We have cloned the complete cDNA from mouse paxillin, a 68-kDa adapter protein found in focal adhesions. We found that paxillin was degraded by caspases in Ba/F3 cell apoptosis induced by withdrawal of interleukin-3 (IL-3), a survival factor for this cell, and by ionizing radiation. Also, paxillin was degraded in vitro by incubation with recombinant caspase-3. Western blot analyses of degradation products of overexpressed green fluorescent protein-tagged paxillin and site-specific mutants demonstrated that Asp-102 and Asp-301 were early caspase cleavage sites, and Asp-5, Asp-146, Asp-165, and Asp-222 were late cleavage sites. Overexpression of paxillin delayed apoptosis of Ba/F3 after IL-3 withdrawal. Furthermore, this anti-apoptotic effect of paxillin was augmented by a triple mutation in aspartic acids at caspase cleavage sites. These results suggest that paxillin plays a critical role in cell survival signaling and that the cleavage of paxillin by caspases might be an important event for focal adhesion disassembly during cell apoptosis, contributing to detachment, rounding, and death.

When cells adhere to the extracellular matrix, integrin receptors initiate signals to cluster more integrins together and to recruit cytoskeleton proteins (such as talin, tensin, vinculin, zyxin, and α-actinin), adapters (such as paxillin, Crk-associate substrate (p130CAS)1 and Crk), and kinases (such as focal adhesion kinase (FAK), and Src) to their cytoplasmic tails.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF293882 and AF293883.

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1 The abbreviations used are: p130CAS, Crk-associated substrate; Crk, chicken tumor virus no. 10 (CT10) regulator of kinase; IL-3, interleukin-3; FAK, focal adhesion kinase; LD, consensus sequence of LD motifs (LDLXXL); LIM, zinc finger motifs originally described in homeo-box-containing proteins such as Lin-11, Isl-1, and Mec-3; ARF-GAP, ADP-ribosylation factor-GTPase-activating protein; CALM, cell adhesion kinase; z-DEVD-fmk, benzoxycarbonyl-Asp-Glu-Val-Asp-fluoromethoxy ketone; z-VAD-fmk, benzoxycarbonyl-Asp-Glu-Val-Asp-fluoromethoxy ketone; ALLN, acetyl-leucyl-leucyl-norleucinal; AEBSF, 4-[(2-aminoethyl)benzenesulfonyl] fluoride; GFP, green fluorescence protein; PARP, poly-ADP-ribose polymerase; Ac-DEVD-pNA, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; PBS, phosphate-buffered saline; PI, propidium ioddie; DTT, dithiothreitol.

Linkage of Caspase-mediated Degradation of Paxillin to Apoptosis in Ba/F3 Murine Pro-B Lymphocytes*

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Apoptosis, programmed cell death, is accompanied by a succession of characteristic changes in cellular morphology such as detachment from substratum, rounding, cytoplasm shrinkage, membrane blebbing, chromatin condensation, nuclear shrinkage and fragmentation, and DNA fragmentation (41). It is clear that all these processes are dependent on proteolytic cleavages by caspases, a family of proteases that are activated in cascade and degrade many key cellular proteins during apoptosis. However, the basic mechanisms responsible for these phenomena and how they are integrated or interdependent have not been explored in detail. Recent studies have shown that focal adhesions are disassembled and some of their constituents, such as FAK (42, 43) and p130CAS (44), are cleaved by caspases during apoptosis. However, cleavage of these particular components does not appear to be critical for focal adhesion disassembly.
during apoptosis, because release of FAK from focal adhesion precedes cleavage of FAK by caspase (45). It is more likely that caspase cleavages of components upstream of FAK are crucial for focal adhesion disassembly. One plausible candidate for the caspase target that is critical to focal adhesion disassembly is paxillin, because binding to LD motifs and tyrosine phosphorylation sites at the N-terminal half of paxillin is essential for recruiting other critical focal adhesion proteins, such as FAK (21–23), vinculin (23–24), Crk (23, 40), p130CAS (46), and actopaxin (26), to the focal adhesion complex.

IL-3 is a survival and proliferation factor for hematopoietic cells. Ba/F3, a murine pro-B cell line, is dependent on IL-3 for not only survival and growth but also for its polarized and elongated shape (48). Ba/F3 cells express a considerable amount of paxillin, and cell adhesion and migration are essential for lymphocyte functions (37). In this report, we show that paxillin is degraded by caspase during IL-3 withdrawal and radiation-induced Ba/F3 apoptosis. We studied the kinetics of caspase-mediated cleavage of paxillin and its cleavage sites in detail, and we demonstrate that paxillin is anti-apoptotic in IL-3 withdrawal-induced apoptosis of Ba/F3 cells.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Active recombinant caspase-3 was purchased from Biovision (Milpitas, CA). Cell-permeable caspase inhibitors (z-VAD-fmk and z-DEVD-fmk), colorimetric caspase-3 substrate (Ac-DEVD-pNA), N-acetyl-leucyl-leucyl-norleucinal (ALLN), and N-acetyl-Leu-Leu-methioninal (ALLM), proteasome inhibitor (clasto-Lactacystin β-lactone), aprotinin, leupeptin, and 4-(2-aminoethyl)benzenesulfonyl fluoride (ABESF) were purchased from Calbiochem (La Jolla, CA), and CHAPS was obtained from Sigma Chemical Co. (St. Louis, MO). Alexa 647-conjugated annexin V was from Molecular Probes (Eugene, OR). Antibodies for paxillin (clone 349), green fluorescence protein (GFP) (monoclonal and polyclonal), actin (clone AC-40), and poly(A)DP-rybosyl polymerase (PARP) (polyclonal) were from Transduction Laboratories (San Diego, CA), CLONTECH (San Francisco, CA), Sigma, and Santa Cruz Biotechnologies (Santa Cruz, CA), respectively. Secondary antibodies, horseradish peroxidase-conjugated anti-rabbit and -mouse IgG, were from Amersham Biosciences, Inc. (Piscataway, NJ). Enhanced chemiluminescence substrate kits, Supersignal West Pico and Supersignal West Dura, and the Chemiluminescence Detection System (Pierce, Rockford, IL) were used for Western blot analysis. Immunoprecipitation from Ba/F3 cell extracts as described above. The 5' and 3' portions of paxillin were amplified by PCR using the initial clones for the two halves of paxillin and restriction sites for BglII and EcoRI, respectively. This cDNA was then cloned into pEGFP-C2 and pEGFP-N1 to create GFP-paxillin and Paxillin-GFP fusion proteins, respectively. All clones were verified by DNA sequencing. A QuikChange site-directed mutagenesis kit was used for in vitro mutagenesis of potential caspase-targeting potential aspartic acids (see below) according to the manufacturer's instructions.

Stable Overexpression of Wild-type and Mutant Paxillin—cDNAs encoding GFP fusion proteins with paxillin and their mutants were transfected into Ba/F3 cells by electroporation using Electro Square Porator ECM830 (BTX Division of Genetronics, Inc., San Diego, CA). In detail, Ba/F3 cells (4 × 10⁶) were washed once with 5 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.4) containing 250 mM sucrose and 1 mM EDTA (pH 7.4) and seeded in 20 ml of RPMI 1640 containing 10% heat-inactivated fetal calf serum. Cells were incubated at 37 °C overnight for 12 h and then cloned into 96-well plates by limiting dilution under selection with 400 μg/ml G418. Clones that showed green fluorescence under the fluorescent microscope were selected for expansion, and the expression level of paxillin and GFP-paxillin fusion proteins was assayed by Western blot analysis using anti-paxillin or anti-GFP antibodies.

Western Blotting Analysis and Immunoprecipitation—In 100-mm tissue culture dishes were washed twice by centrifugation at 4 °C and resuspended in cold PBS (pH 7.4). Appropriate amounts of cold PBS buffer containing 20 mM Tris-Cl, 150 mM NaCl, 10 mM Tris-Cl (pH 7.2), 0.5 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 10 mM Tris-Cl (pH 7.2), 0.5 mM EDTA, 0.5 mM EGTA, and 0.1% CHAPS, and 10% sucrose, for 1 h at 37 °C, and 0.5% DMSO was added to the supernatant and continuously inverted overnight at 4 °C.

Caspase Assay and Caspase Reaction (49)—Cells (10 μg of protein) were incubated with 250 μM caspase-3 substrate (Ac-DEVD-pNA) in 100 μl of 25 mM HEPES buffer (pH 7.5) containing 1 mM EDTA, 2 mM dithiothreitol (DTT), 0.1% CHAPS, and 10% sucrose, for 1 h at 37 °C, and 0.5% DMSO was added to the supernatant and continuously inverted overnight at 4 °C.

Propidium Iodide Staining and Confocal Microscopy—Cells (1 × 10⁶) were washed once with PBS containing 1 g/liter sucrose and then fixed and permeabilized overnight in 1 ml of 70% ethanol. After centrifugation at 3000 × g for 10 min, cell pellets were resuspended in PBS containing 1 g/ml PI and incubated at room temperature for 1 h. One drop of this suspension of stained cells was put on a slide glass under a coverslip. Confocal fluoromicroscopic images were acquired on an LSM 510 confocal laser-scanning microscope (Zeiss, Thornwood, NY). Annexin V Conjugation Assay—Cells (1 × 10⁶) were washed once with PBS containing 1 g/liter sucrose and resuspended in 100 μl of binding buffer containing 10 mM HEPES (pH 7.5), 140 mM NaCl, and 2.5 mM CaCl₂ (50). Cells were incubated at room temperature for 15 min after addition of 5 μl of Alexa 647 (excitation: 647 nm; emission: 665 nm)-conjugated annexin V stock solution and 1 μg/ml PI and analyzed

A complete cDNA clone for mouse paxillin β cDNA was also obtained from this library and will be reported elsewhere.
with the FACSCalibur system (BD PharMingen immunocytometry system, San Jose, CA).

Protein Assay—A bicinchoninic acid (BCA) protein assay kit was used, and bovine serum albumin was used as protein standard (51).

RESULTS

Decrease of Paxillin during Ba/F3 Cell Apoptosis Induced by IL-3 Withdrawal and/or γ-Irradiation—Ba/F3 cells are dependent on IL-3 for their survival and proliferation in culture, and IL-3 withdrawal-induced apoptosis of Ba/F3 cells has been well studied (47, 52–54). Ba/F3 cells are sensitive to ionizing radiation. Exposure to 4 Gy of γ-radiation in the absence of IL-3 is enough to induce apoptosis in this cell line (53), but IL-3 protects this cell from irradiation-induced apoptosis (55–56). Ba/F3 cells also depend on IL-3 for cell shape (48). They have an elongated shape under optimum growth conditions, although every culture contains a low percentage of round cells. The entire cell population becomes round immediately after removal of IL-3. During the study of regulation of Ba/F3 cell shape by IL-3, we noticed that paxillin protein levels decreased following IL-3 withdrawal. Western blot analysis of cell extracts with monoclonal anti-paxillin antibody revealed two protein bands, and the intensity of both bands decreased after IL-3 withdrawal. (Fig. 1A). The upper 68-kDa band is paxillin, and the lower 48- or 50-kDa band could be Hic-5 (16) or leupaxin (17), previously described paxillin-like proteins. The lower band was not detected by commercial antibody for Hic-5 (Transduction Laboratories, San Diego, CA, data not shown). Thus, we presume that this band is leupaxin, for which no commercial antibody is available. Paxillin was degraded more rapidly after exposure to 20 Gy of γ-radiation after withdrawal of IL-3 (Fig. 1B). However, in neither case did we see the appearance of smaller breakdown products concurrent with disappearance of p68 paxillin or p48 presumed leupaxin (Fig. 1). Because the cell extracts used in this Western blot analysis included only the Triton X-100-soluble fraction, we also checked the insoluble fraction as described under “Experimental Procedures.” No significant band was detected in the insoluble fraction using the same paxillin antibody (data not shown). We assumed 1) that Ba/F3 cells underwent apoptosis after IL-3 withdrawal and γ-irradiation, 2) that paxillin was cleaved by proteases that were activated during the apoptosis, and 3) that this cleavage destroyed the epitope site for the monoclonal paxillin antibody or left too small epitope-bearing fragments to be detected in Western blot analyses.

IL-3 withdrawal-induced apoptosis of Ba/F3 cells was analyzed with annexin V conjugation, to detect early apoptotic cells, and with parallel PI staining to detect late apoptotic cells (Fig. 2A). Under normal conditions of culture, more than 99% of the Ba/F3 cell population was negative for both annexin V and PI staining (Fig. 2A, 0 h). After 6 h without IL-3, a considerable proportion (19%) of the cells was converted to annexin V-positive, but the major population (80%) of the annexin V-positive cells remained negative for PI staining (early apoptotic cells). The percentage of annexin V-positive cells increased to 81% of total cells after 24-h incubation. At this time point, most of annexin V-positive cells (95%) were converted to positive for PI staining (late apoptotic cells). Apoptosis was much more rapid in cells exposed to radiation in addition to IL-3 withdrawal. Characteristic early apoptotic changes in nuclear morphology (Fig. 2B) were revealed by PI staining of ethanol-fixed cells 8 h after IL-3 withdrawal and γ-irradiation. Virtually all the nuclei appeared apoptotic, namely, shrunken and fragmented, and the chromatin stained brighter and more homogenously (8 h), compared with nuclei in control cells (0 h). The percentage of apoptotic nuclei is plotted in Fig. 2C, along with a plot showing the caspase-3 activity of cell extracts at various times after IL-3 withdrawal and γ-irradiation. The numbers of apoptotic nuclei and the caspase-3 activities appeared to increase in parallel, but apoptotic nuclear changes lagged the increase in caspase activity by about 1 h. Western blot analysis for PARP, a typical caspase-3 substrate that is degraded during apoptosis (49), showed the gradual decrease of 116-kDa intact protein and the gradual increase of an 85-kDa protein, a well known caspase-cleavage fragment of PARP (Fig. 2D). We concluded that Ba/F3 cells underwent apoptosis after IL-3 withdrawal and/or γ-irra-

![Fig. 1. Decrease of paxillin in Ba/F3 cells after IL-3 withdrawal with and without γ-irradiation. Ba/F3 cells were deprived of IL-3 for the indicated times with (B) or without (A) exposure to 20 Gy of γ-radiation. Paxillin and actin were detected by sequential Western blot analyses of the same blotted membrane using ECL (20 μg of protein). Protein molecular mass markers are shown on the left.](image-url)

![Fig. 2. Apoptotic changes in Ba/F3 cells after IL-3 withdrawal and γ-irradiation. Ba/F3 cells were incubated in the absence of IL-3 for the indicated times without (A) or with (B–D) 20 Gy of γ-irradiation. A, annexin V conjugation with parallel PI staining of IL-3-withdrawn cell extracts was analyzed with flow cytometry. B, confocal microscopic images after PI staining with ethanol permeabilization showed apoptotic changes in nuclear morphology after 8 h of incubation. C, apoptotic and normal nuclei (total 300 cells per time point) were counted under the fluorescent microscope after PI staining at various times after irradiation. These values are plotted as percentages of apoptotic nuclei in total number of cells (○). Caspase-3 activities of the cells at the same time points (extracts containing 10 μg of protein) were measured using a colorimetric caspase-3 substrate as described under "Experimental Procedures" (●). D, PARP and its fragments in cell extracts (20 μg of protein) at each time point were detected by Western blot analysis using ECL.](image-url)
were assayed by Western blot analyses of 20 $\mu$g of protein from each cell extract. Control cells were maintained in IL-3 and not irradiated.

diation, and these apoptotic changes were accompanied by an activation of caspase activity and a decrease in paxillin.

**One or More Caspases Were Responsible for the Decrease in Paxillin during Ba/F3 Cell Apoptosis**—We used several protease inhibitors to determine which protease(s) might be involved in the loss of paxillin during Ba/F3 apoptosis. We selected three types of proteases, caspases, proteasome, and calpain, for testing. Caspases are the main executioner proteases in apoptosis (57), but proteasomes and calpains have also been implicated in apoptosis in many cell systems, with significant cross-talk with caspase pathways (58, 59). The general caspase inhibitor, z-VAD-fmk (30 $\mu$M), completely blocked apoptotic nuclear changes and paxillin decrease following IL-3 withdrawal and $\gamma$-irradiation (Fig. 3, lane 3), whereas the proteasome inhibitor, clasto-lactacystin $\beta$-lactone (5 $\mu$M), only partially inhibited apoptotic nuclear changes and completely inhibited paxillin loss (Fig. 3, lane 4). However, the combination of two calpain inhibitors, ALLN and ALLM (50 $\mu$M each), did not lead to any significant abrogation of either apoptotic nuclear changes or paxillin decrease after IL-3 withdrawal and $\gamma$-irradiation (Fig. 3, lane 6). We next tested whether caspase-3, a principal “executioner” caspase isoform in most apoptosis model systems, could directly digest paxillin in vitro. To this end, immunoprecipitated paxillin (Fig. 4A) and GFP-paxillin fusion protein (Fig. 4B) were incubated with 100 $\mu$g of purified active caspase-3. Both proteins were effectively degraded by caspase-3, and this degradation was blocked completely by 10 $\mu$M caspase-3-specific inhibitor, z-DEVD-fmk (Fig. 4). A 27-kDa fragment was detected following GFP-paxillin degradation by caspase-3, and this degradation product disappeared by addition of 10 $\mu$M caspase-3-specific inhibitor, z-DEVD-fmk (Fig. 4B). These data did not rule out a contribution by proteasomes to paxillin’s degradation, but, collectively, they strongly indicated that caspase activity (possibly caspase-3) was involved in both apoptosis and paxillin breakdown. We then embarked on a detailed investigation of caspase-mediated paxillin cleavage during apoptosis.

Cloning of Murine Paxillin cDNA into GFP-tagged Construct—It is known that the mouse has only two paxillin splice variants, $\alpha$ and $\beta$ (15), whereas the human has an additional $\gamma$-isoform (14). However, cloning and sequencing of full-length mouse cDNA of paxillin has not been reported. For the detailed study of paxillin cleavage site by caspases, we cloned the murine paxillin $\alpha$ and $\beta$ cDNAs by PCR as described under “Experimental Procedures” and reported the open reading frame sequences to GenBank™ (accession numbers AF293882 and AF293883). As expected, murine paxillin showed high homology to human and chicken paxillins. Murine paxillin $\alpha$ and $\beta$ were composed of 557 and 591 amino acids, respectively, the same number of amino acids in the corresponding human isoforms (14). Also, they were variably spliced at the same sites as in human paxillins. Murine paxillin $\alpha$ showed 89 and 95% identity in cDNA and amino acid sequences, respectively, to human paxillin $\alpha$, and 80 and 89% to chicken paxillin. Because no endogenous paxillin breakdown products were observed by anti-paxillin probing of Western blots (Fig. 1), we adopted enhanced green fluorescence protein (GFP) as a tag that is big enough to be detected in the Western blot with even the tiniest of paxillin breakdown products. Expression constructs for GFP-paxillin and paxillin-GFP fusion proteins were made by cloning this cDNA into pEGFP-C2 and pEGFP-N1, respectively. To confirm the expression of fusion proteins, after transfection of these constructs into COS-7 cells by electroporation, green fluorescence was observed under the fluorescence microscope, and 95-kDa fusion proteins were detected by Western blot analysis using anti-paxillin and anti-GFP antibodies (data not shown).

Overexpressed GFP-paxillin and Paxillin-GFP Fusion Proteins Were Cleaved during Ba/F3 Apoptosis, and the Cleavages Were Inhibited by Caspase Inhibitor—GFP-paxillin and paxillin-GFP fusion proteins (95 kDa) were stably overexpressed in Ba/F3 cells (Fig. 5, 0 h). As with endogenous paxillin (Fig. 1), these fusion proteins also decreased after IL-3 withdrawal (Fig. 5, A and C) or the combination of IL-3 withdrawal and $\gamma$-irradiation (Fig. 5, B and D). Western blots developed with anti-GFP antibody detected the appearance of 27- and 38-kDa fragments (Fig. 5, A and B) in GFP-paxillin-overexpressing cells and a 50-kDa fragment (Fig. 5, C and D) in the paxillin-GFP-overexpressing cells. The intensities of the 27- and 50-kDa fragments increased in a reciprocal fashion relative to the decrease of the original protein. However, the 38-kDa fragment from GFP-paxillin appeared earlier than the 27-kDa fragment, transiently increased, and finally decreased as the intensity of the 27-kDa fragment increased. This result indicated that the
proteolytic cleavage that gave rise to the 38-kDa fragment is an event earlier than that which generated the 27-kDa fragment. All these and all the following results that use stable overexpressing cell lines were reproduced in at least three independent clones with virtually identical results. Fragments smaller than 27 kDa (size of GFP component) were not observed. In addition, we confirmed that GFP remained intact for at least 27 hours (not transient) appearance of the expected 27-kDa fragment and a stable (not transient) appearance of the expected 38-kDa fragment as the final product of cleavage. These results indicated that Asp-5 is the specific cleavage site that generated the 27-kDa fragment. This makes it the most N-terminal caspase site in paxillin. The second set of mutations was built on the D5E mutant, adding mutations at Asp-67 and Asp-102 in GFP-paxillin, replacing them with alanine (D5E/D67A and D5E/D102A). The D5E/D102A double mutant showed differences in cleavage fragments, lacking the 38-kDa fragment but producing a new ladder of four fragments, 48 kDa, 50 kDa, 56 kDa, and 68 kDa (Fig. 7B). This result indicated that Asp-102 is the specific cleavage site responsible for generating the 38-kDa fragment seen in Figs. 5B and 7A, and that cleavage at this site occurred earlier or more effectively than the other sites for the newly appearing four fragments. A third set of mutations was introduced into the D5E/D102A double mutant of GFP-paxillin at Asp-146, Asp-165, Asp-222, or Asp-301 to yield the following triple mutants (3rd-D146E, 3rd-D165E, 3rd-D222E, and 3rd-D301A). All four triple mutants were stably overexpressed in Ba/F3 cells. These stable cell lines were incubated for 6 h after IL-3 withdrawal and γ-irradiation, and cleavage fragments from each mutant were analyzed by anti-GFP Western blotting and compared with apoptotic fragments seen in the D5E/D102A double mutant (Fig. 7C). The ladder of fragments from each triple mutant was different, lacking one band from the four-fragment ladder of the D5E/D102A double mutant. These results indicated that Asp-146, Asp-165, Asp-222, and Asp-301 were the specific cleavage sites for the 48-, 50-, 56-, and 68-kDa fragments, respectively. The effects of caspase inhibitor on cleavages at these four sites were studied next. As shown in Fig. 7D, 30 μM z-VAD-fmk completely inhibited the proteolytic breakdown of the D5E/D102A double mutant into its characteristic four fragments. An additional single aspartic acid replacement was introduced into the paxillin-GFP fusion protein.
of six caspase-cleavage sites in mouse paxillin by site-directed mutagenesis and stable overexpression in Ba/F3 cells. Mouse GFP-paxillin a fusion protein cDNA was mutated by replacing aspartic acid (D) with alanine (A) or glutamic acid (E) at six different sites: D5E, D102A, D146E, D165E, D222E, and D301A. A: single mutant, D5E; B and D: double mutant, D5E/D102A; C: four triple mutants with the third mutation (as indicated), introduced into the D5E/D102A double mutant. A different single mutation was introduced into paxillin-GFP fusion protein cDNA (panel E: D301A). All amino acid numbers are based on order of mouse paxillin α amino acids (K.-O. Chay et al., GenBankTM AF293882). Ba/F3 cell lines stably overexpressing mouse paxillin mutants as GFP fusion proteins were deprived of IL-3 for the indicated times after exposure to 20 Gy of γ-radiation. Fusion proteins and their fragments were detected by Western blot analyses of 20 μg of protein from each cell extract using anti-GFP polyclonal antibody.

(paxillin-GFP D301A) and overexpressed in Ba/F3. This mutant also showed differences from the apoptotic fragments generated from wild-type paxillin-GFP: missing the characteristic 50-kDa fragment (Fig. 5D), instead producing a 70-kDa new major fragment and a ladder of larger minor fragments (Fig. 7E). These results indicated that Asp-301 was the first cleavage site (generating smallest fragment) from the C terminus of paxillin. This conclusion was confirmed by the data from the 3rd-D301A triple mutant of GFP-paxillin, which indicated that Asp-301 is the cleavage site farthest (making the largest fragment) from the N terminus of paxillin (Fig. 7C, lane 4). When considered in their entirety, these data indicated that there are no other caspase cleavage sites. These mutants and their characteristic fragments of GFP-paxillin permitted the localization of the epitope site for anti-paxillin monoclonal antibody (clone 349), which is the most widely used antibody for paxillin studies. This was done by comparing the anti-GFP and anti-paxillin Western blot analyses of the D5E/D102A double mutant of GFP-paxillin and its fragments (Fig. 8A). The anti-paxillin antibody did not recognize the 48-kDa fragment, whereas the anti-GFP antibody recognized all the fragments. These data showed that the epitope for this antibody was lost when paxillin was cleaved at Asp-146, indicating that it was located between Asp-146 and Asp-165. Fig. 8B shows a schematic diagram of the six identified cleavage sites and the epitope site for clone 349 anti-paxillin antibody. The two early cleavage sites are designated LD and LIM motifs, respectively (7–10).

**Paxillin Overexpression**—Paxillin has been implicated in integrin-mediated cell migration, adhesion, and survival pathways (7–10). We tested whether paxillin was involved in the survival signal pathway of this cell using the IL-3 withdrawal-induced apoptosis model system and the annexin V conjugation assay for apoptotic cells. Non-transfected Ba/F3 control cells and stable overexpression cell lines of GFP-paxillin fusion proteins (clone 2) and its mutant (clone 12), in which the three main caspase-cleavage sites had been modified to a non-cleavable form (3rd-D301A), showed different time courses of apoptosis (Fig. 9A). Before IL-3 withdrawal, more than 95% of Ba/F3 control and overexpressing cells were negative for annexin V conjugation (Fig. 9, A and B). After 4 h of IL-3 withdrawal from Ba/F3 control cells, annexin V-positive cells appeared. The number of annexin V-positive cells increased gradually to 52% after 12 h and to 81% after 24 h in a sigmoid pattern (Fig. 9A). This time course of Ba/F3 apoptosis is well matched to one reported previously (52). But, the appearance and increase in number of annexin V-positive cells were delayed significantly in cells with stable overexpression cells of GFP-paxillin. The number of annexin V-positive increased only to 22% after 12 h and to 69% after 24 h. These data suggested that paxillin has anti-apoptotic effects in this IL-3-withdrawal system. Ba/F3 cells with stable overexpression of the triple mutant GFP-paxillin (3rd-D301A) showed a time course of apoptosis similar to that of wild-type overexpressing cells for 12 h. However, apoptosis in this cell line showed a prominent delay between 12 and 24 h after IL-3 withdrawal. Only 17 and 52% of cells were positive for annexin V at 12 and 24 h, respectively. We also compared the percentage of apoptotic nuclei of these cells after ethanol permeabilization and PI staining. After 24 h of IL-3 withdrawal, Ba/F3 control cells showed 82 ± 2% apoptotic nuclei, whereas Ba/F3 cells with stable overexpression of the wild-type (clone 2) and the triple mutant GFP-paxillin (3rd-D301A, clone 12) showed only 67 ± 3% and 38 ± 3% apoptotic nuclei, respectively. These data suggested that prolonged pres-
ence of overexpressed paxillin (due to loss of caspase cleavage sites) resulted in a prolonged anti-apoptotic effect. But, the meaning of these data was limited, because they were from cloned cell lines. To confirm the anti-apoptotic effect of paxillin and its mutant overexpression, we analyzed two additional clones from each group and two separate clones of GFP-overexpressing cells (Fig. 9B). Both additional clones of GFP-overexpressing cells (clones 2 and 3) showed an extent of apoptosis after 12 and 24 h of IL-3 withdrawal that was similar to that of Ba/F3 (Control) cells. Two additional clones of wild-type GFP-paxillin-overexpressing cells (clones 5 and 6) showed the significant delay in apoptosis seen for clone 2, and two other clones from the triple mutant-overexpressing cells (3rd-D301A, clones 13 and 15) showed a further delay seen in the designed clone 12 (Fig. 9A), which was particularly evident after 24 h of IL-3 withdrawal. Because three clones from each group showed consistent delays in apoptosis, we concluded that overexpressed paxillin has inhibitory effects on IL-3 withdrawal-induced apoptosis of Ba/F3 cells. Fig. 9C shows the expression levels of endogenous paxillin, GFP, and GFP-paxillin fusion proteins in all the cells used. The small variations in extent of delayed apoptosis of each clone appeared to be proportional to their expression levels of overexpressed paxillin.

**DISCUSSION**

IL-3 is produced by activated T cells and acts as a survival and proliferation factor for many hematopoietic cells (60). IL-3 may play an important role in the expansion of hematopoietic populations during inflammation. On the other hand, attenuation of an inflammatory response may require depletion of IL-3 and the resultant apoptotic death of the expanded IL-3-dependent cells (61). The murine pro-B cell, Ba/F3, is dependent on IL-3 for its survival and proliferation (47). IL-3 also protects this cell from radiation-induced apoptosis (55, 56). The signal pathway for the anti-apoptotic effect of IL-3 seems to be separate from the growth-promoting signal pathway, because treatment Ba/F3 with genistein, a protein-tyrosine kinase inhibitor, abrogated the IL-3-induced DNA synthesis but not the IL-3-induced anti-apoptotic effect (62). Ba/F3 is dependent on IL-3 for its cell shape as well as its survival and proliferation (48). In normal culture in the presence of appropriate amount of IL-3, Ba/F3 cells, like most cultured lymphocytes, have irregular, elongated, and polarized shapes. They have actin-rich membrane ruffles at one side of cells and tubulin-rich tail at the other side. Removal of IL-3 results in the rounding and depolarization of these cells. Salgia et al. (63) showed the tyrosine phosphorylation of paxillin in response to IL-3 in 32D cells, a murine promyelocytic line, which is also dependent on IL-3 for survival and proliferation. They also showed that IL-3 induced a transient interaction between paxillin and vinculin and transient localization of these focal adhesion proteins to pseudopodia of 32D cells. IL-3 also induced tyrosine phosphorylation of paxillin in Ba/F3 with concomitant changes in cellular morphology.3

In this report, we report that paxillin decreased rapidly in Ba/F3 cells following IL-3 withdrawal (Fig. 1A). This decrease in paxillin was more rapid and complete following addition of another apoptotic insult, γ-irradiation, to the IL-3 withdrawal (Fig. 1B). In both cases, no breakdown products of paxillin could be detected (Fig. 1). We assumed that paxillin was cleaved into one or more small fragments by some protease(s) during IL-3 withdrawal-induced apoptosis of Ba/F3 cells. To test this hypothesis, we first studied the apoptotic changes in Ba/F3 cells after IL-3 withdrawal and/or γ-irradiation (Fig. 2). Several parameters for apoptosis such as PS exposure, nuclear changes, activation of caspase-3, and cleavage of PARP indicated that Ba/F3 cells underwent apoptosis after IL-3 withdrawal and/or γ-irradiation. Furthermore, the time course of paxillin decrease coincided with the changes in these apoptotic parameters. We used this combination of IL-3 withdrawal and γ-irradiation as an apoptotic insult to study the details of caspase cleavage of paxillin, because this system gave more rapid results and might reduce the possibility of further degradation of paxillin cleavage products by other nonspecific proteases.

3 L. Y. Romanova, K.-O. Chay, M. V. Blagosklonny, and J. F. Mushinski, manuscript in preparation.
To identify the protease(s) involved, we used protease inhibitors specific for caspase, proteasome, and calpain, all of which can be activated during apoptosis (57–59). Pan-caspase inhibitor (30 μM z-VAD-fmk) blocked the decrease of paxillin and apoptotic changes in nuclear morphology completely (Fig. 3). The same concentration of caspase-3 specific inhibitor (z-DEVD-fmk) showed similar effects on paxillin decrease but slightly less extensive (80%) inhibition of nuclear change (data not shown). Cross-talk between the caspase and proteasome systems is complex. The role of proteasomes in apoptosis seems to be bidirectional. Inhibition of proteasomes induced apoptosis in most cells, but in some others, it protected from apoptosis (58). Recently, Hirsch et al. (64) reported that the proteasome is involved in the premitochondrial arm of apoptosis, upstream of the caspase cascade. Although lactacystin (5 μM clasto-lactacystin β-lactone) protected partially Ba/F3 cells from apoptosis (Fig. 3) and inhibited paxillin degradation, these effects might be from an upstream effect that decreased caspase activation, rather than direct degradation of paxillin by proteasome. A ladder of polyubiquitinated paxillin was not observed in Western blot of lactacystin-treated samples, suggesting that ubiquitin/proteasome pathway utilization is minimal in this system. Furthermore, Figs. 5–7 showed that overexpressed paxillin and its mutants were degraded into products with discreet and considerable size, which is unlikely due to the action of proteasomes, which chop proteins into small and different size of oligopeptides (65). Another family of proteases that are known to cleave proteins into discreet sizable products during apoptosis and other physiological processes are calpains (59, 66). Although cleavage of proteins by calpains is sequence-specific, calpains do not have strict specificity for substrate sequences (59, 67), whereas caspases are absolutely specific for aspartic acid residue at the cleavage site (P1 position) (57). Treatment with calpain-specific inhibitors (ALLN + AMLM, 50 μM of each) did not inhibit either paxillin decrease or nuclear changes in Ba/F3 cell apoptosis (Fig. 3). So, although caspases are activated in apoptosis of some cells, our data suggested that calpain was not critical to paxillin cleavage in Ba/F3 cell apo-

As the third step, we focused on the precise action of caspase. In vitro incubation of immunoprecipitated paxillin with a purified active form of caspase-3 resulted in the decrease of paxillin, and it was reversed by caspase-3-specific inhibitor (Fig. 4A). The same phenomena were observed for the GFP-paxillin fusion protein obtained from transient expression in COS-7 cells (Fig. 4B).

We demonstrated that paxillin is cleaved at six different aspartic acid residues, Asp-5, Asp-102, Asp-146, Asp-165, Asp-222, and Asp-301 (Fig. 8). Intriguingly, all six sites were found only in the N-terminal half of paxillin, and the C-terminal half of paxillin remained intact until the late stage of apoptosis (Fig. 5D). As stated above, the N-terminal half of paxillin provides binding motifs for a variety of focal adhesion proteins (21–29), whereas the C-terminal half of paxillin provides focal adhesion targeting motifs for paxillin (30, 31). The consequences of the intact C-terminal half of paxillin in apoptotic cell remains to be studied. We simply classified the cleavage sites into two groups, early (Asp-102 and Asp-301) and late cleavage sites (Asp-5, Asp-146, Asp-165, and Asp-222) (Fig. 8), based on the order of appearance of corresponding products (discussed under “Results”). But the kinetics of cleavage may be more complicated, and a more detailed study of kinetics with the different caspase isozymes remains to be undertaken.

We also demonstrated that overexpressed paxillin can inhibit apoptosis by comparing the time course of apoptosis of Ba/F3 control cells with Ba/F3 cells overexpressing GFP alone and GFP-paxillin. This result was reinforced by the additional data that overexpression of a non-cleavable mutant of paxillin, with mutations at two early sites, augmented the anti-apo-

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