

# Caspase-mediated Cleavage of Focal Adhesion Kinase pp125<sup>FAK</sup> and Disassembly of Focal Adhesions in Human Endothelial Cell Apoptosis

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## Summary

Normal endothelial and epithelial cells undergo apoptosis when cell adhesion and spreading are prevented, implying a requirement for antiapoptotic signals from the extracellular matrix for cell survival. We investigated some of the molecular changes occurring in focal adhesions during growth factor deprivation-induced apoptosis in confluent monolayers of human umbilical vein endothelial cells. Among the first morphologic changes after initiation of the apoptotic process are membrane blebbing, loss of focal adhesion sites, and retraction from the matrix followed by detachment. We observe a specific proteolytic cleavage of focal adhesion kinase (pp125<sup>FAK</sup>), an important component of the focal adhesion complex, and identify pp125<sup>FAK</sup> as a novel substrate for caspase-3 and caspase-3-like apoptotic caspases. The initial cleavage precedes detachment, and coincides with loss of pp125<sup>FAK</sup> and paxillin from focal adhesion sites and their redistribution into the characteristic membrane blebs of apoptotically dying cells. Cleavage of pp125<sup>FAK</sup> differentially affects its association with signaling and cytoskeletal components of the focal adhesion complex; binding of paxillin, but not pp130<sup>Cas</sup> (Cas, Crk-associated substrate) and vinculin, to the COOH terminally truncated pp125<sup>FAK</sup> is abolished. Therefore, caspase-mediated cleavage of pp125<sup>FAK</sup> may be participating in the disassembly of the focal adhesion complex and actively interrupting survival signals from the extracellular matrix, thus propagating the cell death program.

Anchorage of cells to the extracellular matrix and the resulting integrin-mediated signaling events are thought to play a crucial role in cell survival (1). Endothelial and epithelial cells undergo apoptosis when cell anchorage is prevented in vitro or in vivo (2–6). However, recent evidence suggests that the shape of the cell and the rigidity of the nucleocytoskeletal architecture, both intricately connected to integrin engagement, have a major impact on the cell's decision between life and death (7). The focal adhesion complex is the multifunctional structure that the cell uses for integrating integrin-mediated signaling events from the matrix with the dynamic cytoskeletal meshwork that mechanically couples the cell with the extracellular matrix. Appropriate orientation of the signaling machinery of the cell is dependent on the structure of the focal adhesion complex, which provides an intricate interrelationship between the extracellular matrix, the cytoskeleton, and signaling cascades (8–11).

A central member of the focal adhesion complex is the

tyrosine kinase pp125<sup>FAK</sup> (FAK, focal adhesion kinase<sup>1</sup>; reference 12), which has been implicated in the integration of signals from integrins, oncogenes, and neuropeptides (13). Although pp125<sup>FAK</sup> has been shown to be dispensable for the assembly of focal adhesions, it has a key role in the assembly of various signaling proteins recruited to focal adhesions and the downstream events initiated by these components (14). pp125<sup>FAK</sup> has also been shown to play an important role in cell survival; constitutively active forms of pp125<sup>FAK</sup> can rescue epithelial cell lines from apoptosis in suspension (15). Inversely, fibroblasts undergo apoptosis when the interaction of pp125<sup>FAK</sup> with the cytoplasmic do-

<sup>1</sup>Abbreviations used in this paper: Ac-DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-aldehyde; FAK, focal adhesion kinase; FRNK, pp125<sup>FAK</sup>-related nonkinase; GF, growth factor; HUVEC, human umbilical vein endothelial cell; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; SH, Src homology domain; ZVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone.

main of the beta 1 integrin is inhibited by microinjection of a peptide identical to the pp125<sup>FAK</sup> binding site on the integrin, or by microinjection of an antibody interfering with this binding (16). Proteolytic alteration of pp125<sup>FAK</sup> during c-myc-induced apoptosis has been suggested (17), but the nature of this change remains unclear. Evidence has also been provided for proteolytic degradation of pp125<sup>FAK</sup> during platelet aggregation (18).

In this study, we characterize a distinct pattern of specific proteolytic cleavage of pp125<sup>FAK</sup> during apoptosis of human umbilical vein endothelial cells (HUVECs) induced by growth factor (GF) deprivation. Further, we identify the enzymes responsible for this cleavage to be members of the family of death proteases (caspases), related to the mammalian interleukin 1 $\beta$ -converting enzyme (ICE), and the product of the *Caenorhabditis elegans ced-3* gene, which are instrumental in the execution phase of apoptosis (19). Evidence for the influence of this cleavage on potential downstream events in the apoptotic process is also provided.

## Materials and Methods

**Protein Analysis and Immunoprecipitations.** Cells were lysed in 50 mM Tris/HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 10% glycerol, 5 mM EDTA, 50 mM NaF, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM  $\beta$ -glycerophosphate, PMSF, leupeptin, and aprotinin. Lysates were separated on 10% SDS-PAGE, proteins transferred to Immobilon membrane (Millipore, Bedford, MA), and immunoblotted with specific antibodies. All immunoblots were visualized by enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL). The following antibodies were used: polyclonal antibodies to pp125<sup>FAK</sup> (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antibodies to pp125<sup>FAK</sup>, paxillin, p130<sup>Cas</sup> (Cas, Crk-associated substrate) (Transduction Labs., Lexington, KY), vinculin (Sigma Chemical Co., St. Louis, MO), and poly(ADP-ribose) polymerase (PARP) (Enzyme Systems Products, Dublin, CA). Proteins associated with pp125<sup>FAK</sup> were detected by immunoprecipitation with the NH<sub>2</sub>-terminal pp125<sup>FAK</sup> antibody, followed by immunoblotting with specific antibodies.

**Immunocytochemistry, Cell Fractionation Analysis, and Inhibition of Apoptosis by Benzoyloxycarbonyl-Val-Ala-Asp Fluoromethyl Ketone.** HUVECs were plated on gelatin-coated chamber slides, grown for 48 h at 37°C, and starved for 4 h. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed two times with PBS, and permeabilized in 0.5% NP-40 in PBS for 10 min. After another two washes, quenching was performed by 3  $\times$  5 min incubations with 50 mM NH<sub>4</sub> acetate. Fixed cells were incubated with monoclonal antibodies to pp125<sup>FAK</sup> (1:50) or paxillin (1:200) in 0.1% BSA/PBS for 1 h at room temperature followed by incubation with a fluorescein isothiocyanate-labeled anti-mouse IgG antibody (Cappel, Durham, NC). Actin was stained using phalloidin (1:1,000, Sigma Chemical Co.). Cell fractionation was performed as described for cyclin A (20) using 0.1% digitonin to gently solubilize the plasma membranes. In experiments using the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (ZVAD-fmk; Alexis Biochemicals, San Diego, CA), cells were preincubated with the inhibitor for 1 h and then exposed to GF deprivation in the presence of the inhibitor.

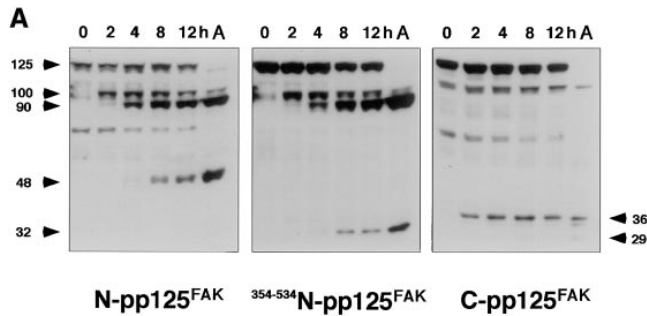
**In Vitro Cleavage of pp125<sup>FAK</sup>.** When endogenous pp125<sup>FAK</sup> from cell lysates was used as a substrate, control cells were lysed

on ice in 10 mM Hepes/KOH, pH 7.4, 2 mM EDTA, 5 mM dithiothreitol, 1% NP-40, and protease inhibitors leupeptin and aprotinin as previously described (21). Lysates were cleared by centrifugation at 27,000 *g* for 5 min, and 50  $\mu$ g cell lysate was incubated with 500 ng (10 pmol) of the individual caspases at 37°C for 2 h in a total volume of 10  $\mu$ l in reaction buffer (50 mM Hepes/KOH, pH 7.4, 0.1 M NaCl, 0.1% 3-[3-cholamidopropyl]dimethyl-ammonio-1-propanesulfonate (CHAPS), and 10% sucrose). In vitro transcription and translation of pp125<sup>FAK</sup> were performed using the TNT<sup>®</sup> coupled reticulocyte lysate system (Promega, Madison, WI) and [<sup>35</sup>S]methionine (1,000 Ci/mmol; Amersham Corp.) according to the manufacturer's instructions. The expression plasmid for pp125<sup>FAK</sup> was a gift from Dr. J.T. Parsons (University of Virginia, Charlottesville, VA). 1/500 of the reaction was used as a substrate and incubated with 250 ng (5 pmol) of the individual caspases, or with 10  $\mu$ g apoptotic or control cell lysates, respectively, and prepared as described above, in the presence or absence of 10 nM *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO; Bachem Bioscience, King of Prussia, PA) in a total volume of 9  $\mu$ l in reaction buffer at 37°C for 1.5 h. The reactions were stopped in all cases by the addition of 4 $\times$  sample buffer. Purified caspase-3, -7, and -6 were a gift from Drs. K. Orth and V.M. Dixit (University of Michigan, Ann Arbor, MI).

## Results and Discussion

**Time-dependent Cleavage of pp125<sup>FAK</sup> in Apoptotic Endothelial Cells.** HUVECs undergo apoptosis when deprived of GFs. Among the first morphologic changes after initiation of the apoptotic process are membrane blebbing, loss of focal adhesion sites, and retraction from the substratum followed by detachment. After detachment, the apoptotic cells appear in the culture medium as "floaters", displaying characteristic morphologic (membrane blebbing, nuclear condensation, and fragmentation; reference 22) and biochemical (DNA laddering, caspase activation, cleavage of PARP) features of apoptosis (data not shown). Apoptotic floaters are observed as early as 4 h after GF withdrawal and after 12–16 h account for 40–45% of the total cell population, whereas the remaining adherent cells are viable ("viable cells") and would survive and proliferate if supplemented with GFs in the culture medium (data not shown).

To determine whether alterations in a central member of the focal adhesion complex, the tyrosine kinase pp125<sup>FAK</sup>, coincide with apoptosis and the detachment of apoptotic cells from the matrix, we probed lysates from HUVECs exposed to GF deprivation for increasing periods of time with different antibodies to pp125<sup>FAK</sup> (Fig. 1 A). pp125<sup>FAK</sup> undergoes distinct proteolytic changes with time, and completely disappears in the pure apoptotic cell population (the floaters). Three different NH<sub>2</sub>-terminal proteolytic fragments of  $\sim$ 100, 90, and 48 kD are recognized with a polyclonal NH<sub>2</sub>-terminal antibody. The 100-kD fragment appears as early as 2 h after GF deprivation, decreases in intensity over the next 10 h, and is almost totally absent in the floaters. The 90-kD fragment appears first at 2 h, but increases in intensity with time, and constitutes the principal high molecular weight proteolytic pp125<sup>FAK</sup> fragment present in the floaters. The 48-kD fragment first appears at

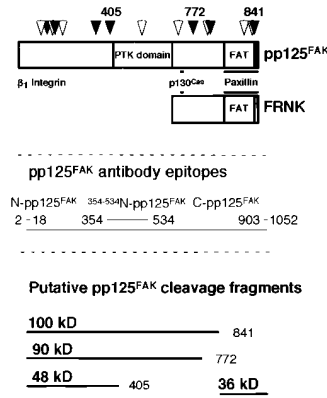


**Figure 1.** Appearance of proteolytic fragments of pp125<sup>FAK</sup> during GF deprivation-induced apoptosis. (A) HUVECs were deprived of GF for the indicated times and cell lysates of pooled cell populations (0–12 h) or only apoptotic floaters at 12 h (A) were immunoblotted for pp125<sup>FAK</sup> with three different antibodies: a polyclonal antibody to residues 2–18 at the NH<sub>2</sub> terminus (N-pp125<sup>FAK</sup>), a monoclonal antibody to residues 354–534 (354-534N-pp125<sup>FAK</sup>), and a polyclonal antibody to residues 903–1,052 at the COOH terminus (C-pp125<sup>FAK</sup>). Arrowheads, approximate molecular weights of the fragments (kD). (B) Cleavage of PARP during endothelial apoptosis was analyzed as described in A and detected using a monoclonal antibody to PARP.

4 h, and its levels also increase with time. A monoclonal antibody to amino acids 354–534 of pp125<sup>FAK</sup> also recognizes both the 90- and 100-kD fragments, but fails to detect the 48-kD fragment. This monoclonal antibody recognizes an additional 32-kD fragment, which appears first at 4 h, suggesting generation of an internal proteolytic pp125<sup>FAK</sup> fragment devoid of both a COOH and an NH<sub>2</sub> terminus. A polyclonal antibody to the COOH terminus of pp125<sup>FAK</sup> does not recognize the 100-, 90-, 48-, or 32-kD fragments, but detects two different fragments: a principal fragment of 36 kD, and a smaller one of 29 kD, with the main COOH-terminal fragment of 36 kD appearing as soon as 2 h after GF deprivation, which then remains constant with time. Substantial amounts of the 100-kD pp125<sup>FAK</sup> cleavage fragment are present after just 2 h of GF deprivation, and appearance of the 100-kD fragment of pp125<sup>FAK</sup> precedes cleavage of PARP. Significant amounts of cleaved PARP product are detectable 4–8 h after GF deprivation (Fig. 1 B).

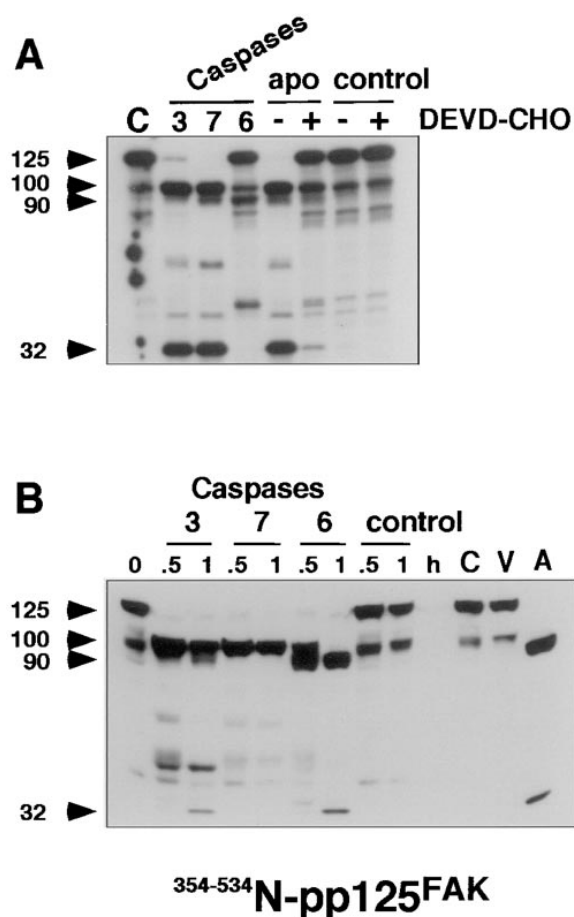
**Caspase-mediated Cleavage of pp125<sup>FAK</sup>.** Analysis of the amino acid sequence of pp125<sup>FAK</sup> reveals several putative cleavage sites for caspases (23, 24): seven sites for caspase-3-like caspases, which contain the conserved motif DXXD, and eight sites with the sequence (IVL)XXD, which are cleaved preferentially by caspase-6-like caspases (Fig. 2). To investigate whether cleavage by caspases is involved in the generation of the proteolytic pp125<sup>FAK</sup> fragments in endothelial cell apoptosis, we incubated in vitro translated pp125<sup>FAK</sup> with purified caspase-3, -6, and -7, a caspase recognizing the same substrate motif as caspase-3 (Fig. 3 A). We further tested whether cell lysates from apoptotic cells contain endogenously activated caspases that could cleave in vitro translated pp125<sup>FAK</sup> (Fig. 3 B). Both recombinant caspase-3 and -7 cleave pp125<sup>FAK</sup> into two main fragments, which are ~100 and 36 kD. The apoptotic cell lysates, used as a source of active endogenous caspases, generate a cleavage pattern identical to the one observed with recombinant caspase-3 and -7. This pp125<sup>FAK</sup> cleaving activity in apoptotic cell lysates was entirely inhibited in the presence of 10 nM Ac-DEVD-CHO, a specific inhibitor of caspase-3-like caspases (25), suggesting active endogenous caspases to be responsible for the cleavage. Cell lysates from control cells do not cleave pp125<sup>FAK</sup>, with or without the caspase inhibitor. Caspase-6 is much less efficient in cleaving the native in vitro translated pp125<sup>FAK</sup> molecule. The cleavage fragments generated by caspase-6 also differ from those resulting from caspase-3-mediated cleavage, and the predominant fragment is ~90 kD. A fragment of the same size is also seen as a result of pp125<sup>FAK</sup> cleavage by apoptotic cell lysates (Fig. 3 B), and appears to correspond to the final, ~90-kD fragment in apoptotic cells (Fig. 1).

We next compared, side by side, the in vitro-generated pp125<sup>FAK</sup> cleavage fragments with those observed during HUVEC apoptosis by incubating recombinant caspases with cell lysates from control cells as a source of endogenous pp125<sup>FAK</sup>, which also provides the molecular environment in which pp125<sup>FAK</sup> cleavage occurs (Fig. 3 B). Both caspase-3 and -7 cleave endogenous pp125<sup>FAK</sup> completely and generate the upper pp125<sup>FAK</sup> cleavage fragment of 100 kD. Surprisingly, under these experimental conditions, caspase-6 is as effective as caspase-3 in completely cleaving endogenous pp125<sup>FAK</sup> and generating the initial 100-kD fragment, and then proceeds further to efficiently generate the lower 90-kD fragment. The 90-kD fragment appears to be derived from the 100-kD fragment, since the 100-kD fragment disappears completely after 90 min, whereas the 90-kD fragment increases in intensity. Since caspase-6 can activate caspase-3 (26), it is possible that exogenously added caspase-6 activates endogenous caspase-3, which then initiates the first cleavage of pp125<sup>FAK</sup> and acts together with caspase-6 to further degrade pp125<sup>FAK</sup> to the final 90-kD fragment in a manner similar to the indirect cleavage of U1-70 kD and PARP mediated by caspase-6 through its activation of caspase-3 (26). Using both the antibody to the internal epitope (354-534N-pp125<sup>FAK</sup>; Fig. 3 B) and the NH<sub>2</sub>-terminal pp125<sup>FAK</sup> antibody (data not



**Figure 2.** Schematic diagram of pp125<sup>FAK</sup> and FRNK. The domains of pp125<sup>FAK</sup> and FRNK responsible for interaction with different binding partners are indicated, and the putative cleavage sites for caspase-3 (black arrowheads) and caspase-6 (white arrowheads) are shown. The putative fragments resulting from caspase-mediated cleavage observed during GF deprivation-induced endothelial cell apoptosis and the epitopes of the antibodies used for analysis are illustrated diagrammatically in relation to pp125<sup>FAK</sup>.

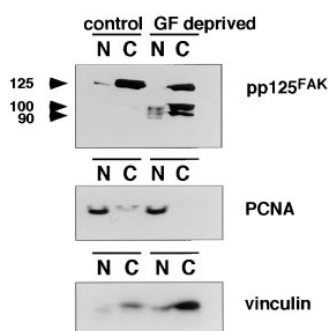
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**Figure 3.** pp125<sup>FAK</sup> is a substrate for caspases. (A) In vitro-translated and [<sup>35</sup>S]methionine-labeled pp125<sup>FAK</sup> was incubated with reaction buffer alone (C), caspase-3, -7, -6, and 10 μg apoptotic (apo) or control (control) cell lysates in the presence (+) or absence (-) of the caspase inhibitor Ac-DEVD-CHO (10 nM) at 37°C for 1.5 h. The samples were subjected to SDS-PAGE and pp125<sup>FAK</sup> cleavage fragments were detected by autoradiography. (B) Cell lysates from control cells were used as a source of endogenous pp125<sup>FAK</sup> and incubated with caspase-3, -7, -6, and reaction buffer alone (control) for the indicated times. Caspase-generated pp125<sup>FAK</sup> cleavage fragments were compared to those present in cells exposed to GF deprivation for 12 h (C, control, V, viable, and A, apoptotic floaters) by immunoblotting using the monoclonal antibody to an internal (amino acids 354–534) pp125<sup>FAK</sup> epitope.

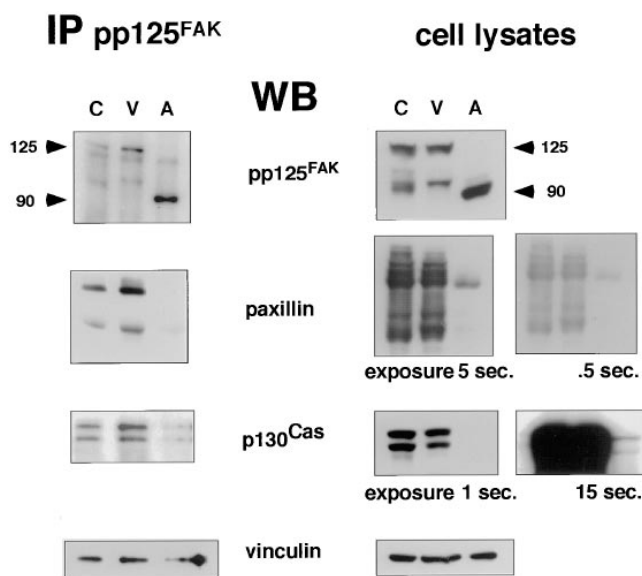
shown), we compared the pp125<sup>FAK</sup> cleavage fragments in the in vitro cleavage reactions with those in straight cell lysates from control, viable, and apoptotic cells, and observed that the purified caspases generate an identical pattern to that observed in the HUVEC apoptosis time course (Fig. 3 B). From these observations we conclude that caspase-3 and/or caspase-3-like caspases are responsible for the initial cleavage of pp125<sup>FAK</sup> to a 100-kD fragment, and that they, together with caspase-6 and/or caspase-6-like caspases, participate in the further degradation of the molecule (see Fig. 2).

**Cleavage Products of pp125<sup>FAK</sup> Are Observed in Both the Nuclear and Cytoplasmic Fractions of Apoptotic Cells.** Caspase cleavage often results in altered cellular localization of substrates after cleavage (21). We, therefore, asked whether the sub-



**Figure 4.** Intracellular distribution of pp125<sup>FAK</sup> in GF deprivation-induced apoptosis. The nuclear (N) and cytoplasmic (C) fractions of control cells and cells deprived of GF for 5 h were immunoblotted for pp125<sup>FAK</sup>, PCNA, and vinculin. The nuclear and cytoplasmic fractions were normalized to contain equal cell equivalents (400,000/lane).

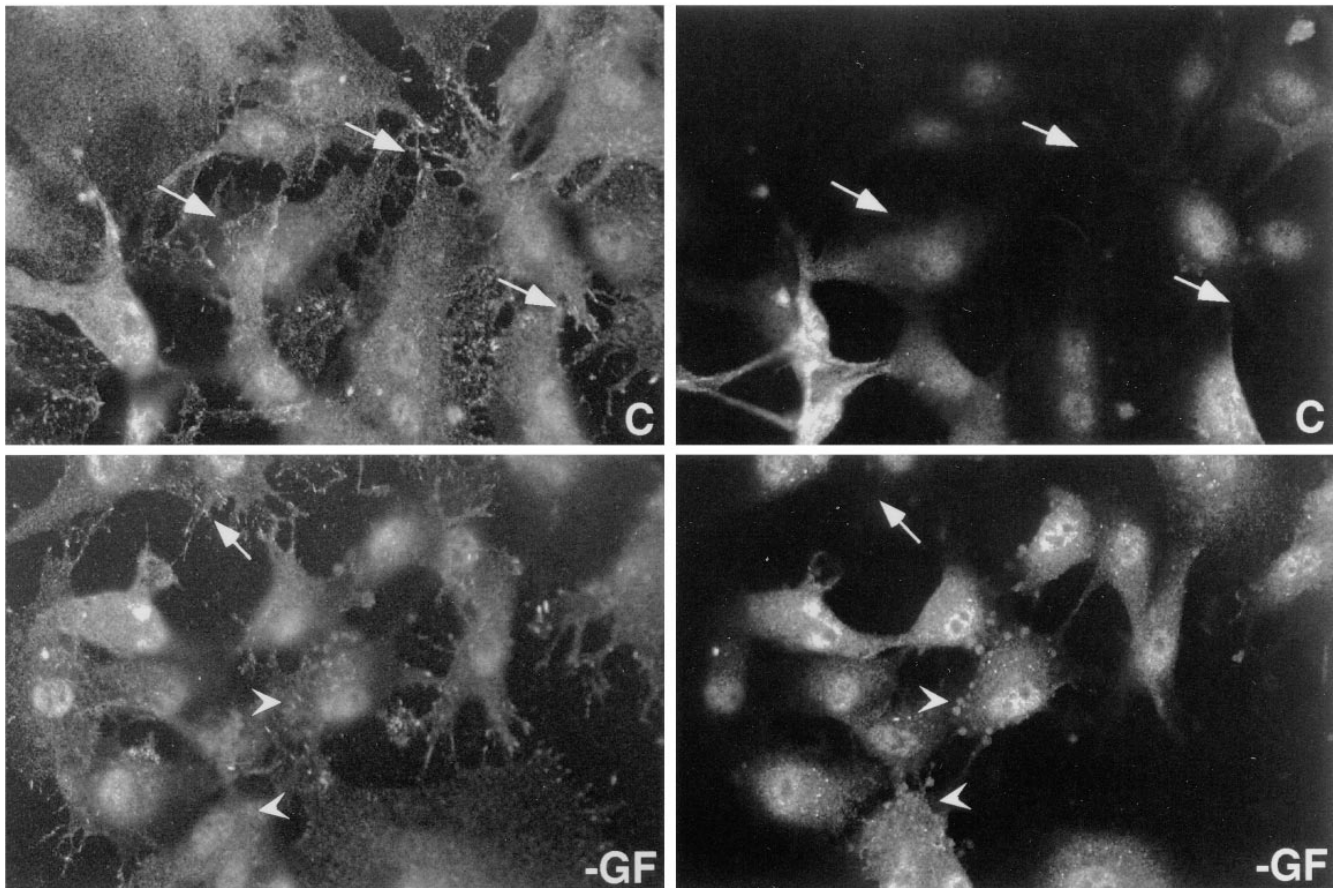
cellular localization of pp125<sup>FAK</sup> is altered in cells undergoing apoptosis. We isolated nuclear and cytoplasmic extracts from control cells and cells exposed to GF deprivation for 5 h, a time when the majority of the cells are viable and most of the cells undergoing apoptosis remain attached. Although full-length pp125<sup>FAK</sup> is detected mainly in the cytoplasmic fractions of control and GF-deprived cells, the 100- and 90-kD cleavage fragments of pp125<sup>FAK</sup> in GF-deprived cells are detected both in the cytosolic and in the nuclear fraction, 67 and 34% of the total, respectively (Fig. 4), suggesting entry of pp125<sup>FAK</sup> fragments into the nucleus after cleavage. Probing the same lysates for proliferating cell nuclear antigen (PCNA) and vinculin as controls show mainly nuclear PCNA and cytoplasmic vinculin localization, consistent with minimal cross-contamination between nuclear and cytoplasmic compartments using a digitonin-based protocol (20). We have no explanation for this putative nuclear translocation of pp125<sup>FAK</sup> fragments, and analysis of



**Figure 5.** Differential association of apoptotic pp125<sup>FAK</sup> cleavage fragments with components of the focal adhesion complex. pp125<sup>FAK</sup> was immunoprecipitated from control (C), viable (V) and apoptotic (A) cells and associated paxillin, p130<sup>Cas</sup>, and vinculin detected by immunoblotting (left). Western blots for the same proteins present in 50 μg of total cell lysates are shown (right).

bottom of cell

4  $\mu$  above bottom

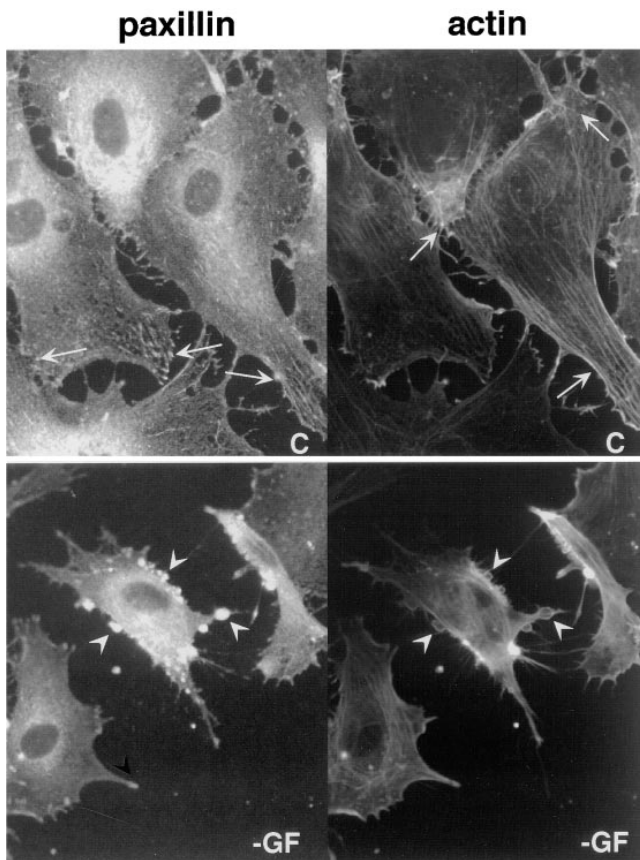


**Figure 6.** Loss of pp125<sup>FAK</sup> from focal adhesions and its appearance in the membrane blebs of retracting cells. Confocal microscopy of immunostaining for pp125<sup>FAK</sup> on control cells (C) and cells deprived of GF for 4 h (-GF), as seen at the bottom of the cell and 4  $\mu$ m above the basal level. pp125<sup>FAK</sup> immunostaining is observed in focal adhesions in control and viable cells (arrows), but is lost from focal adhesions and redistributes into the membrane blebs of retracting cells during the process of their detachment from the substratum (arrowheads).

the COOH-terminal fragment of pp125<sup>FAK</sup> generated by caspase-mediated cleavage did not reveal any obvious nuclear export signal, although partial homology is observed in the sequence <sup>884</sup>LxxLxxL<sup>891</sup>.

**Cleavage of pp125<sup>FAK</sup> Alters Its Interaction with Components of the Focal Adhesion Complex.** Kinetic analysis of pp125<sup>FAK</sup> cleavage in GF-deprived HUVECs reveals caspase-mediated proteolysis early in the process of apoptosis. We, therefore, examined how this cleavage may affect pp125<sup>FAK</sup> interaction with other focal adhesion components. The first proteolytic cleavage leads to loss of  $\sim$ 200 COOH-terminal amino acids (see Fig. 2), the domain that contains the focal adhesion targeting sequence (COOH-terminal residues 904–1,040), required for efficient localization to focal adhesions, as well as the paxillin binding site (COOH-terminal 148 residues), which overlaps the focal adhesion targeting sequence, but is functionally separate (27). We therefore examined the ability of the COOH-terminally truncated pp125<sup>FAK</sup> in apoptotic cells to bind paxillin. Immunoprecipitation of pp125<sup>FAK</sup> from control, viable, and

apoptotic cells shows that the NH<sub>2</sub>-terminal 90-kD pp125<sup>FAK</sup> fragment can be efficiently immunoprecipitated from apoptotic cells (Fig. 5). Immunoblotting for paxillin on the same blots shows paxillin to coimmunoprecipitate with pp125<sup>FAK</sup> in control and viable cells, but to be virtually absent from the 90-kD pp125<sup>FAK</sup> cleavage fragment in apoptotic cells, despite the presence of diminished, but substantial amounts of paxillin in total cell lysates from apoptotic cells (Fig. 5). The COOH terminus of pp125<sup>FAK</sup> also contains a proline-rich region, P<sup>712</sup>PKPSR, which has been shown to mediate the binding of p130<sup>Cas</sup> through its Src homology domain (SH)3 domain (28) (Fig. 2). This region would still be on the principal 90-kD NH<sub>2</sub>-terminal pp125<sup>FAK</sup> fragment in apoptotic cells (predicted to end at the proposed cleavage site DQTD<sup>772</sup>S), and should therefore be available for interaction with p130<sup>Cas</sup>. Indeed, we see residual p130<sup>Cas</sup> to be associated both with the native pp125<sup>FAK</sup> protein and the 90-kD NH<sub>2</sub>-terminal pp125<sup>FAK</sup> fragment in apoptotic cells, in spite of the fact that no p130<sup>Cas</sup> is detected in straight lysates from apoptotic cells



**Figure 7.** Loss of paxillin from focal adhesions and its accumulation in membrane blebs of retracting cells is accompanied by disassembly of the actin cytoskeleton. Double staining for paxillin and actin was performed on control cells (C) and on cells deprived of GF for 4 h (-GF), and the images were evaluated by confocal microscopy. *Arrows*, paxillin localized in focal adhesions and actin stress fibers in the control cells; *arrowheads*, membrane blebs devoid of actin and highly enriched with paxillin on individual retracting and detaching cells after GF withdrawal.

(Fig. 5). Vinculin, another component of the focal adhesion complex, can also be detected in pp125<sup>FAK</sup> immunoprecipitates, and shows no changes between control, viable, and apoptotic cells, and total protein levels of vinculin in the same lysates are quite similar (Fig. 5). Immunoblotting of pp125<sup>FAK</sup> and paxillin immunoprecipitates with an antiphosphotyrosine antibody shows no tyrosine phosphorylation, and no c-Src is associated with the complex (data not shown). These data suggest that cleavage of pp125<sup>FAK</sup> results in a selective modification of the pattern of molecules it associates with, and thus may affect both its cytoskeletal and signaling functions.

**Dissociation of Molecular Components of Focal Adhesions and Membrane Blebbing.** Among the hallmarks of apoptosis in virtually all known apoptotic systems is the phenomenon of plasma membrane blebbing. The sudden onset of surface blebbing, together with cytoplasmic fragmentation, condensation, and exfoliation is one of the earliest morphologic events in apoptosis. We observe membrane blebbing very early in the process of GF deprivation-induced apop-

tosis of endothelial cells, always preceding the detachment of apoptotic cells as floaters. Membrane blebbing has been recently described to be independent of caspases during apoptosis induced by oncogenes, DNA damage, and expression of the proapoptotic bcl-2 homologue bak in Rat-1 fibroblasts. The broad-spectrum cell permeable caspase inhibitor ZVAD-fmk could prevent cleavage of nuclear lamins and PARP, as well as DNA fragmentation, but was unable to prevent membrane blebbing (29). In contrast, we observe ZVAD-fmk to protect HUVEC from apoptosis, and to be equally effective in dose dependently inhibiting cleavage of known endogenous substrates, including pp125<sup>FAK</sup>, and apoptotic membrane blebbing (data not shown). A possible explanation for this divergence could be the different methods used to induce apoptosis, which may affect the point in the signaling cascade where apoptosis is initiated in the two systems. In addition, we are studying apoptosis in normal diploid cells, which may have distinct pathways from transformed cell lines.

To test whether the molecular changes in pp125<sup>FAK</sup> that alter its interaction with components of the focal adhesion complex correlate with changes in the cellular architecture and composition of focal adhesions, we examined the distribution of pp125<sup>FAK</sup>, paxillin, and actin in cells undergoing apoptosis 4 h after GF deprivation. At this time point, the majority of the cells are viable and individual cells begin to exhibit membrane blebbing but remain attached to the substratum. Using confocal microscopy, we see single GF-deprived cells to lose pp125<sup>FAK</sup> immunostaining in the focal adhesions at the bottom of the cell (Fig. 6), and these same cells exhibit membrane blebbing with immunoreactivity for pp125<sup>FAK</sup> observed in the membrane blebs on the apical surface 4  $\mu\text{m}$  above the basal section (Fig. 6). We also observe enhanced immunoreactivity for pp125<sup>FAK</sup> in the cell nuclei of retracting and blebbing cells, in accordance with the cell fractionation studies. Immunostaining for paxillin (Fig. 7) also shows loss of paxillin from focal adhesions, but a distinct feature of these cells is a dramatic redistribution and enrichment for paxillin in the membrane blebs. No actin filaments are visible in the blebbing cells (Fig. 6), and the membrane blebs frequently show an actin ring surrounding the bleb.

**Does Dissolution of Adherence Complexes Accelerate Apoptosis?** In conclusion, we have identified pp125<sup>FAK</sup> as a new cleavage substrate of the apoptotic caspases and suggest that the membrane blebbing observed in GF deprivation-induced apoptosis of HUVECs results, at least in part, from the disassembly of the regular architecture of the focal adhesions and from disruption of molecular interactions within focal adhesions. Besides the changes in focal adhesions, we also observe a dissolution of adherens junctions in apoptotic endothelial cells, including caspase cleavage of  $\beta$ -catenin and plakoglobin, and modifications of vascular endothelial cadherin (Herren, B., B. Levkau, E.W. Raines, and R. Ross, manuscript submitted). Both processes may contribute to the active interruption of extracellular signals required for cell survival. It is possible that this controlled disengage-

ment of cell–matrix and cell–cell interactions results in profound changes in cell shape providing a cell geometry permissive for subsequent irreversible apoptotic events (7). Chen et al. (7) used different patterns on microfabricated surfaces to alter the extent of cell spreading while retaining a constant cell–matrix interaction area. They observed that the more rounded endothelial cells had a higher apoptotic index. It is also possible that initiation of apoptosis in large vessel endothelial cells may be more dependent on modulation of cell adherence than other cell types; once endothelial cells are detached and swept into the circulation, a mechanism to prevent their survival and proliferation at distal sites is critical.

Activation of caspases, such as caspase-3, are generally thought to trigger the final degradative phase of apoptosis (30), but they constitute an amplified protease cascade whose sequence and end points have not been fully defined. Although a few targets of caspase-mediated proteolysis during apoptosis have been identified, their relationship to the apoptotic process is also not clear. Our identification of caspase cleavage of pp125<sup>FAK</sup> and adherens junction components early in the process of endothelial apoptosis suggests the possibility that initial cleavage of the molecular elements required for maintenance of extracellular–cytoskeletal interactions and cell shape may be critical for the progression of the cell death program to the final execution stage, particularly in cell types so dependent on cell–cell and cell–matrix interactions for survival as endothelial cells. Of interest is also the question of whether the main COOH-

terminal pp125<sup>FAK</sup> fragment we observe in apoptotic cells, which is structurally very similar to the pp125<sup>FAK</sup>-related nonkinase (FRNK; see Fig. 2), acts in a manner similar to FRNK. FRNK competitively blocks the formation of focal adhesions (31), and thus, if the COOH-terminal pp125<sup>FAK</sup> cleavage fragment is also a competitive inhibitor, it may further promote disassembly of the focal adhesion in cells induced to undergo apoptosis.

Whether cleavage of pp125<sup>FAK</sup> through the apoptotic caspases is an initiating event in the disassembly of the focal adhesion complex or merely participates in its progression remains unanswered, although the altered pattern of molecular partners that pp125<sup>FAK</sup> can associate with after cleavage suggests a role in this process. The correct assembly and interaction of molecules in the focal adhesion complex, occurring mainly via SH2 and SH3 domains, may be necessary for the mediation of survival signals from the extracellular matrix. This hypothesis is supported by the ability of SH2 domains from a number of signaling molecules to inhibit the “initiation phase” of apoptosis in a cell-free apoptotic system using extracts from *Xenopus* eggs (32). The identification of pp125<sup>FAK</sup> as a target of the apoptotic caspases, together with previous reports on the necessity of pp125<sup>FAK</sup> for survival (15, 16), suggest the possibility that, once initiated, the apoptotic program may be accelerated by interrupting pp125<sup>FAK</sup>-mediated survival signals from the extracellular matrix through the proteolytic destruction of the mediator.

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