Cleavage of Focal Adhesion Kinase by Caspases during Apoptosis*

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Apoptotic cells undergo characteristic morphological changes that include detachment of cell attachment from the substratum and loss of cell-cell interactions. Attachment of cells to the extracellular matrix and to other cells is mediated by integrins. The interactions of integrins with the extracellular matrix activates focal adhesion kinase (FAK) and suppresses apoptosis in diverse cell types. Members of the tumor necrosis family such as Fas and Apo-2L, also known as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), induce apoptosis in both suspension and adherent cells through the activation of caspases. These caspases, when activated, cleave substrates that are important for the maintenance of nuclear and membrane integrity. In this study, we show that FAK is sequentially cleaved into two different fragments early in Apo-2L-induced apoptosis. We also demonstrate that FAK cleavage is mediated by caspases and that FAK shows unique sensitivity to different caspases. Our results suggest that disruption of FAK may contribute to the morphological changes observed in apoptotic suspension and adherent cells.

Apoptosis or programmed cell death is a physiological process that is important for the elimination of transformed cells, the elimination of self-reactive lymphocytes, and for the organization of developing tissues (1). Apoptosis causes characteristic morphological changes that include membrane blebbing, cellular shrinkage, and chromatolysis that lead to cellular detachment from the substratum in adherent cells or a loss of cell-cell contact in suspension cells (2). Cellular attachment to the extracellular matrix (ECM) is mediated by the association of integrins with extracellular matrix components such as fibronectin, collagen, and vitronectin, whereas cell-cell interactions are mediated by the association of integrins with members of the immunoglobulin gene superfamily such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (3). Recent studies suggest that integrins and ECM also promote cell survival. Inhibition of integrin function, for example, leads to loss of cell-cell adhesion and apoptosis in colon epithelial cells, loss of integrin interaction with ECM causes apoptosis of epithelial and endothelial cells (“anoikis”), and ECM suppresses apoptosis in mammary epithelial cells (4–7). Cells attach to the ECM through focal adhesions which are sites of tight structural attachment of the integrins on the cell membrane to the ECM. The focal adhesion can mediate signal transduction events that affect cellular migration, proliferation, differentiation, and survival (8). The cytoplasmic domain of the β subunit of integrins targets the integrin to the focal adhesion (for review see Ref. 9). Binding of integrins to ECM or clustering of integrins results in activation of specific kinases such as the tyrosine kinase focal adhesion kinase (FAK/ pp125FAK) (10).

FAK is a tyrosine kinase that localizes to focal adhesions and associates temporally and spatially with integrins (for reviews see Refs. 11 and 12). FAK also associates with other components of focal adhesions such as paxillin, p130Cas, GRB2, pp60src, pp59Csk, talin, and phosphatidylinositol 3′-kinase (13–23). FAK is expressed at low levels in normal cells but is overexpressed in some cancers such as breast and colon cancer (24). The precise function of FAK is not known, but it may regulate the assembly of focal adhesions in spreading or migrating cells or it may participate in a signal transduction pathway to inform the nucleus that a cell is bound to the ECM, which may suppress apoptosis. The first hypothesis is supported by a study that demonstrates that a truncated isoform of FAK (pp41/43FRNK), which is identical to the COOH-terminal domain of full-length FAK, inhibits cell spreading and migration (25). Richardson and Parsons (25) observed that overexpression of FRNK inhibits tyrosine phosphorylation of FAK, suggesting that FRNK may act as a competitive inhibitor of FAK. The latter hypothesis is supported by recent studies which report the following: 1) FAK suppresses anoikis in epithelial cells; 2) inhibition of FAK in fibroblasts results in apoptosis; 3) attenuation of FAK induces apoptosis in tumor cells; and 4) FAK is cleaved early in myc-induced apoptosis of chick embryo fibroblasts and FAK cleavage and apoptosis is inhibited by plating the cells on fibronectin or collagen (7, 26–28).

It is clear that mammalian cytokine proteases (now designated caspases) related to the Caenorhabditis elegans cell death gene CED-3 are the effectors of the apoptotic signaling pathway triggered by members of the tumor necrosis factor family (reviewed in Ref. 29). Members of the tumor necrosis factor family such as Fas ligand and Apo-2L, also known as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), induce apoptosis in cells which express their cognate receptors (30–32). Apo-2L is more effective in killing adherent cell lines, but apoptosis induced by either ligand is inhibitable by CrmA, a cowpox-derived inhibitor of caspases (33, 34). Sig-

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The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; PARP, poly(A)DP-ribose polymerase; mAb, monoclonal antibody; FMK, fluoromethyl ketone; ICE, interleukin-1β-converting enzyme.
Apo-2L and Fas Induce FAK Cleavage—Recent studies demonstrate that Apo-2L is more effective in inducing apoptosis of adherent cells than Fas, but both are similarly effective in inducing apoptosis of suspension cells (33, 34). FAK has recently been shown to play a role in suppressing apoptosis (7, 26–28). We, therefore, examined the effect of Apo-2L on FAK in suspension and adherent cells. Apo-2L induced sequential cleavage of FAK into two fragments in Jurkat T cells (Fig. 1A). The first cleavage product of approximately 85 kDa appeared 2 h after the addition of Apo-2L, and the second cleavage product of approximately 77 kDa appeared at 4 h (Fig. 1A). At 24 h after the addition of Apo-2L, the 85 kDa fragment disappeared, but the 77-kDa fragment remained (Fig. 1A). The disappearance of the 85-kDa fragment later in Apo-2L-induced apoptosis suggests that either the 77-kDa fragment is cleaved further into the 77-kDa fragment or the 77-kDa fragment may be derived from cleavage of intact FAK and the 85-kDa fragment is unstable. FAK cleavage occurred early in apoptosis and correlated temporally with PARP cleavage (Fig. 1A). Similar results were obtained with an agonistic cross-linking Fas antibody, but FAK cleavage and apoptosis occurred 2–3 h later (data not shown).

We then examined the effect of Apo-2L on FAK in the H460 non-small cell lung cancer adherent cell line. Apoptosis in H460 cells after the addition of Apo-2L followed the same time course that was observed in Jurkat T cells (Fig. 1B). Apo-2L induced
cleavage of FAK into a fragment of approximately 85 kDa 3 h after the addition of Apo-2L (Fig. 1B). In H460 cells, interestingly, no second 77-kDa cleavage product was observed at 5 or 24 h after the addition of Apo-2L (Fig. 1B and data not shown). We did observe, however, that FAK was cleaved into the 85- and 77-kDa fragments in the adherent MCF-7 breast cancer cell line (data not shown). An anti-Fas cross-linking mAb also induced apoptosis and FAK cleavage in H460 cells, but it was temporally later and quantitatively less than Apo-2L (data not shown). The chemotherapeutic agent doxorubicin (2 μM), which induces apoptosis in Jurkat cells, was treated with 50 ng/ml soluble Apo-2L and assayed as described in A. Note that the second 77-kDa product was not detected in H460 cells. These studies were repeated four times with similar results.

A recent study reported that FAK is cleaved during c-Myc-induced apoptosis of chick embryo fibroblasts. Cell death and FAK cleavage were suppressed by plating the cells on ECM components such as collagen and fibronectin (26). FAK cleavage was also inhibited by a β1-specific integrin antibody. Crouch et al. (26) concluded that c-myc-induced cell death in chick embryo fibroblasts requires disruption of the integrin signaling pathways, which are mediated, at least in part, by the interaction of FAK with its downstream effectors. We examined if plating the H460 adherent cell line on ECM inhibits Apo-2L-induced apoptosis and FAK proteolysis. H460 cells were plated on an ECM mixture of fibronectin, vitrogen, and bovine serum albumin. Focal adhesions were detected in the H460 cells plated on ECM by immunofluorescent staining with an anti-vinculin monoclonal antibody (data not shown). There was no significant increase in cell survival or inhibition of FAK proteolytic activity after plating the cells on ECM (data not shown). These data coupled with our observation that FAK is cleaved in suspension cells suggests that integrin signaling through ECM is not involved in suppressing apoptosis induced by Apo-2L or Fas. 

FAK Cleavage Is Suppressed by Cysteine Protease Inhibitors—We then tested the effects of tetrapeptide cysteine protease inhibitors on FAK cleavage and cell survival after Apo-2L treatment of Jurkat cells. ZVAD-FMK (40 μM) completely blocked FAK cleavage in vitro and significantly increased cell survival (Fig. 2). ZVAD-FMK is a general inhibitor of cysteine proteases and Fas-induced apoptosis (43). DEVD-CHO inhibited formation of the second 77-kDa cleavage product at lower concentrations (40 μM), but it did not affect formation of the first 85-kDa fragment at that concentration. At higher concentrations DEVD-CHO (200 μM) suppressed formation of both FAK cleavage products and increased cell survival. DEVD-CHO preferentially inhibits activation of YAMA/CPP32-like caspases (44, 45). YVAD-CHO did not suppress FAK cleavage or augment survival even at a concentration of 200 μM (Fig. 2 and data not shown). YVAD-CHO is more effective as an inhibitor of ICE and ICE-like proteases, but YVAD-CHO is less effective than YVAD-CMK as an ICE-like protease inhibitor (44, 46). We then tested the ability of these inhibitors to suppress cleavage of purified FAK by apoptotic extract in vitro. DEVD-CHO and ZVAD-FMK, but not YVAD-CHO, at 0.5 μM concentrations or more inhibited formation of both FAK cleavage products by apoptotic extract in vitro (Fig. 2B). These data suggest that DEVD-CHO is less potent as a caspase inhibitor in vitro because of reduced cellular permeability. CPP32/YAMA-like caspases appear to mediate the cleavage of FAK, and in vivo the second 77-kDa cleavage event is more sensitive to a
DEVD-inhibitable caspase(s) than the first 85-kDa cleavage event. Similar results were also obtained with these inhibitors following activation of the Fas pathway in Jurkat cells (data not shown).

**Differential Sensitivity of FAK to Caspase-3 (CPP32, Yama, Apopain), Caspase-6 (Mch2), Caspase-7 (Mch3, ICE-LAP3, CMH-1), and Caspase-8 (MACH, FLICE, Mch5) in Vitro.**—Our *in vivo* findings suggested that FAK cleavage is mediated by DEVD-sensitive caspase(s). To identify the caspase(s) involved in FAK cleavage, we examined the effect of different purified caspases on baculovirus expressed FAK *in vitro*. The addition of purified caspase-3 or caspase-7 to FAK generated an 85-kDa FAK cleavage product that migrated with the same mobility as the first cleavage product which was observed in apoptotic extracts *in vivo* (Fig. 3A). Neither caspase-3 nor caspase-7 generated the second cleavage product *in vitro*, but each generated other fragments that probably represent specific caspase-3- and caspase-7-sensitive cleavage sites in FAK (Fig. 3A). Caspase-6, interestingly, partially cleaved FAK into a 77-kDa cleavage product that migrated with the same mobility as the second cleavage product observed with the apoptotic extract *in vivo*, but it did not generate levels of the first cleavage product that were above control (Fig. 3A). Caspase-6 induced only partial FAK cleavage so that we examined if cleavage to the 88-kDa FAK fragment would facilitate generation of the 77-kDa fragment by caspase-6 *in vitro*. Incubation of purified FAK with caspase-3 or caspase-7 followed by incubation with caspase-6 did generate more of the 77-kDa product than caspase-6 alone (data not shown).

Caspase-6, but not caspase-3 or caspase-7, has recently been shown to cleave lamin A (47, 48). Caspase-8 has recently been shown to be capable of proteolytically activating caspases such as caspase-3, caspase-4, caspase-7, and caspase-9 in the absence of naive extract (49, 50). Caspase-6 and caspase-2 were efficiently cleaved by caspase-8 only in the presence of naive extract (50). We tested if caspase-8 alone or in the presence of naive extract cleaves FAK *in vitro*. Caspase-8 induced partial FAK cleavage to form the 85-kDa product, but it induced more FAK cleavage in the presence of naive extract suggesting that caspase-8 activates other caspases such as caspase-3 and caspase-7 in naive extract (Fig. 3). We used twice as much caspase-8 in the assay because caspase-3, caspase-6, and caspase-7 are twice as active as caspase-8.2

These data shown above were generated with an antibody that recognizes the FAK kinase domain. To help us map the potential cleavage sites in FAK we reprobed the blots with an antibody that recognizes the FAK carboxyl terminus. The COOH-terminal antibody recognized a FAK cleavage product of approximately 33 kDa after FAK was incubated with caspase-3, caspase-7, or with caspase-8 plus naive extract *in vitro* (Fig. 3B). The 33-kDa fragment is the predicted size of a COOH-terminal fragment that would be detected after the first FAK cleavage *in vitro*. The same antibody detected a cleavage product of approximately 41 kDa after incubation with caspase-6, but the band was visible only after a longer gel exposure (data not shown). To detect the COOH-terminal FAK fragment in intact cells, we immunoprecipitated with a polyclonal FAK antibody raised against the kinase and COOH-terminal domain of FAK followed by immunoblotting with a COOH-terminal FAK antibody (Fig. 3C). We detected a band of 33 kDa in apoptotic extract that was the same size as the FAK cleavage fragment generated by caspase-3 or caspase-7 *in vitro* (Fig. 3C).

Based on the size of these cleavage products that we observed with the kinase domain antibody and COOH-terminal antibody, we predict that the first cleavage in FAK occurs after the P1 aspartate in DQTD (amino acid 772) and the second cleavage occurs after the aspartate in VSWS (amino acid 704) (see Fig. 4 for schematic of FAK cleavage). The first cleavage would, therefore, generate a 33-kDa COOH-terminal fragment leaving an intact 85-kDa fragment. The second cleavage would remove an additional 8 kDa from the 85-kDa fragment, but the 33-kDa carboxyl-terminal fragment would not change. Our data also suggest that the first FAK cleavage may be caused by caspase-3 or caspase-7 and the second cleavage by caspase-6 or by a related caspase.

Caspase-7 Is More Effective Than Caspase-3 at Cleaving FAK *in Vitro*.—Caspase-3 and caspase-7 both cleave substrates with

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2 Guy Salvesen, personal communication.
The sequence of the caspase-3/caspase-7-like and caspase-6-like cleavage sites are predicted from the size of the FAK cleavage products generated in vitro and in vivo. The first cleavage would occur after Asp^772 to generate a 33-kDa COOH-terminal FAK fragment, and the second cleavage would occur after Asp^782 to generate a 41-kDa COOH-terminal fragment. The relative positions of the SH2, kinase, and focal adhesion targeting domains in FAK are also shown.

have observed that Jurkat cells have basal FAK kinase activity, and kinase activity is markedly reduced in apoptotic extract from Apo-2L-treated Jurkat cells, which supports our hypothesis that FAK function is compromised during Apo-2L-induced apoptosis. Richardson and Parsons (25) identified a naturally occurring truncated isoform of FAK (pp41/43FRNK) that is identical to the COOH-terminal domain of full-length FAK. Overexpression of FRNK inhibited tyrosine phosphorylation of FAK and caused a delay in cell spreading suggesting that FRNK may be a competitive inhibitor of FAK. It is possible that the COOH-terminal FAK fragment that is generated during Apo-2L- and Fas-induced apoptosis will also act as a competitive inhibitor of FAK. It will be interesting, therefore, to determine if overexpression of the COOH-terminal FAK fragment which is generated during Apo-2L- and Fas-induced apoptosis will also perturb FAK function and perhaps induce apoptosis.

Our observation that FAK is cleaved in suspension cells and that cell-cell interactions are disrupted during apoptosis suggests that FAK may play a role in maintaining cell-cell interactions in suspension cells. In summary, our data which show early cleavage of FAK into at least two fragments during Apo-2L- and Fas-triggered apoptosis suggest that disruption of FAK contributes to the morphological changes observed in apoptotic suspension and adherent cells.

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