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Anchorage-Independent Phosphorylation of p130^{Cas} Protects Lung Adenocarcinoma Cells From Anoikis

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Abstract The regulation and function of the signaling adaptor protein p130^{Cas} in tumor cell anchorage-independent survival, or anoikis resistance, were investigated in human lung adenocarcinoma cells. The tyrosine phosphorylation and function of p130^{Cas} during cell detachment were analyzed in tumor cells and compared with that of normal epithelial cells. Cell detachment trigged rapid dephosphorylation of p130^{Cas} in the nontumorigenic and anoikis-sensitive normal epithelial cells, but had no effect on the tyrosine phosphorylation of p130^{Cas} in the anoikis-resistant lung adenocarcinoma cells. Further analysis revealed that the total tyrosine kinase activities associated with p130^{Cas} in the lung tumor cells are anchorage-independent and are significantly higher than that in the normal cells, in which the p130^{Cas}-associated tyrosine kinase activities are anchorage-dependent. Analysis of two known p130^{Cas}-associated tyrosine kinases FAK and Src indicated that the regulation of tyrosine phosphorylation of FAK and Src are altered in the tumor cells. Inhibition of Src specifically abolished phosphorylation of p130^{Cas} and induced anoikis. Furthermore, overexpression of dominant-negative forms of p130^{Cas} also induced apoptosis. Taken together, these data suggest that p130^{Cas} mediates a cell survival signal from cell–matrix interaction. Alterations in tumor cells that lead to constitutive phosphorylation of p130^{Cas} can prevent cells from anoikis, hence contribute to tumor cell anchorage independence and metastasis. J. Cell. Biochem. 87: 439–449, 2002.

Key words: p130^{Cas}; Src; FAK; anoikis; lung adenocarcinoma cells

The interaction of cells with the extracellular matrix (ECM) proteins plays a fundamental role in regulating their proliferation, differentiation, migration, and survival. Prohibition of cell–ECM interaction causes apoptosis of many types of cells, particularly epithelial and endothelial cells [Meredith et al., 1993; Frisch and Francis, 1994; Frisch and Screaton, 2001]. This type of apoptosis was termed anoikis [Frisch and Francis, 1994]. Anoikis is physiologically important for maintaining homeostasis and architecture of epithelia of various organs. Epithelial cells accidentally detached from their appropriate ECM environment are eliminated by anoikis to prevent them from growing in inappropriate sites.

Malignant tumor cells, more than 80% of which are derived from epithelial cells, are characterized by their ability to survive and grow independent of anchorage. They are anoikis resistant [Wei et al., 2001]. This property of tumor cells is critical for tumor cell metastasis since metastatic cells have to survive in blood circulation before they reattach and spread at distant sites. Although certain cytoplasmic oncogenes, such as Src and Ras, have been shown to be capable of preventing normal epithelial cells from anoikis [Frisch and Francis, 1994; Khwaja et al., 1997], the mechanism of anoikis resistance of many human tumor cells is essentially unknown.

The molecular mechanism of anoikis is not fully understood. Accumulating evidences suggest that cell–ECM interaction is one of the essential cell survival signals to suppress otherwise a default cell death pathway. Cell

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detachment from their ECM activates this default endogenous cell death pathway as a result of de-inhibition. The cell survival signals generated from ECM are mainly mediated by cell surface ECM receptor integrins [Frisch and Ruoslahti, 1997]. Ligation of integrins with ECM proteins leads to phosphorylation and activation of the focal adhesion tyrosine kinase FAK. FAK interacts with a number of intracellular signaling proteins, including Src, Grb2, Shc, PI 3K, paxillin, and $p130^{Cas}$ [Schaller and Parsons, 1994, 1995; Schlaepfer et al., 1994; Polte and Hanks, 1995; Richardson and Parsons, 1995; Schlaepfer et al., 1999]. Activation of FAK leads to increased tyrosine phosphorylation and complex formation of these signaling mediators, which subsequently activate a set of kinases, such as PI 3K and Akt that are crucial for protecting cells from death [Khwaja et al., 1997]. Disruption of integrin–EMC interaction inactivates FAK, leading to loss of FAK-mediated cell survival signals and cell death. Activated FAK has been shown to prevent epithelial cells from anoikis [Frisch et al., 1996]. Therefore, FAK and its downstream signaling partners play important roles in regulating anoikis.

One of the important downstream signaling components of FAK pathway is $p130^{Cas}$ (Crk-associated substrate), a member of a family of structurally related signaling adaptor proteins. $p130^{Cas}$ was originally identified as a major tyrosine-phosphorylated protein in v-Crk [Mayer and Hanafusa, 1990; Matsuda et al., 1990; Birge et al., 1992] or v-Src [Reynolds et al., 1989; Kanner et al., 1990, 1991] transformed cells. It contains multiple structural motifs of adapter proteins, which include a SH3 domain, several proline-rich regions, and a cluster of 15 tyrosine phosphorylation sites that are SH2 binding motifs [Sakai et al., 1994]. Although FAK physically binds to $p130^{Cas}$ and have a role in mediating $p130^{Cas}$ phosphorylation [Polte and Hanks, 1995; Vuori et al., 1996; Cary et al., 1998], the direct tyrosine kinases for $p130^{Cas}$ are Src family kinases [Hamasaki et al., 1996; Sakai et al., 1997]. $p130^{Cas}$ binds to FAK via its SH3 domain [Polte and Hanks, 1995; Burnham et al., 1996; Harte et al., 1996; Astier et al., 1997], and to Src and Lyn via its C-terminal proline-rich sequence and the phosphorylated tyrosine 668 [Manie et al., 1997; Nakamoto et al., 1997]. Tyrosine-phosphorylated $p130^{Cas}$ binds to a number of SH2-domain-containing proteins, which include Crk, Src, PI-3-kinase, Nck, PLCγ, via its distinct SH2 binding motifs [Sakai et al., 1994; Burnham et al., 1996; Vuori et al., 1996]. These multiple interactions allow $p130^{Cas}$ function as a signal assembly protein to integrate and coordinate a variety of signals to control cellular activities. Multiple factors were found which can stimulate tyrosine phosphorylation of $p130^{Cas}$, ranging from ECM proteins such as fibronectin [Nojima et al., 1995; Petch et al., 1995; Vuori and Ruoslahti, 1995; Schlaepfer et al., 1997], peptide growth factors NGF [Ribon and Saltiel, 1996], EGF [Ojaniemi and Vuori, 1997], and PDGF [Casamassima and Rozengurt, 1997], neuropeptides bombasin, vasopressin, endothelin, and bradykinin, to bioactive lipids LPA and SPC [Seufferlein and Rozengurt, 1994; Casamassima and Rozengurt, 1997]. Functionally, $p130^{Cas}$ has been shown to be involved in 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; Nakamoto et al., 1997], actin organization [Nakamura et al., 1998], and cell migration [Cary et al., 1998; Klemke et al., 1998].

Recently, a number of experiments suggest that $p130^{Cas}$ is also involved in regulation of cell death. Dephosphorylation and cleavage of $p130^{Cas}$ have been found closely associated with apoptosis induced by a variety of agents. [Chan et al., 1999; Weng et al., 1999; Kook et al., 2000; Weyant et al., 2000; Harrington et al., 2001; Lesay et al., 2001; Shim et al., 2001; Wang et al., 2001]. Overexpression of wild-type $p130^{Cas}$ protected 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 exact role of $p130^{Cas}$ in regulation of apoptosis, however, has not been clearly defined. In this report, we analyzed the regulation and function of $p130^{Cas}$ in anoikis and anoikis resistance. We found that tyrosine phosphorylation of $p130^{Cas}$ was anchorage-dependent in normal anoikis-sensitive normal epithelial cells but became anchorage-independent in anoikis-resistant human lung adenocarcinoma cells. The constitutive phosphorylation of $p130^{Cas}$ protects tumor cells from anoikis. Our data provide the first direct evidence to demonstrate how alteration of focal adhesion components may contribute to oncogenic transformation of tumor cells.

MATERIALS AND METHODS

Cell Culture and Anoikis Assay

Human bronchial epithelial cell line HBE4-E6/E7, Madin–Darby canine kidney epithelial cell line (MDCK), 293, and lung adenocarcinoma cell lines A549, H1792, and SK-LU-1 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, counted, 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, drying in tissue culture hood, and repeating 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 both fragmented genomic DNA and RNA, were extracted with a 0.6-ml solution of 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris (pH 7.4), pheno-chloroform extracted three times, and ethanol precipitated and analyzed on a 1.5% agarose gel. The gel was incubated in RNase A-containing solution (5 μg/ml) to digest away the RNAs in the gel before photographing.

Antibodies, Immunoblotting, and Immunoprecipitation

A mouse monoclonal antibody to phosphotyrosine (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibodies against p130^{Cas}, phospho-FAK, and FAK were purchased from Transduction Laboratory (San Diego, CA). A rabbit polyclonal antibody to phospho-Src at amino acid residue 418 was purchased from BioSource, Inc. (Camarillo, CA). The mouse monoclonal Anti-body to Src was purchased from Santa Cruze Biotech, Inc. (Santa Cruze, CA). Rabbit polyclonal antibodies to phospho-Akt, Akt, phospho-MAPK, and MAPK were purchased from New England BioLabs (Beverly, MA). Protein A-Agarose beads were purchased from Santa Cruze Biotech, Inc. All commercially purchased antibodies were used as recommended by the manufacturers.

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, Hercules, CA). Proteins were resolved by SDS–PAGE (7.5%) and transferred to a nitrocellulose membrane. For immunoblotting with monoclonal antibody 4G10, the membranes were preincubated for 20 min at room temperature with 3% (w/v) nonfat dry milk in PBS; for immunoblotting with other antibodies, the membrane was incubated for 1 h at room temperature in a solution containing 5% nonfat dry milk and 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% (v/v) Tween 20. After preincubation, the membranes were incubated with primary antibodies at 4°C overnight. The membranes were then washed three times with the blocking solution with no milk and incubated for 1 h at room temperature with horseradish peroxidase- conjugated secondary antibodies (Promega, Madison, WI), followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ).

For immunoprecipitation, cells were lysed in either the modified RIPA buffer or in a low stringency buffer [20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 5 mM EGTA (pH 8.0), 0.2 mM phenylmethylsulfonyl fluoride, leupeptin (5 g/ml), aprotinin (5 g/ml), and 1 mM Na_3VO_4] and the cell lysates containing 500–1,000 μg of protein in a volume of 500 μl were used for experiments. Immunoprecipitation was carried out at 4°C. After pre-clearing, the cell lysates were incubated with various antibodies for 2 h, followed by 2 h incubation with protein A-Agarose beads with rotation. After washing three times with the cell lysis buffer, the protein A-Agarose beads were boiled in 50 μl of Laemmli sample buffer.

Plasmid Constructs

A full-length and four deletion mutant constructs of $p130^{Cas}$ (Cas-ΔSD: deletion of substrate domain from amino acid residue 119 to 445; Cas-ΔSDP: deletion of part of the substrate domain from amino acid residue 249 to 445; Cas-ΔSrcBD: deletion of the Src binding domain from amino acid residue 446 to 945; Cas–FAK: FAK binding domain from amino acid residue 1 to 118) were subcloned in frame into a EGFP c2 expression vector (Clontech, CA) to generate green fluorescent protein (GFP) fusion proteins. All the constructs were verified by restriction enzyme digestion mapping. The GFP–Cas fusion proteins produced from these constructs were analyzed by transfection and immunoblotting.

Transfection and Apoptosis Assay

Cells (293, MDCK, 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). One microgram of plasmid DNA 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 each construct. Cells were 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 Src Inhibitor

The specific Src inhibitor, PP2 was purchased from Calbiochem (La Jolla, CA) and dissolved in DMSO. In experiments that cells were treated with the Src inhibitor, the same volumes of DMSO were added to the controls.

In Vitro Kinase Assay

For assessing the $p130^{Cas}$-associated tyrosine kinase activities, cells were lysed in NP40 buffer containing 0.5% NP-40, 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), aprotinin (5 μg/ml), and 1 mM Na_3VO_4. $p130^{Cas}$ was immunoprecipited with a rabbit polyclonal antibody to $p130^{Cas}$ from Santa Cruze Biotech, Inc. The bacterial-expressed $p130^{Cas}$–GST fusion protein was used as the substrate. After washing, the above $p130^{Cas}$ immunoprecipitates were suspended in 60 μl kinase buffer containing 10 μg $p130^{Cas}$–GST fusion protein, 200 μM ATP, 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, and incubated at 30°C for 30 min. After kinase reaction, the proteinA-Agarose beads of the $p130^{Cas}$ immunoprecipitates were removed from the samples by centrifugation and the supernatants were added with equal volume of 2× Laemmli sample buffer and resolved by 7.5% SDS–PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblotting with the anti-phosphotyrosine antibody 4G10.

RESULTS

Tyrosine Phosphorylation of $p130^{Cas}$ Is Anchorage-Dependent in Anoikis-Sensitive Normal Epithelial Cells, but Anchorage-Independent in Anoikis-Resistant Tumor Cells

We and others previously reported that normal epithelial cells, such as MDCK cells, undergo rapid apoptosis when they were detached and cultured in suspension, termed anoikis [Frisch and Francis, 1994], whereas many tumor cells remained alive under the same culturing condition [Wei et al., 2001]. We also observed that protein tyrosine kinases are critical in transducing cell adhesion-generated cell survival signals and in protecting tumor cells from anoikis [Wei et al., 2001]. In a separate study, we noticed that signaling adaptor protein $p130^{Cas}$ and its tyrosine phosphorylation play an important role in protecting cells from activation of tyrosinephosphatase-induced apoptosis [Weng et al., 1999]. To explore a potential role of $p130^{Cas}$ in anoikis and anoikis resistance, we analyzed the regulation of tyrosine-phosphorylation of $p130^{Cas}$ upon cell detachment in anoikis-sensitive non-transformed epithelial cells (MDCK and HBE4-E6/E7) and compared to that of anoikis-resistant human lung adenocarcinoma cells (A549, NCI-H1792, and NCI-H23). All three lung adenocarcinoma cells are resistant to anoikis [Wei et al., 2001]. Detachment induced dephosphorylation of

p130^{Cas} in the two non-transformed epithelial cells (Fig. 1A). On the contrary, the tyrosine phosphorylation of p130^{Cas} in the three anoikis-resistant lung tumor cells remained largely unaffected by cell detachment (Fig. 1A). The dephosphorylation of p130^{Cas} in the non-transformed cells was rapid, within 2 h after cell detachment (Fig. 1B and data not shown). At 5 h, most of the p130^{Cas} protein was dephosphorylated in MDCK cells. Cell death was detectable by 5 h after cell detachment and became evident at 7 h (Fig. 1B). The phosphorylation of p130^{Cas} in the anoikis-resistant A549 cells remained unchanged after detachment (Fig. 1B). Therefore, the regulation of p130^{Cas} tyrosine phosphorylation is anchorage-dependent in anoikis-sensitive normal epithelial cells but anchorage-independent in the anoikis resistant lung tumor cells. The distinct difference in the requirement of anchorage for p130^{Cas} phosphorylation in anoikis sensitive and resistant cells suggests a possible functional role of p130^{Cas} phosphorylation in regulation of anoikis sensitivity.

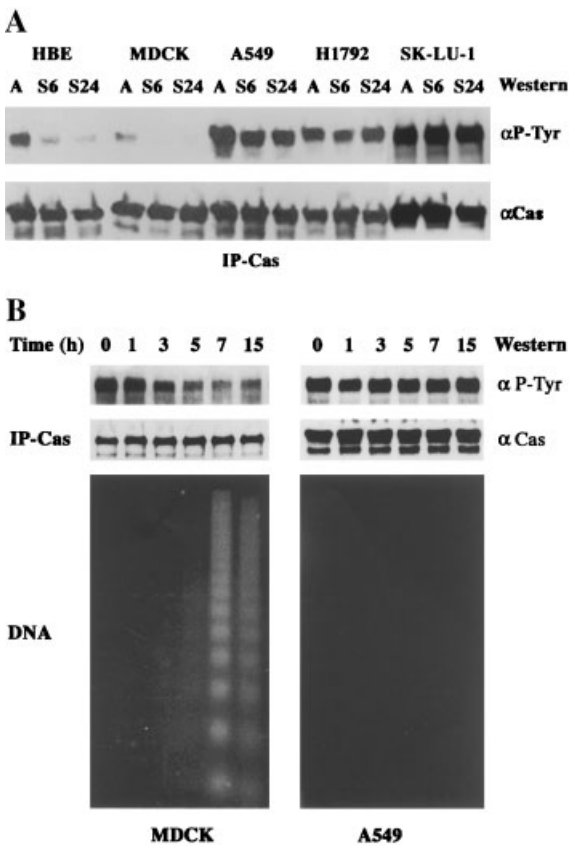

Fig. 1. Effect of cell detaching on tyrosine-phosphorylation of p130^{Cas} and cell death. **A**: Confluent normal (MDCK and HBE) and human lung adenocarcinoma (A549, H1792, and SK-LU-1) cells from regular tissue culture dishes were trypsinized and cultured in suspension on poly-HEMA-coated dishes. A: attached cells. S6: cells cultured in suspension for 6 h. S24: cells cultured in suspension for 24 h. For analyzing tyrosine-phosphorylation of p130^{Cas}, p130^{Cas} protein was immunoprecipitated by anti-Cas antibody and analyzed by immunoblotting using anti-phosphorylated tyrosine (αP-Tyr) and anti-p130^{Cas} (αCas) antibodies sequentially. **B**: Time course of p130^{Cas} dephosphorylation and cell death. Confluent MDCK and A549 cells from regular tissue culture dishes were trypsinized and cultured in suspension on poly-HEMA-coated dishes for indicated time periods. Half of the cell lysates were used for protein phosphorylation analysis and the other half were used for DNA analysis. For analyzing DNA, cytosolic DNA were extracted as described in Methods and Materials and analyzed by agarose gel electrophoresis.

p130^{Cas}-Associated Tyrosine Kinase Activities Are Anchorage Dependent in Normal Epithelial Cells but Independent in Lung Tumor Cells

The constitutively phosphorylated p130^{Cas} in the lung tumor cells could be due to altered kinase and/or phosphatase activities. Since our previous studies suggest that altered tyrosine kinase activities are responsible for anoikis resistance of the lung tumor cells [Wei et al., 2001], we analyzed tyrosine kinase activities associated with p130^{Cas} in an in vitro kinase assay using immunoprecipitated p130^{Cas} from normal MDCK and lung tumor A549 and H1792 cells. A bacteria-expressed p130^{Cas}–GST fusion protein was used as a substrate. p130^{Cas}-associated tyrosine kinase activities were detectable in the p130^{Cas} immunoprecipitates from both attached normal and tumor cells (Fig. 2). The p130^{Cas}-associated tyrosine kinase activity is higher in the two tumor cell lines than that in the MDCK cells. After detachment of cells, the p130^{Cas}-associated kinase activity in the MDCK cells was diminished, whereas the levels of p130^{Cas}-associated kinase activity in the two detached tumor cells were essentially unaffected (Fig. 2). These data suggest that a p130^{Cas}-associated tyrosine kinase(s) is regulated by cell adhesion in non-tumor cells. The regulation of this kinase activity is likely altered in the tumor cells and may be responsible for the anchorage-independent phosphorylation of p130^{Cas} in the tumor cells.

Src Is Responsible for the Anchorage-Independent Phosphorylation of p130^{Cas} in the Lung Tumor Cells

Both FAK and Src physically interact with p130^{Cas} and regulate tyrosine phosphorylation

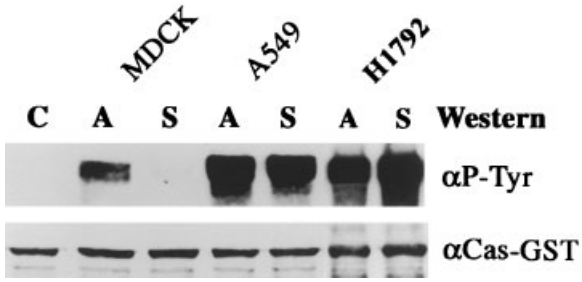

Fig. 2. In vitro kinase assay of $p130^{Cas}$-associated tyrosine kinase activity. MDCK, A549, and H1792 cells were cultured as attached (A) or as suspension (S) cultures for 15 h. Cells were then lysed and $p130^{Cas}$ were immunoprecipitated with anti-Cas antibody. The immunocomplexes were mixed with equal amounts of purified bacteria-expressed Cas–GST fusion protein in kinase assay buffer and incubated for 30 min. After kinase reaction, the proteins were separated by SDS–PAGE and analyzed by immunoblotting using anti-phosphorylated tyrosine antibody (αP-Tyr) and anti-GST antibodies (αCas-GST) sequentially. (C) is a control without addition of $p130^{Cas}$ immunocomplex.

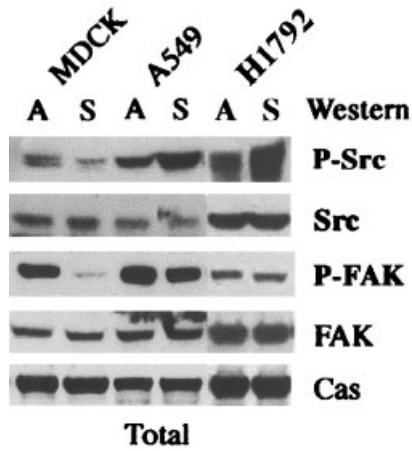

Fig. 3. Anchorage dependence of Src and FAK phosphorylation/activation. MDCK, A549, and H1792 cells were cultured as attached (A) or as suspension (S) cultures for 15 h. Cells were then lysed in protein lysis buffer. Cell lysates were resolved by SDS–PAGE and analyzed by immunoblotting using the antibodies against phosphorylated Src Tyr-418 (P-Src), phosphorylated FAK Tyr-397 (P-FAK), Src, or FAK as indicated.

of $p130^{Cas}$. Src is a key kinase involved in mediating cell adhesion-dependent phosphorylation of $p130^{Cas}$ in normal cells [Hamasaki et al., 1996]. To determine whether FAK or Src is responsible for the anchorage-independent phosphorylation of $p130^{Cas}$ in the tumor cells, we examined the phosphorylation of FAK and Src in response to cell detachment in these cell lines. The phosphorylation of both FAK Tyr-397 and Src Tyr-418, which are the indicatives of the activated kinases, are regulated by cell adhesion in normal cells. Cell detachment decreased phosphorylation of FAK significantly in MDCK cells, but had much less or no effect on the phosphorylation of FAK in A549 and H1792 cells (Fig. 3 and data not shown). Phosphorylation of Src Tyr-418 was decreased in detached MDCK cells, and interestingly, dramatically increased in the two tumor cells after cell detachment (Fig. 3). These data suggest that the phosphorylation of both FAK and Src, and, therefore, their activities, are altered in the tumor cells and may contribute to the anchorage-independent phosphorylation of $p130^{Cas}$ in the tumor cells.

To further understand the role of Src and FAK in the constitutive phosphorylation of $p130^{Cas}$ in the tumor cells, we inhibited Src activity by using a Src-specific inhibitor PP2 and analyzed its effect on phosphorylation of $p130^{Cas}$ and FAK. Treatment of A549 cells with PP2 specifically abolished phosphorylation of Src and $p130^{Cas}$, without affecting the phosphorylation of FAK (Fig. 4A). The treatment of Src inhibitor also completely abolished the $p130^{Cas}$-associated tyrosine kinase activity in the in vitro kinase assay (data not shown). Taken together, these data strongly suggest that Src is the key kinase that is responsible for the constitutive phosphorylation of $p130^{Cas}$ in the tumor cells.

Inhibition of Src and $p130^{Cas}$ Phosphorylation Induces Anoikis in A549 Tumor Cells

To test whether constitutive phosphorylation of $p130^{Cas}$ is critical to the anoikis resistance of the lung tumor cells, we altered its activity by two different methods. We first inhibited phosphorylation of $p130^{Cas}$ by the Src inhibitor and analyzed its effect on cell death of the lung tumor cells. We observed a dose-dependent inhibition of $p130^{Cas}$ phosphorylation and the induction of cell death by the Src inhibitor, as indicated by PARP cleavage and by DNA fragmentation analysis (Fig. 4). At 50 μM concentration of PP2, the phosphorylation of $p130^{Cas}$ was completely inhibited and the cell death began to occur, suggesting that phosphorylation of $p130^{Cas}$ by Src may be essential for transmitting the cell survival signal.

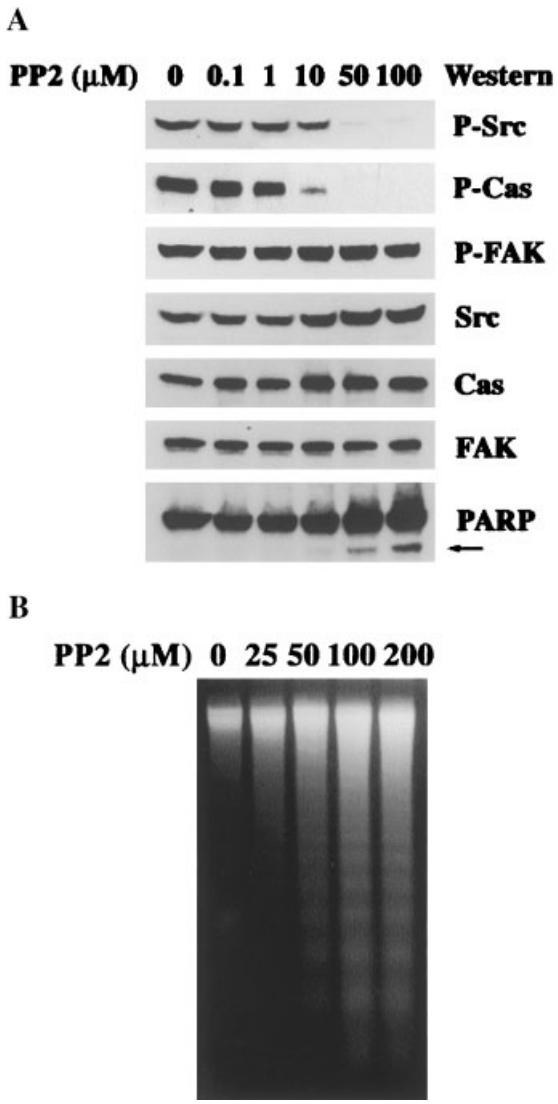

Fig. 4. Effect of Src inhibitor PP2 on tyrosine-phosphorylation of Cas, FAK, and Src and on cell death. A549 cells were cultured in suspension for 15 h in the presence of different concentrations of PP2 as indicated. The cells were then harvested and lysed. **A**: The cell lysates were resolved by SDS–PAGE and analyzed by immunoblotting using antibodies against phosphorylated Src Tyr-418 (P-Src), phosphorylated FAK (P-FAK), Src, Cas, FAK, or PARP. Phosphorylated Cas (P-Cas) was analyzed by immunoprecipitation of Cas first, followed by immunoblotting with anti-phosphotyrosine antibody 4G10. Cell death is indicated by the cleavage of PARP. The arrow indicates the cleavage product of PARP. **B**: Cytosolic DNA were extracted and analyzed by agarose gel electrophoresis. Cell death is indicated by the appearance of the fragmented DNA.

Activity of p130cas and Its Interaction With Src is Critical for Tumor Cell Survival

To examine if the function of p130cas is critical for the survival of tumor cells, we transiently transfected MDCK and lung tumor A549 cells with various mutant forms of p130^{Cas}–GFP fusion protein (Fig. 5A), some of which were previously shown to act as dominant-negative forms of p130^{Cas} and could induce apoptosis in fibroblasts [Almeida et al., 2000]. The expressions of these plasmids were first tested in 293 cells. All p130^{Cas}–GFP fusion proteins were detected by western blotting analysis (Fig. 5B). We then transfected A549 cells with these plasmids. Twenty four hours after transfection, the green-fluorescent transfected cells were examined for viability. About 40% of the cells transfected with the Cas-ΔSD mutant, which has the entire substrate domain of p130^{Cas} deleted, were apoptotic (Fig. 5C). The Cas-ΔSDP mutant, which has the substrate domain partially deleted, also induced cell death, but to a less extent. A reasonable hypothesis for these results is that these mutants compete with endogenous p130^{Cas} for interaction with Src.

To examine if the interaction of Src with p130^{Cas} is essential for it to support cell survival in tumor cells and for mutants of p130^{Cas} to act as dominant negative mutants, we generated two truncation mutants, which have the Src binding sites deleted. Transfection of these two mutants into A549 cells failed to induce cell death (Fig. 5C), suggesting that Src–p130^{Cas} interaction is critical for p130^{Cas} to transmit the cell survival signal and unproductive interaction of p130^{Cas} mutant with Src is critical for them to inhibit survival signaling. Similar data were also obtained from using MDCK and 293 cells (data not shown). Taken together, these data strongly suggest that p130^{Cas} and its phosphorylation are important for cell survival. The constitutive phosphorylation of p130^{Cas} by Src in A549 cells is critical for their anoikis resistance.

DISCUSSION

p130^{Cas} is a multifunctional signaling adaptor protein. It integrates and relays signals generated from a variety of extracellular stimuli and regulate 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; Nakamoto et al., 1997], actin organization [Nakamura et al., 1998], and cell migration [Cary et al., 1998; Klemke et al., 1998].

Evidences linking $p130^{Cas}$ to regulation of cell death/survival have been emerging recently. A number of experiments have shown that $p130^{Cas}$ undergo dephosphorylation or cleavage 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], cell detachment [Lesay et al., 2001], UV irradiation [Chan et al., 1999], tyrosine phosphatase [Weng et al., 1999], to serum withdraw [Almeida et al., 2000]. More direct evidences 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 this report, we analyzed the regulation and function of $p130^{Cas}$ in anoikis of normal epithelial cells and compared with that of anoikis-resistant human lung adenocarcinoma cells. We found that the phosphorylation of $p130^{Cas}$ is anchorage-dependent in the normal anoikis-sensitive epithelial cells, but anchorage-independent in the anoikis-resistant lung tumor cells. Further, we found that inhibition of the phosphorylation of $p130^{Cas}$ or overexpression of dominant-negative forms of $p130^{Cas}$ induced apoptosis in the tumor cells. Our data, together with others, strongly support a critical role of $p130^{Cas}$ in mediating cell survival signals generated from cell adhesion. Inactivation of $p130^{Cas}$ is an important step to fully execute a cell death program. Alterations in the regulation of $p130^{Cas}$ may contribute to resistance to apoptosis of tumor cells.

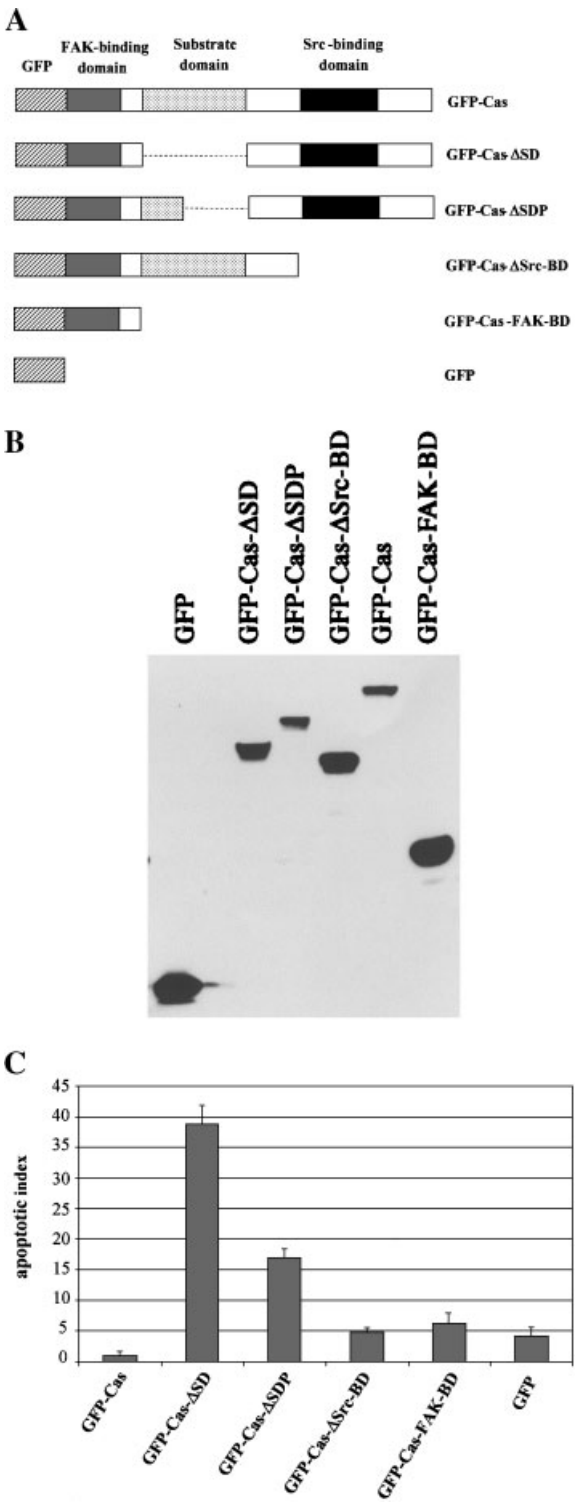

Fig. 5. Cell death induced by transfection of $p130^{Cas}$ mutants. **A**: Schematic representation of GFP–Cas fusion protein and its mutant forms. The GFP portion and the various functional domains of $p130^{Cas}$ are indicated. GFP–Cas: fusion protein of GFP and wild type $p130^{Cas}$; GFP–Cas-ΔSD: fusion protein of GFP and substrate domain-deletion mutant of $p130^{Cas}$; GFP–Cas-ΔSDP: fusion protein of GFP and partial substrate domain-deletion mutant of $p130^{Cas}$; GFP–Cas-ΔSrc–BD: fusion protein of GFP and Src binding domain-deletion mutant of $p130^{Cas}$; GFP–Cas–FAK–BD: fusion protein of GFP and FAK binding domain of $p130^{Cas}$; GFP: GFP alone. **B**: Immunoblot showing the protein expression of the GFP–Cas fusion constructs in 293 cells. **C**: Cell death induced by transfection of GFP–Cas fusion constructs. A549 cells cultured in regular tissue culture dishes were transfected with the indicated constructs. Twenty four hours after transfection, cells were stained with DABI. Total green fluorescent cells and green fluorescent dead cell were scored and calculated. The numbers were presented as the percentage of green fluorescent dead cells.

The mechanism responsible for the constitutive phosphorylation of p130^{Cas} in the tumor cells has yet to be fully understood. Either altered tyrosine kinases or phosphatases of p130^{Cas} could contribute to the constitutive phosphorylation of p130^{Cas}. Our data suggest that it is a p130^{Cas}-associated tyrosine kinase that may be altered in the tumor cells and is responsible for the anchorage-independent phosphorylation of p130^{Cas}. Both Src and FAK are physically associated with p130^{Cas}. The functional importance of Src in the constitutive phosphorylation of p130^{Cas} in the tumor cells was analyzed by using its specific inhibitor. Inactivation of Src by its inhibitor specifically and completely abolished the phosphorylation of p130^{Cas}, confirming that Src is the key tyrosine kinase in the constitutive phosphorylation of p130^{Cas} in the tumor cells.

Elevated levels of Src activity have been reported in a number of human cancers, including lung cancer [Biscardi et al., 1999]. In certain breast cancer cells, Src is activated by increased levels of a Src tyrosine-530-specific phosphatase [Egan et al., 1999]. We did not observe decreased phosphorylation at the tyr-530 of Src in the lung tumor cells (data not shown). We, however, observed an increased phosphorylation at the tyrosine-418 of Src upon cell detachment in the tumor cells. Therefore, increased Src tyrosine phosphatase activity may not be the mechanism for the activation of Src upon cell detachment in the lung tumor cells. The mechanism of Src activation in the lung tumor cells remains to be determined.

FAK is also constitutively phosphorylated in some of the lung tumor cells. The mechanism of constitutive phosphorylation of FAK and its role in phosphorylation of p130^{Cas} are not clear at present. FAK apparently does not phosphorylate p130^{Cas} directly since inhibition of Src is sufficient to abolish phosphorylation of p130^{Cas} completely. This, however, does not exclude the role of the constitutively phosphorylated FAK in physically linking Src and p130^{Cas} together to facilitate the phosphorylation of p130^{Cas} by Src.

p130^{Cas} interacts with a number of signaling proteins that are involved in regulation of phosphorylation/activation of JNK, Erk, and PI 3K, all of which are important regulators of cell survival/death [Vuori et al., 1996; Schlaepfer et al., 1997; Zhu et al., 1998; Blaukat et al., 1999; Oktay et al., 1999; Almeida et al., 2000; Cho and Klemke, 2000; Xing et al., 2000; Yoshizumi et al., 2000]. We previously reported that phosphorylation of Erk and Akt were decreased in MDCK cells but increased in A549 cells upon cell detachment. However, inhibition of Erk and Akt phosphorylation/activation did not induce cell death [Wei et al., 2001], suggesting that signaling molecules other than Erk and Akt may mediate the cell survival signals from p130^{Cas}. We recently also analyzed the regulation of phosphorylation of JNK and p38 MAPK by cell adhesion in A549 cells. No phosphorylated JNK or p38 was detected in this cell line (unpublished data). Since functional p130^{Cas} is essential for the anoikis resistance of A549 cell, we propose that p130^{Cas} transmits a cell survival signal through a novel signaling molecule, which remains to be identified.

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