Caspase-mediated Cleavage of Focal Adhesion Kinase pp125FAK 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 (pp125FAK), an important component of the focal adhesion complex, and identify pp125FAK as a novel substrate for caspase-3 and caspase-3–like apoptotic caspases. The initial cleavage precedes detachment, and coincides with loss of pp125FAK and paxillin from focal adhesion sites and their redistribution into the characteristic membrane blebs of apoptotically dying cells. Cleavage of pp125FAK differentially affects its association with signaling and cytoskeletal components of the focal adhesion complex; binding of paxillin, but not pp130Cas (Cas, Crk-associated substrate) and vinculin, to the COOH terminally truncated pp125FAK is abolished. Therefore, caspase-mediated cleavage of pp125FAK 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.

A central member of the focal adhesion complex is the tyrosine kinase pp125FAK (FAK, focal adhesion kinase; reference 12), which has been implicated in the integration of signals from integrins, oncogenes, and neuropeptides (13). Although pp125FAK has been shown to be dispensible 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). pp125FAK has also been shown to play an important role in cell survival; constitutively active forms of pp125FAK can rescue epithelial cell lines from apoptosis in suspension (15). Inversely, fibroblasts undergo apoptosis when the interaction of pp125FAK with the cytoplasmic do-

2Abbreviations used in this paper: Ac-DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-aldehyde; FAK, focal adhesion kinase; FRNK, pp125FAK-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, benzoxycarbonyl-Val-Ala-Asp fluoromethyl ketone.
main of the beta 1 integrin is inhibited by microinjection of a peptide identical to the pp125FAK binding site on the integrin, or by microinjection of an antibody interfering with this binding (16). Proteolytic alteration of pp125FAK 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 pp125FAK during platelet aggregation (18).

In this study, we characterize a distinct pattern of specific proteolytic cleavage of pp125FAK 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 1β-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 Na3VO4, 10 mM β-glycerophosphate, PM SF, 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 pp125FAK (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antibodies to pp125FAK, paxillin, p130Cas (Crk-associated substrate) (Transduction Labs., Lexington, KY), vinculin (Sigma Chemical Co., St. Louis, MO), and poly(A)-polymerase (PARP) (Enzyme Systems Products, Dublin, CA). Proteins associated with pp125FAK were detected by immunoprecipitation with the NH2-terminal pp125FAK antibody, followed by immunoblotting with specific antibodies.

Immunocytochemistry. Cell Fractionation Analysis, and Inhibition of Apoptosis by Benzyloxy carbonyl-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 × 5 min incubations with 50 mM NH4 acetate. Fixed cells were incubated with monoclonal antibodies to pp125FAK (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 capase inhibitor benzoylcarbonyl-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 pp125FAK. When endogenous pp125FAK from cell lysates was used as a substrate, control cells were lysed on ice in 10 mM Hapes/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 μg 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 μl in reaction buffer (50 mM Hapes/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 pp125FAK were performed using the TNT coupled reticulocyte lysate system (Promega, Madison, WI) and [35S]methionine (1,000 Ci/mmol; Amersham Corp.) according to the manufacturer’s instructions. The expression plasmid for pp125FAK was a gift from Dr. J.T. Parsons (University of Virginia, Charlottesville, VA). 1/1000 of the reaction was used as a substrate and incubated with 250 ng (5 pmol) of the individual caspases, or with 10 μg 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 μl in reaction buffer at 37°C for 1.5 h. The reactions were stopped in all cases by the addition of 4× sample buffer. Purified caspase-3, -7, and -6 were a gift from Drs. K. O’Rth and V.M. Dixit (University of Michigan, Ann Arbor, MI).

Results and Discussion

Time-dependent Cleavage of pp125FAK in A Poptotic Endothelial Cell. 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 pp125FAK, 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 pp125FAK (Fig. 1 A). pp125FAK undergoes distinct proteolytic changes with time, and completely disappears in the pure apoptotic cell population (the floaters). Three different NH2-terminal proteolytic fragments of ~100, 90, and 48 kD are recognized with a polyclonal NH2-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 pp125FAK fragment present in the floaters. The 48-kD fragment first appears at
totic 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 pp125FAK 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 pp125FAK, with or without the caspase inhibitor. Caspase-6 is much less efficient in cleaving the native in vitro translated pp125FAK 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 pp125FAK 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 pp125FAK cleavage fragments with those observed during HUVEC apoptosis by incubating recombinant caspases with cell lysates from control cells as a source of endogenous pp125FAK, which also provides the molecular environment in which pp125FAK cleavage occurs (Fig. 3 B). Both caspase-3 and -7 cleave endogenous pp125FAK completely and generate the upper pp125FAK cleavage fragment of 100 kD. Surprisingly, under these experimental conditions, caspase-6 is as effective as caspase-3 in completely cleaving endogenous pp125FAK 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 pp125FAK and acts together with caspase-6 to further degrade pp125FAK 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-pp125FAK; Fig. 3 B) and the NH2-terminal pp125FAK antibody (data not
shown), we compared the pp125FAK cleavage fragments in the in vitro reactions with those in straight cell lysates from control and apoptotic cells, and observed that the purified caspases generate an identical pattern to that observed in the HUVEC apoptosis time course (Fig. 3B). From these observations we conclude that caspase-3 and/or caspase-3–like caspases are responsible for the initial cleavage of pp125FAK 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 pp125FAK 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 subcellular localization of pp125FAK 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 pp125FAK is detected mainly in the cytoplasmic fractions of control and GF-deprived cells, the 100- and 90-kD cleavage fragments of pp125FAK 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 pp125FAK 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 pp125FAK fragments, and analysis of

Figure 3. pp125FAK is a substrate for caspases. (A) In vitro–translated and [35S]methionine–labeled pp125FAK 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 pp125FAK cleavage fragments were detected by autoradiography. (B) Cell lysates from control cells were used as a source of endogenous pp125FAK and incubated with caspase-3, -7, -6, and reaction buffer alone (control) for the indicated times. Caspase-generated pp125FAK 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) pp125FAK epitope.

Figure 4. Intracellular distribution of pp125FAK 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 pp125FAK, PCNA, and vinculin. The nuclear and cytoplasmic fractions were normalized to contain equal cell equivalents (400,000/lane).

Figure 5. Differential association of apoptotic pp125FAK cleavage fragments with components of the focal adhesion complex. pp125FAK was immunoprecipitated from control (C), viable (V) and apoptotic (A) cells and associated paxillin, p130Cas, and vinculin detected by immunoblotting (left). Western blots for the same proteins present in 50 μg of total cell lysates are shown (right).
the COOH-terminal fragment of pp125FAK generated by caspase-mediated cleavage did not reveal any obvious nuclear export signal, although partial homology is observed in the sequence LxxLxxL.

Cleavage of pp125FAK Alters Its Interaction with Components of the Focal Adhesion Complex. Kinetic analysis of pp125FAK cleavage in GF-deprived HUVECs reveals caspase-mediated proteolysis early in the process of apoptosis. We, therefore, examined how this cleavage may affect pp125FAK interaction with other focal adhesion components. The first proteolytic cleavage leads to loss of ~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 pp125FAK in apoptotic cells to bind paxillin. Immunoprecipitation of pp125FAK from control, viable, and apoptotic cells shows that the NH2-terminal 90-kD pp125FAK fragment can be efficiently immunoprecipitated from apoptotic cells (Fig. 5). Immunoblotting for paxillin on the same blots shows paxillin to coimmunoprecipitate with pp125FAK in control and viable cells, but to be virtually absent from the 90-kD pp125FAK 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 pp125FAK also contains a proline-rich region, P712PKPSR, which has been shown to mediate the binding of p130Cas through its Src homology domain (SH)3 domain (28) (Fig. 2). This region would still be on the principal 90-kD NH2-terminal pp125FAK fragment in apoptotic cells (predicted to end at the proposed cleavage site DQTD725S), and should therefore be available for interaction with p130Cas. Indeed, we see residual p130Cas to be associated both with the native pp125FAK protein and the 90-kD NH2-terminal pp125FAK fragment in apoptotic cells, in spite of the fact that no p130Cas is detected in straight lysates from apoptotic cells.

Figure 6. Loss of pp125FAK from focal adhesions and its appearance in the membrane blebs of retracting cells. Confocal microscopy of immunostaining for pp125FAK on control cells (C) and cells deprived of GF for 4 h (-GF), as seen at the bottom of the cell and 4 μm above the basal level. pp125FAK 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).
Among the hallmarks of apoptosis in 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 pp125FAK, 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 pp125FAK 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 pp125FAK, 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 pp125FAK immunostaining in the focal adhesions at the bottom of the cell (Fig. 6), and these same cells exhibit membrane blebbing with immunoreactivity for pp125FAK observed in the membrane blebs on the apical surface 4 μm above the basal section (Fig. 6). We also observe enhanced immunoreactivity for pp125FAK 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 pp125FAK 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 β-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 pp125FAK 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 pp125FAK fragment we observe in apoptotic cells, which is structurally very similar to the pp125FAK-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 pp125FAK 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 pp125FAK 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 pp125FAK 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 pp125FAK as a target of the apoptotic caspases, together with previous reports on the necessity of pp125FAK for survival (15, 16), suggest the possibility that, once initiated, the apoptotic program may be accelerated by interrupting pp125FAK-mediated survival signals from the extracellular matrix through the proteolytic destruction of the mediator.

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