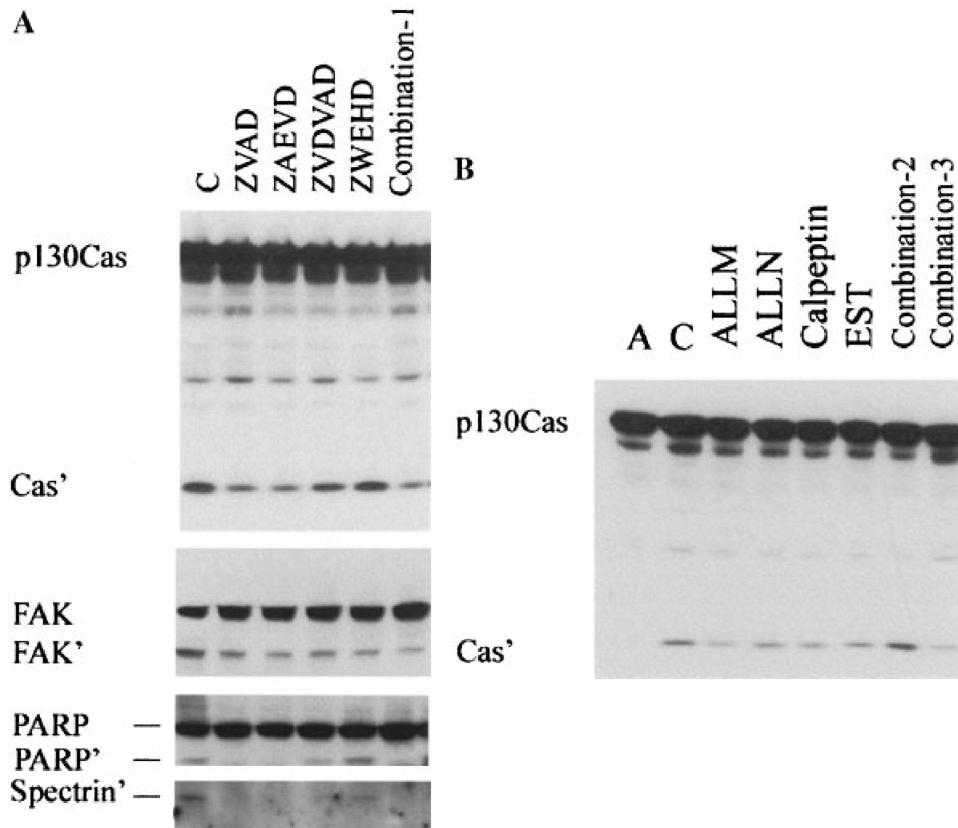

Fig. 4. Effects of caspases and calpain inhibitors on the cleavage of p130^{Cas}. MDCK cells were incubated in suspension for 5 h with or without 25 μM caspases inhibitors ZVAD, ZAEVD, ZVDVAD, or ZWEHD (**A**); or 25 μM calpain inhibitors ALLM, ALLN, calceptin, or EST (**B**). Combination-1 is a mixture of all the caspases inhibitors at a concentration of 25 μM each (A); Combination-2 is a mixture of all calpains inhibitors at a concentration of 25 μM each (B); Combination-3 is a mixture of both the caspases and calpains inhibitors at a concentration of 25 μM each (B). Total cell lysates were resolved in 7.5% SDS–PAGE and analyzed by Western blotting using mAbs to p130^{Cas}, FAK, PARP, or spectrin. The cleavage products are indicated. A: attached cells without addition of inhibitors (B); **C**: cells cultured in suspension without addition of inhibitors (A and B).

anoikis, there are other proteases involved, particularly in the early stage of p130^{Cas} cleavage.

Calpain, a calcium-activated proteolytic enzyme, has also been shown to be involved in the cleavage of p130^{Cas} in etoposide-induced apoptosis in Rat-1 and L929 cells [Shim et al., 2001] and in cleavage of FAK in degraded-collagen-treated smooth muscle cells [Carragher et al., 1999]. Therefore, we investigated possible involvement of calpain in the cleavage of p130^{Cas} in anoikis by using various calpain specific inhibitors, including ALLM, ALLN, calceptin, and EST. Similar to the caspase inhibitors, the calpain inhibitors also partially inhibited the cleavage of p130^{Cas} in MDCK cells, suggesting that calpains are also involved in the cleavage of p130^{Cas} (Fig. 4B). Combination of both inhibitors to caspases and calpain still could not completely block the cleavage of p130^{cas} (Fig. 4B), suggesting that additional proteases are involved in the cleavage of p130^{Cas}, particularly at the early stage of anoikis.

To compare the cleavage of p130^{Cas} in anoikis with that in other types of apoptosis, we examined the time-course of p130^{Cas} cleavage in cisplatin-induced apoptosis of MDCK cells. As shown in Figure 5A, cleavage of p130^{Cas}, as well as FAK, occurred simultaneously with the cleavage of PARP and spectrin. Treatment of caspase inhibitors completely blocked the cleavage of PARP and spectrin, but only partially inhibited the cleavage of p130^{Cas} and FAK (Fig. 5B). These data suggest that the cleavages of focal adhesion proteins p130^{Cas} and FAK are different from that of PARP and spectrin. There are proteases other than caspases and calpain that are involved in the cleavage of p130^{Cas}. These proteases may be activated before the activation of caspases and calpain upon cell detachment. The early cleavage of p130^{Cas} by

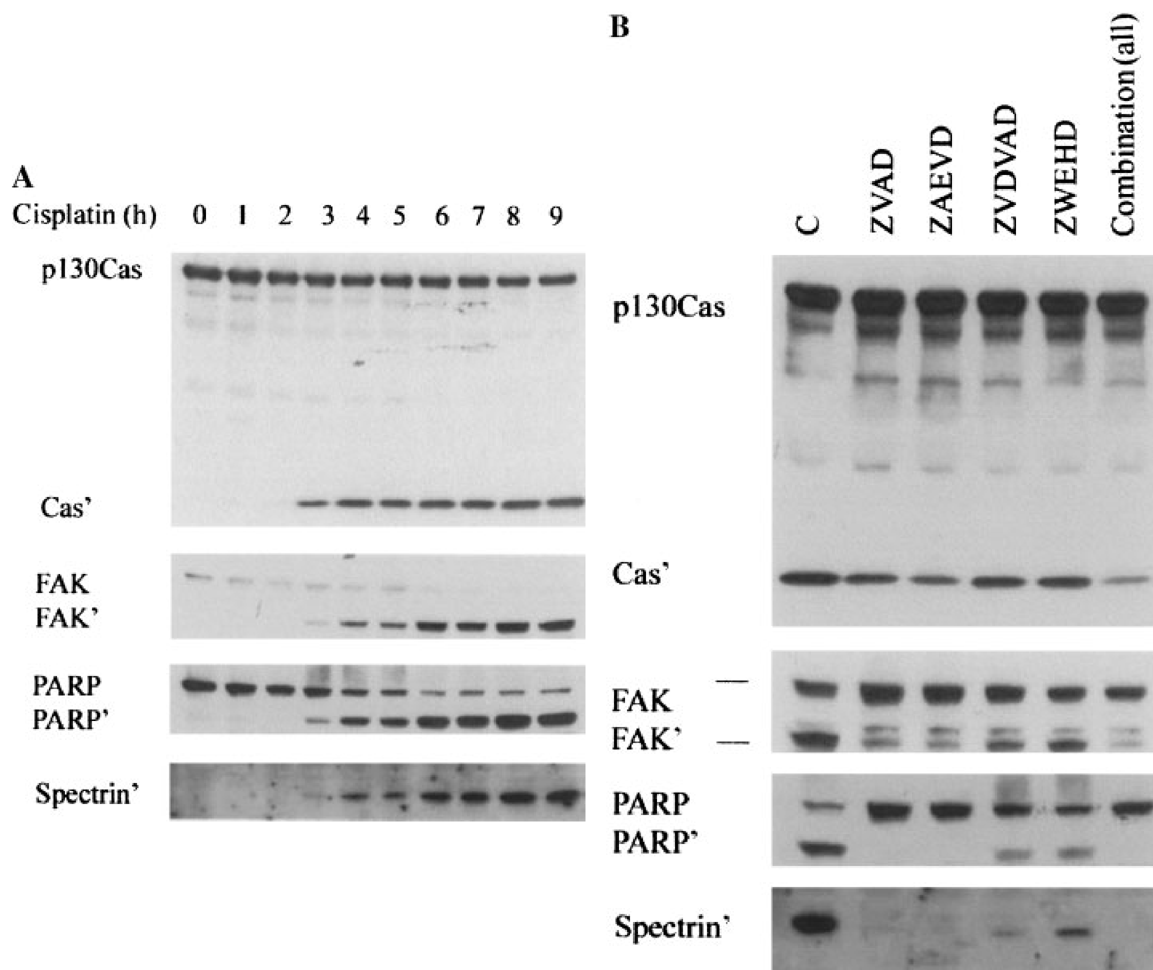

Fig. 5. **A**: Cleavage of p130^{Cas} in cisplatin-induced cell death. MDCK cells cultured on regular tissue culture dishes were exposed to cisplatin (0.1 mM) for the indicated time-periods and lysed with RIPA buffer. The total cell lysates were resolved in 7.5% PAGE and analyzed by Western blotting using antibodies against p130^{Cas}, FAK, PARP, and spectrin. The cleavage products are indicated. **B**: Effects of caspase inhibitors on the cleavage of p130cas, FAK, PARP, and spectrin in cisplatin-induced apoptosis. MDCK cells on regular tissue culture dishes were pre-treated with the caspase inhibitors at 100 μM for 30 min and then exposed to cisplatin of 0.1 mM for 4 h. The total cell lysates were resolved in 7.5% PAGE and analyzed by Western blotting using antibodies against p130^{Cas}, FAK, PARP, and spectrin.

the unidentified proteases may function to initiate anoikis.

Cleavage of p130^{Cas} Is Functionally Involved in the Initiation of Anoikis

The data presented above indicate that cleavage of p130^{Cas} is closely associated with cell death and may have a role in the ignition of anoikis. To find out whether the cleavage of p130^{Cas} is functionally involved in executing cell death, we tested the possibility that the p130^{Cas} cleavage product may participate in the initiation of anoikis. We constructed a cDNA expression plasmid encoding a GFP (green fluorescence protein) p130^{Cas} C-terminal segment fusion protein, which mimics the p130^{Cas} cleavage product, and expressed this protein in the anoikis-resistant A549 cells. Cell death was analyzed with DAPI staining. As shown in Figure 6, 35% of the A549 cells transfected with p130^{Cas}–GFP fusion protein were dead when cultured in suspension for 24 h. The cells transfected with the vector alone, however, had only 2% cell death. These data suggest that cleavage of p130^{Cas} was likely the cause, rather than the consequence of cell death and that the cleavage of p130^{Cas} may participate in the initiation of anoikis.

To further understand the function and mechanism of p130^{Cas} cleavage in inducing cell

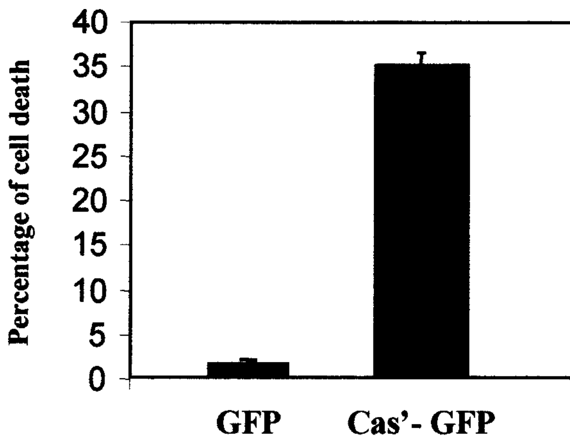

Fig. 6. Effects of overexpression of p130^{Cas} cleavage product on cell death. Anoikis-resistant tumor cell A549 was transfected with the GFP vector alone or the expression construct of GFP–p130^{Cas} C-terminal segment fusion protein and cultured in suspension for 24 h. A549 cells were analyzed for cell death with DAPI staining.

death, we analyzed the effect of overexpression of the p130^{Cas} cleavage product on phosphorylation of Akt and MAPK, two important cell survival signal mediators. As shown in Figure 7, overexpression of the p130^{Cas} cleavage product had no effect on Akt phosphorylation/activation, but reduced phosphorylation of MAPK, suggesting that the p130^{Cas} cleavage product may induce cell death through a pathway that lies upstream of MAPK, but independent of Akt.

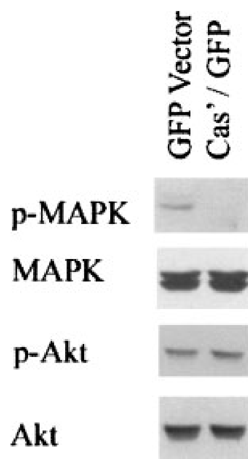

Fig. 7. Effects of p130^{Cas} cleavage on MAPK and Akt phosphorylation. 293 cells were transfected with the above constructs (over 80% of the cells were transfected). The transfected cells cultured in suspension for 24 h were resolved by 7.5% SDS–PAGE and analyzed by Western blotting analysis using rabbit antibodies to phospho-MAPK, phospho-Akt, MAPK, and Akt.

DISCUSSION

p130^{Cas} is a multifunctional signaling adapter protein. It integrates and relays signals generated from a variety of extracellular stimuli and regulates a number of cellular activities including cell transformation [Burnham et al., 1996; Nojima et al., 1996; Salgia et al., 1996; Honda et al., 1998], cell adhesion [Nojima et al., 1996; Vuori et al., 1996; Nakamura et al., 1998], actin organization [Nakamura et al., 1998], and cell migration [Cary et al., 1998; Klemke et al., 1998]. Recently, there are also evidence linking p130^{Cas} to the regulation of cell death/survival. It was reported that p130^{Cas} undergoes dephosphorylation during apoptosis induced by various agents ranging from anti-cancer drugs [Kook et al., 2000; Weyant et al., 2000; Shim et al., 2001], collagen gel overlay [Wang et al., 2001], adenosine/homocysteine [Harrington et al., 2001], UV irradiation [Chan et al., 1999], tyrosine phosphatase [Weng et al., 1999], to serum withdraw [Almeida et al., 2000]. Results from our own study indicate that anchorage-independent phosphorylation of p130^{Cas} prevents lung tumor cells from anoikis [Wei et al., 2002]. More direct evidence supporting an active role of p130^{Cas} in apoptosis came from overexpression experiments, in which overexpression of wild-type p130^{Cas} protects cells from tyrosine phosphatase-induced apoptosis [Weng et al., 1999] and increases survival of migratory cells [Cho and Klemke, 2000], whereas overexpression of dominant-negative forms of p130^{Cas} blocks FAK-mediated cell survival [Chan et al., 1999; Almeida et al., 2000].

In addition to phosphorylation regulation, proteolytic cleavage has also been shown to play an important role in regulating signaling proteins and cell signal transduction. It has been reported that caspase-dependent cleavage of the hematopoietic specific adapter protein Gads alters signaling from the T cell receptor by disrupting cross talks between SLP-76 and LAT [Berry et al., 2001]. FAK cleavage by calpain attenuates its kinase activity and induces its subcellular redistribution [Cooray et al., 1996]. Cleavage of FAK by caspase generates a FRNK-like polypeptide which prevents FAK from localizing to focal adhesions and may play an important role in the execution of apoptosis by disabling the anti-apoptotic function of FAK [Gervais et al., 1998]. Fyn and Lyn, two of the Src family members, were also reported to

be cleaved during induction of apoptosis. The cleavage of Fyn and Lyn alters their kinase activities and represents a new mechanism for the regulation of Src kinases [Luciano et al., 2001]. Recently, $p130^{Cas}$ was reported to be cleaved by caspase in etoposide-induced apoptosis of Rat-1 cells [Kook et al., 2000] and/or by calpain [Shim et al., 2001], which may contribute to the disassembly of focal adhesion complexes and interrupt survival signals from the ECM.

Anoikis is a type of apoptosis when adherent cells were denied from attachment to their ECM [Frisch and Francis, 1994]. Resistance to anoikis is a property shared by many malignant tumor cells and is the basis of tumor cell anchorage independence [Wei et al., 2001]. We have found that $p130^{Cas}$ and its anchorage-independent phosphorylation play an important role in protecting tumor cells from anoikis. In this study, we observed that $p130^{Cas}$, in addition to its change in tyrosine phosphorylation, was cleaved in anoikis-sensitive cells, but not in anoikis-resistant tumor cells upon cell detachment. However, when the anoikis-resistant tumor cells were induced to undergo cell death, cleavage of $p130^{Cas}$ was observed, revealing a good correlation between $p130^{Cas}$ cleavage and anoikis. To confirm the role of $p130^{Cas}$ cleavage in anoikis, we expressed a GFP (green fluorescence protein)–$p130^{Cas}$ fusion protein that mimics the $p130^{Cas}$ cleavage product in cultured cells. Expression of this fusion protein in anoikis-resistant tumor cells induced cell death, suggesting that $p130^{Cas}$ cleavage causes cell death and may initiate anoikis.

The mechanism by which $p130^{Cas}$ cleavage product induces apoptosis remains to be understood. $p130^{Cas}$ normally acts as a cell survival signal transducer. Cleavage of $p130^{Cas}$ may terminate its normal role as a survival factor and therefore disrupts the $p130^{Cas}$-mediated cell survival signal transduction. The cleavage products of $p130^{Cas}$ may serve a dual role in regulating cell death. On one hand, the cleavage products may function as dominant-negative agents to interfere with the normal $p130^{Cas}$; on the other hand, the cleavage products may function actively to turn on a death program. PI 3 kinase/Akt pathway and MAP kinase pathway are the two major pathways for cell survival and proliferation downstream of $p130^{Cas}$. In our study, overexpression of the $p130^{Cas}$ cleavage product did not affect the phosphorylation of Akt, but reduced the phosphorylation of MAPK, suggesting that $p130^{Cas}$ may mediate cell survival through MAPK, rather than PI 3K/Akt.

Our finding that the $p130^{Cas}$ cleavage occurs before the activation/cleavage of caspases and PARP during anoikis is intriguing. It suggests that proteases other than caspases are involved in the early stage of $p130^{Cas}$ cleavage and perhaps function in the initiation of anoikis. $p130^{Cas}$ was reported to be cleaved by both caspases and calpain in etoposide-induced apoptosis in Rat-1 and L929 cells [Shim et al., 2001]. By using calpain inhibitors, we found that calpain was also involved in the cleavage of $p130^{Cas}$ during anoikis. Calpain is a ubiquitous cysteine protease and has been shown to be activated by a number of apoptosis-inducing agents, such as IR, etoposide, or staurosporine [Waterhouse et al., 1998; Watters, 1999; Gao and Dou, 2000; Shim et al., 2001]. Calpain is usually activated in the later stage of apoptosis. However, calpain activation was reported to be upstream of caspases in radiation-induced apoptosis [Waterhouse et al., 1998]. Another recent study also showed that calpain activation occurred early in cancer drug-induced apoptosis [Mandic et al., 2002]. Calpain has been found to be localized at focal adhesions in a number of cells and several focal adhesion proteins, such as FAK, talin, vinculin, paxillin, have been identified as calpain substrates [Cooray et al., 1996; Wang, 2000]. It is conceivable that disassembling of focal adhesions during anoikis may somehow activate calpain, which then cleaves the focal adhesion proteins including $p130^{Cas}$. Our data also suggest the involvement of other proteases in the cleavage of $p130^{Cas}$ during anoikis because neither caspases and calpains inhibitors nor their combination could completely block the cleavage of p130cas in anoikis. The identity and the role of these proteases in the early stage of cleavage of $p130^{Cas}$ during anoikis remain to be clarified.

The level of the cleavage product of $p130^{Cas}$ declined at the later stage of anoikis, which is similar to that of FAK and caspase-3. The mechanism and the functional significance of this decline are not clear at present. The cleavage products may be particularly unstable and are vulnerable to nonspecific protein degradation at the later stage of cell death. The decline of $p130^{Cas}$ cleavage at the later stage of anoikis may further emphasize its role in the early stage of anoikis, which acts as a trigger for anoikis and

becomes less important during the later stage of cell death.

In summary, we have demonstrated that $p130^{Cas}$ is cleaved early during anoikis. In addition to caspases and calpain, there are other proteases involved in the cleavage of $p130^{Cas}$. Cleavage of $p130^{Cas}$ contributes to anoikis and may be involved in the initiation of anoikis. These observations provide new insights into the understanding of the function of $p130^{Cas}$ and the mechanisms of anoikis.

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