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Cleavage and nuclear translocation of the caspase 3 substrate Rho GDP-dissociation inhibitor, D4-GDI, during apoptosis

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Abstract

While investigating endonucleases potentially involved in apoptosis, an antisera was raised to bovine deoxyribonuclease II, but it recognized a smaller protein of 26 kDa protein in a variety of cell lines. The 26 kDa protein underwent proteolytic cleavage to 22 kDa concomitantly with DNA digestion in cells induced to undergo apoptosis. Sequencing of the 26 kDa protein identified it as the Rho GDP-dissociation inhibitor D4-GDI. Zinc, okadaic acid, calyculin A, cantharidin, and the caspase inhibitor z-VAD-fmk, all prevented the cleavage of D4-GDI, DNA digestion, and apoptosis. The 26 kDa protein resided in the cytoplasm of undamaged cells, whereas following cleavage, the 22 kDa form translocated to the nucleus. Human D4-GDI, and D4-GDI mutated at the caspase 1 or caspase 3 sites, were expressed in Chinese hamster ovary cells which show no detectable endogenous D4-GDI. Mutation at the caspase 3 site prevented D4-GDI cleavage but did not inhibit apoptosis induced by staurosporine. The cleavage of D4-GDI could lead to activation of Jun N-terminal kinase which has been implicated as an upstream regulator of apoptosis in some systems. However, the results show that the cleavage of D4-GDI and translocation to the nucleus do not impact on the demise of the cell.

Keywords: apoptosis; caspase; D4-GDI; deoxyribonuclease II; protein phosphatase inhibitors; zinc

Abbreviations: AEBSF, 1-(aminoethyl)-benzenesulfonyl fluoride hydrochloride; CHO, Chinese hamster ovary; D4-GDI, Rho GDP-dissociation inhibitor protein; DFF, DNA fragmentation factor; DNase, deoxyribonuclease; EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride; EGFP, enhanced green fluorescent protein; ICE, interleukin 1-β converting enzyme; z-VAD-fmk, carbobenzoxy-valine-alanine-aspartate-fluoromethylketone; SDS, sodium dodecyl sulfate

Introduction

Apoptosis plays a central role in such processes as development, tissue homeostasis, and thymic selection, as well as pathologies ranging from neurodegenerative disease, autoimmune disorders, and viral infection, to cancer. Much research on apoptosis has focused on determining proteins involved in decisions of cell fate, and regulation of the execution phase of cell death involving protease and endonuclease activation. A great deal of this information has been gained from the study of small organisms such as the nematode *C. elegans*, which has a well-defined developmental program during which specific cells die. The characterization of nematodes with mutations in the cell death process has led to identification of genes which regulate apoptosis.[1] The mammalian homologs have been identified for many of these regulatory genes. One gene which is central to the execution phase of cell death is *ced-3*. This gene was found to encode a homolog of the human cysteine protease interleukin 1-β converting enzyme (ICE),[2] and further studies led to the identification of a family of these cysteine proteases now termed caspases.[3]

Caspases have a central role in the execution phase of apoptosis.[2] Numerous substrates have been identified, including poly(ADP-ribose) polymerase,[4] lamin A, lamin B,[5] DNA fragmentation factor (DFF/ICAD),[6] 70 kDa U1 small ribonucleoprotein,[7] and gelsolin.[8] Although many proteins are cleaved during apoptosis, a role has only been identified for a few of them. ICAD has been found to be cleaved by caspase 3 which leads to the release of an endonuclease and subsequent DNA digestion observed during apoptosis.[9,10] Cleavage of gelsolin and lamin reportedly cause some of the morphological changes observed during apoptosis.[8,11]

In the process of investigating the potential role of deoxyribonuclease II (DNaseII) during apoptosis,[12,13] the 31 kDa protein was purified and used to immunize rabbits for the production of a polyclonal antibody. The antiserum was used to probe Western blots of cell lysates. The major species recognized by the antiserum in undamaged cells migrated at 26 kDa. Interestingly, it was observed that this 26 kDa protein was cleaved to a 22 kDa form as cells underwent apoptosis. Therefore this protein was purified and sequenced to confirm its identity. Amino acid sequence analysis of the protein identified it as D4-GDI rather than DNaseII.

D4-GDI is a Rho GDP-dissociation inhibitor protein. This protein functions to keep the Rho protein in its GDP-bound inactive state. The Rho family of small GTPases including Rho, Rac, and Cdc42, have been shown to regulate many cellular processes such as the assembly of the actin cytoskeleton and focal adhesions,[14] oxidant production in

leukocytes, and the stress response through activation of c-Jun N-terminal kinase and p38^{mpk2}.[15]

Previously D4-GDI has been reported to be cleaved at aspartate 19 by caspase 3 during apoptosis in Jurkat T-cells induced with anti-Fas antibody, overexpression of Bax, and staurosporine,[16,17] and in BL60 Burkitt lymphoma cells induced with anti-IgM.[18] We add in this report the observations that D4-GDI is cleaved in ML-1 cells and many other cell types induced to undergo apoptosis by incubation with etoposide, staurosporine, or anti-fas antibody. We also show that protease inhibitors, serine/threonine phosphatase inhibitors and zinc can inhibit this cleavage. In addition, this is the first report to our knowledge of a caspase cleavage triggering translocation of a substrate from the cytoplasm to the nucleus of a cell.

Results

Detection of a protein cleaved during apoptosis

The polyclonal antiserum raised against the 31 kDa bovine DNaseII detected a 30/31 kDa doublet in the commercial DNaseII protein (Figure 1). The antibody also detected the 30/31 kDa doublet in bovine spleen lysates, but only a faint 30 kDa band was visible from the human breast cancer cell lines MDA-468 and T47D, the murine leukemic L1210 cells, and CHO cells. The most prominent band detected in the bovine spleen lysate migrated at 26 kDa (p26). This band was also detected in the human and murine cell lines, but was not detected in CHO cells. In the human myelocytic leukemia cell line ML-1, the p26 was the most abundant species. When ML-1 cells were induced to undergo apoptosis with staurosporine, the major species recognized migrated at 22 kDa (p22). To investigate this further, ML-1 cells were incubated with 20 μg/ml etoposide and assayed every 30 min thereafter. p22 appeared concurrent with DNA fragmentation, while a reduction of p26 was observed (Figure 2a). Hence, p22 appears to result from proteolytic cleavage of p26. Similar results were obtained during apoptosis induced by 1 μM staurosporine (Figure 2b). The cleavage of p26 was also observed in these cells during apoptosis induced by anti-FAS antibody (data not shown). We have also detected this cleavage during apoptosis in Jurkat cells, HL60 cells, MDA-468 and MCF7 human breast cancer cells, murine 32D cells, as well as mouse and rat thymocytes (data not shown).

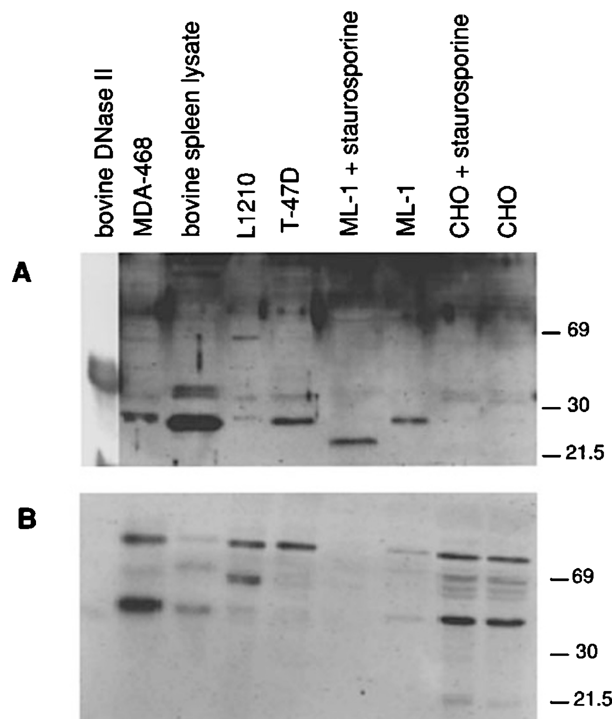

Figure 1 Expression of p26 in a variety of cell lines and bovine spleen. Western analysis was performed of bovine DNaseII, and lysates from bovine spleen and the indicated cell lines. Western blots were probed with serum after immunization with DNaseII (**A**) or with preimmune serum (**B**). The left lane in **A** is a much shorter exposure of the same membrane

Determination of the identity of p26

Due to the uncertainty of the identity of the protein recognized by the antiserum, a scheme was developed to purify and sequence it. Cytosolic fractions of cells were prepared as this was determined to contain p26. The cytosol was then subjected to purification on a phenyl Sepharose column. Fractions containing p26 were identified by Western analysis. The protein was found to bind to the column in 3 M NaCl and was eluted at 1 M NaCl. The 1 M NaCl eluate was concentrated and subjected to reverse phase HPLC. The fractions containing p26 were electrophoresed, transferred to a membrane, excised from the blot, and subjected to N-terminal amino acid sequencing. p26 appeared to be blocked at the amino terminus and was therefore digested with endopeptidase Lys-C. p26 peptides were separated by reverse phase HPLC. Two peptides were subjected to amino terminal sequencing. The amino acid sequence obtained was compared to sequences in the Genbank database. Both peptides were found to be derived from Rho GDP-dissociation inhibitor protein D4-GDI (Figure 3).

To further confirm the identity of p26, purified D4-GDI was used to immunodeplete the antisera. The undepleted antiserum detected p26 and p22 in ML-1 cells, recombinant D4-GDI, and the 31 kDa bovine DNaseII (Figure 4A). Recombinant D4-GDI was capable of depleting the antiserum of its ability to recognize p26, p22, and D4-GDI, while it still recognized the 31 kDa bovine DNaseII, suggesting the antiserum may contain an antibody that recognizes the originally intended antigen (Figure 4B). An antibody raised against D4-GDI also detected p26 and p22 in the normal and apoptotic ML-1 cells respectively, as well as purified D4-GDI. Interestingly, this antibody also detected a small amount of p26 in Sigma bovine DNaseII (Figure 4C). Therefore, a low level of D4-GDI presumably contaminated the DNaseII antigen leading to the production of the antiserum reported here.

Analysis of p26 cleavage during apoptosis

Experiments were performed to determine if the cleavage of D4-GDI was specific to the apoptotic process. ML-1 cells were

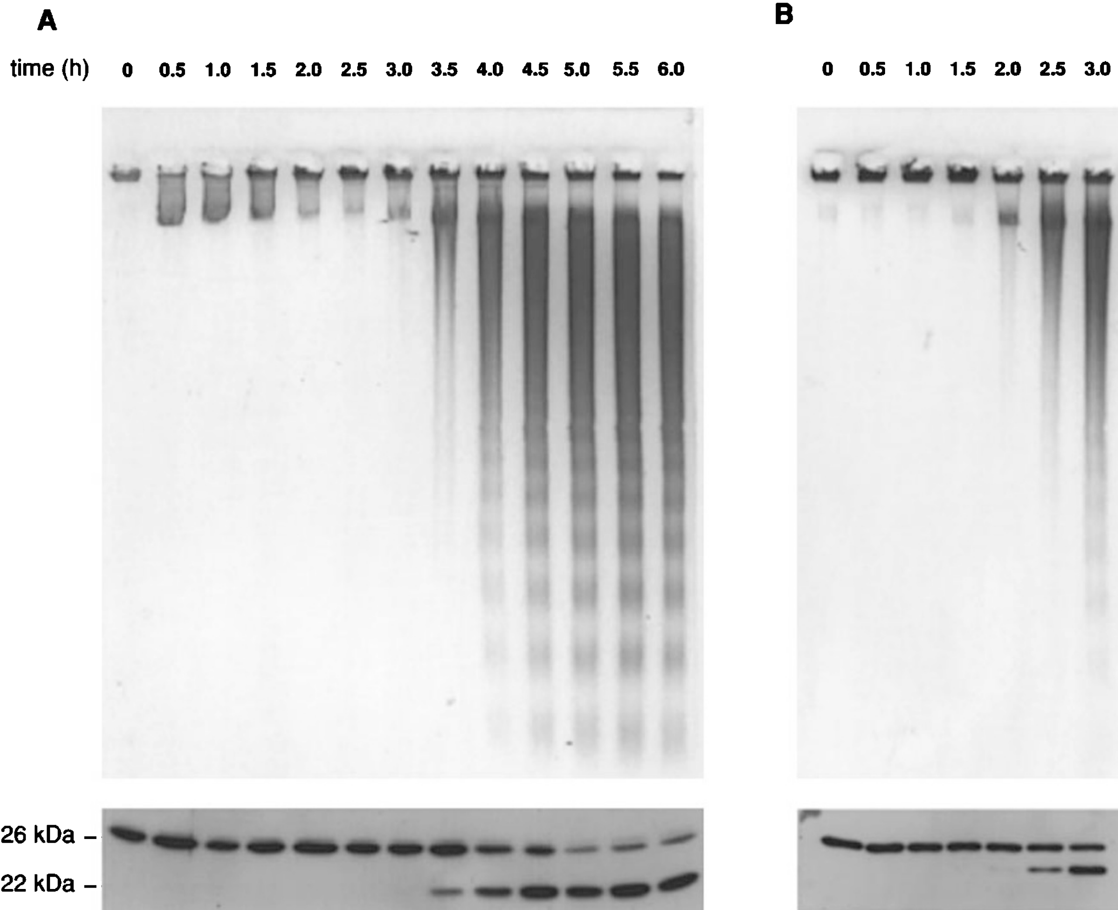

Figure 2 Time course of DNA digestion and cleavage of p26 during apoptosis.(**A**) ML-1 cells were incubated for 30 min with 20 μg/ml etoposide followed by incubation in fresh media for the indicated time. (**B**) ML-1 cells were incubated with 800 nM staurosporine for the indicated time. Cells were harvested and analyzed for DNA digestion (top) or Western analysis using the anti-D4-GDI antiserum developed in this laboratory (bottom)

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MTEKAPEPHVEEDDDDELDSKLNYKPPPQKSLKELQEMDK
DDESLIKYKKTLLGDGPVVTDPKAPNVVVTRLTLVCESAP
GPITMDLTGDLEALKKETIVLKEGSEYRVKIHFKVNRDIV
SGLKYVQHTYRTGVKVDKATFMVGSYGPRPEEYEFLTPVE
EAPKGMLARGTYHNKSFFTDDDKQDHLSWEWNLSIKKEWT
E
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Figure 3 Amino acid sequence of Rho GDP-dissociation inhibitor protein D4-GDI. The underlined amino acids indicate peptides sequenced from the immunoreactive band recognized by the antisera. The asterisks indicate caspase cleavage sites in the protein

incubated with 20 μg/ml etoposide for 30 min, then various inhibitors of apoptosis were added during the following 4 h period. The protein phosphatase inhibitor okadaic acid exhibited concentration-dependent inhibition of the DNA digestion (Figure 5A). The cleavage of p26–p22 was inhibited by the same concentration of okadaic acid that inhibited DNA digestion. The cleavage of p26 and DNA

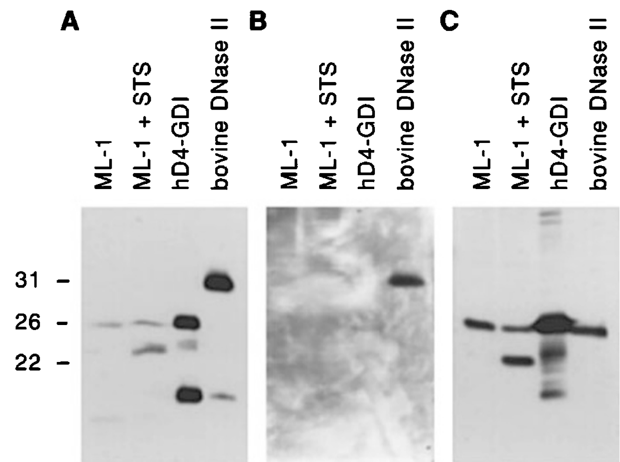

Figure 4 Confirmation of the identity of p26 and p21 as D4-GDI. Triplicate Western blots of ML-1 cells untreated (lane 1) or incubated with 1 μM staurosporine for 3 h (lane 2), recombinant D4-GDI (lane 3), or bovine DNaseII (lane 4), were probed with the antisera produced here (**A**), the same antisera preincubated with recombinant D4-GDI protein (**B**), or authentic anti-D4-GDI antibody (**C**)

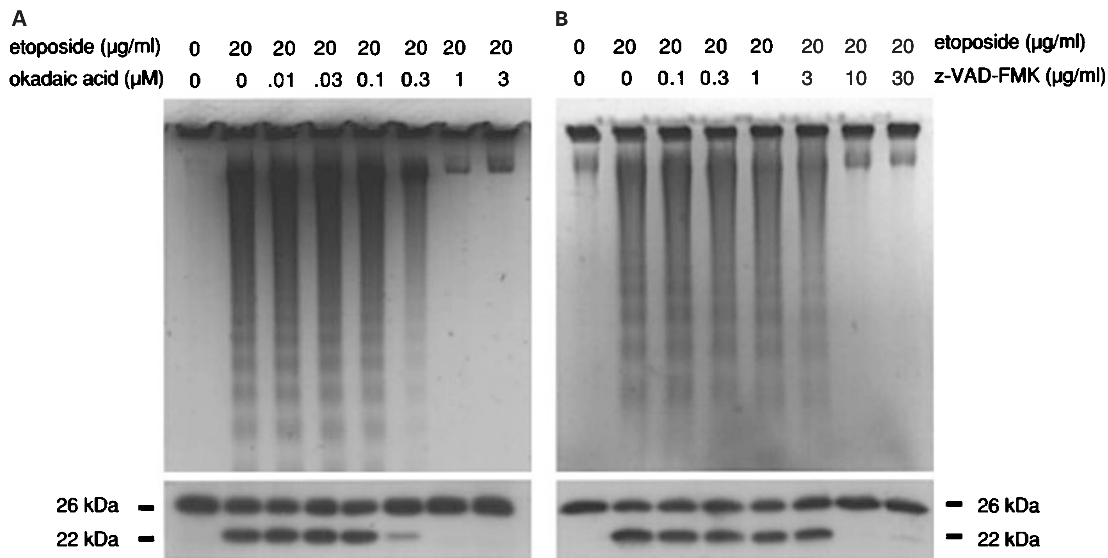

Figure 5 Serine/threonine protein phosphatase inhibitors and caspase inhibitors inhibit D4-GDI cleavage. ML-1 cells were incubated with 20 μg/ml etoposide for 30 min followed by incubation in fresh media with the indicated concentrations of okadaic acid (**A**) or z-VAD-fmk (**B**) for 4 h. Cells were harvested at the end of the incubation and analyzed for DNA digestion (top panels) or by Western analysis using our anti-D4-GDI antibody (bottom panels)

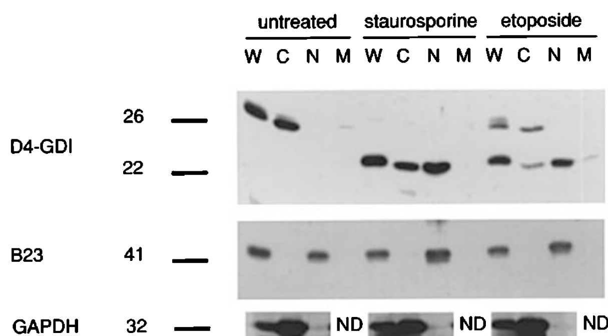

Figure 6 Nuclear translocation of D4-GDI following cleavage. Western blots were performed on lysates from untreated ML-1 cells, ML-1 cells+1 μM staurosporine for 3 h, or ML-1 cells+20 μg/ml etoposide for 30 min, followed by fresh media for 4 h. Samples of whole cells (W), cytosol (C), nuclei (N), and membranes (M) were examined. Blots were probed with antibody to detect D4-GDI (top), B23 nuclear protein (middle), and glyceraldehyde 3 phosphate dehydrogenase as a cytosolic marker (bottom). ND, not determined

digestion were also inhibited by 3 nM calyculin A, 15 μM cantharidin, as well as 100 μM zinc (data not shown). The caspase inhibitor, z-VAD-fmk, also prevented the cleavage of D4-GDI at the same concentration that inhibited DNA digestion (Figure 5B).

We initially believed the antibody was detecting DNaseII, so we examined the subcellular distribution of p26 and p22. p26 was detected in the cytoplasm of untreated ML-1 cells, but none was found in the nucleus or membrane fractions (Figure 6). Following incubation with 1 μM staurosporine, most of the p26 was cleaved to p22, and the majority was detected in the nucleus. Incubation with 20 μg/ml of etoposide for 30 min followed by a 4 h incubation in media induced apoptosis in about 50% of the cells and a comparable proportion of p26 was cleaved to p22. All of the p26 remained in the cytoplasm while the majority of p22 appeared in the nucleus. Little to no p26 or p22 was detected in the membrane fraction. Nuclear and cytoplasmic marker proteins were also analyzed to assure purity of preparations. The B23 nuclear protein marker was retained in the nuclear fractions of the cells while the cytoplasmic marker, glyceraldehyde 3-phosphate dehydrogenase, remained in the cytoplasmic fractions, indicating that cross contamination of the fractions did not occur. Thus it appears that p26 D4-GDI is cleaved in the cytoplasm to p22, and this fragment is then translocated to the nucleus.

Transfection experiments

D4-GDI has recently been reported to be cleaved during apoptosis from a 28 kDa to a 23 kDa species.[17] D4-GDI contains two cleavage sites that have been identified: a caspase 3 cleavage site at position 19, and a caspase 1/ICE cleavage site at position 54. These cleavage sites were identified *in vitro* using recombinant D4-GDI and caspases, and confirmed in recombinant proteins with aspartate to asparagine mutations at each cleavage site. To confirm which cleavage occurs in cells, plasmids encoding wild-type human D4-GDI or D4-GDI with D$>$N mutations at either position 19, 54, or both, were transfected into CHO cells, a cell line in which the antibody does not detect any endogenous p26 or p22. After 2 days, the transfected cells were incubated with 1 μM staurosporine for 6 h to induce apoptosis. Lysates were analyzed by Western blotting (Figure 7). The transfected wild type D4-GDI was detected as a 26 kDa protein and was cleaved to p22 as expected. The D19N mutation at the caspase 3 site inhibited cleavage, whereas the D54N mutation did not inhibit cleavage. The double mutant remained uncleaved. Hence, incubation of CHO cells with staurosporine activates caspase 3 or a related caspase that

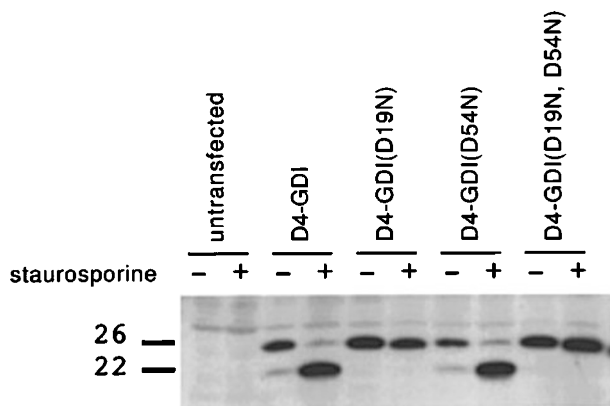

Figure 7 Staurosporine induces cleavage of D4-GDI at the caspase 3 site. Western analysis of untransfected CHO cells, or CHO cells transfected with wild type D4-GDI, D4-GDI(D19N), D4-GDI(D54N), or D4-GDI(D19N,D54N). Cells were incubated in 1 μM staurosporine for 6 h where indicated. Blots were probed with our antisera recognizing D4-GDI

cleaves D4-GDI at aspartate 19, but does not activate caspase 1.

To determine if the cleavage of D4-GDI had any affect on apoptosis in these cells, parallel experiments were performed in which CHO cells were cotransfected with vectors expressing either wild type human D4-GDI or D4-GDI(D19N), and a vector encoding enhanced green fluorescent protein (EGFP). After 48 h, the cells were incubated with 1 μM staurosporine for 6 h to induce apoptosis. Cells were stained with 2 μg/ml Hoechst 33342 for 15 min, which distinguishes normal and apoptotic cells on the basis of condensed chromatin. The per cent EGFP-positive and apoptotic cells were scored. The D4-GDI transfected population exhibited 52.5% (mean of three experiments, S.D. $\pm$2.5%) chromatin condensation in the EGFP-positive cells following incubation with staurosporine. The D4-GDI(D19N) transfected population gave similar results with 51.7% (mean of three experiments, S.D. $\pm$6.7%) chromatin condensation in the EGFP positive cells following incubation with staurosporine. Thus overexpression of a noncleavable form of D4-GDI does not protect CHO cells from apoptosis induced by staurosporine.

Discussion

The studies reported here arose from our attempt to raise an antibody to DNaseII using purified 31 kDa active bovine protein. Western analysis using this antiserum demonstrated its recognition of the 31 kDa bovine protein, and a band denoted p26 (or p22 in apoptotic cell lysates). The p26 was subsequently identified as D4-GDI. Further studies indicated that a small amount of D4-GDI could be detected in the preparation of bovine DNaseII which presumably led to the production of this antibody.

D4-GDI belongs to a family of proteins that function to inhibit the dissociation of GDP from Rho GTPases, which are in turn involved in regulating such cellular processes as cell adhesion, motility, contraction, and cytokinesis. Rho GTPases have been shown to regulate signaling pathways, including the stress-activated kinase JNK, phosphatidylinositol-5′-kinase, phospholipase D, PKN, and p160ROCK.[15]

The GTP-bound form of Rho is the active form. D4-GDI is one of three classes of regulators of Rho proteins and functions by keeping the protein in the GDP-bound inactive state. It is highly expressed in cells of the hematopoietic system but low levels are found in other tissues.[20] D4-GDI knockout mice were viable with normal thymocyte selection and immune responses.[21] The mice did, however, show decreased apoptosis of lymph node cells after IL-2 withdrawal. The normal development and regulation of D4-GDI null mice may be attributed to compensation of the defect by other GDI's such as Rho-GDI, or other regulators of Rho such as Rho-GAP's.

When caspase 1 was purified from THP-1 cells, a second protein copurified and was found to be a cleaved fragment of D4-GDI.[17] *In vitro* studies have shown that D4-GDI can be cleaved by caspase 3 after aspartate 19, and by caspase 1 after aspartate 54.[22] Monocytes release mature IL-1β after cleavage by caspase 1 and D4-GDI was found to be cleaved at the caspase 1 site in these cells. The cleavage of D4-GDI at this site produces a protein that no longer regulates Rho, and the increased Rho activity may lead to the formation of toxic oxygen metabolites and increased inflammatory damage. The cleavage of D4-GDI by caspase 3-like activity has been detected during apoptosis induced by anti-FAS antibody, BAX, and staurosporine in Jurkat cells,[16,17] and anti IgM treatment of BL60 Burkitt lymphoma cells.[18] Inhibitors of caspases have been shown to inhibit this cleavage. Whether the cleavage of D4-GDI at the caspase 3 site activates Rho has yet to be determined.

We have observed cleavage of D4-GDI during apoptosis induced by etoposide, staurosporine, and anti-FAS antibody in ML-1 cells, as well as in many other systems. DNA digestion and D4-GDI cleavage were inhibited by incubation with protein phosphatase inhibitors calyculin A, cantharidin, and okadaic acid, which inhibit apoptosis in many systems.[23,24] The caspase tripeptide inhibitor z-VAD-fmk, as well as zinc, also inhibit D4-GDI cleavage and apoptosis in ML-1 cells. Zinc was originally reported to be an endonuclease inhibitor and was used in many studies for identification of apoptotic endonucleases.[25,26] However, it has recently been shown that zinc inhibits apoptosis upstream of proteolysis rather than at the later step of endonuclease activation.[27]

Many proteins have been shown to be cleaved during apoptosis, but thus far the cleavage of only a few of these substrates have been shown to have an effect on the downstream events in the apoptotic cascade. The mouse homolog of DFF45, ICAD, has been shown to associate with an endonuclease, CAD, in the cytosol. Cleavage of ICAD is thought to release CAD and allow it to translocate to the nucleus.[9,10] However, none of the substrates of the caspases have been shown to translocate to the nucleus, although caspase members themselves must be translocated since many substrates such as the lamins and PARP are nuclear proteins. We show in this paper that D4-GDI is

cleaved in the cytoplasm of the cell, and subsequently translocated to the nucleus. The implications of this, if any, have yet to be established. Potentially, expression of an uncleavable D4-GDI would inhibit Rho activation. This could in turn protect from JNK activation, which has been implicated in apoptosis.[28] However, activation of JNK is more likely to contribute to activation of caspase 3 rather than *vice versa*. This is consistent with the observation that transfection of non-cleavable D4-GDI did not alter the rate of staurosporine-induced apoptosis. We cannot rule out some contribution to apoptosis from endogenous D4-GDI in these cells or other compensatory regulators of Rho function such as Rho-GAP's. However, it is probable that any changes brought about by cleavage of D4-GDI do not impact on the death of the cell.

Much insight into the activation of the caspase cascade and the effector molecules involved has been achieved by the analysis of *in vitro* reconstitution models of apoptosis. One such system involves activation of caspases in cytosolic lysates after addition of cytochrome c and dATP.[29] Methods to detect cytosolic caspase activity in this system include the use of exogenously added caspase substrates, such as radiolabeled caspase-3, and gel electrophoresis to detect caspase cleavage and activation.[30] D4-GDI is a cytoplasmic protein. We have observed cleavage of this protein in cytoplasmic lysates after incubation with cytochrome c and dATP (data not shown). It is clear that D4-GDI cleavage, detectable via Western analysis, is an excellent endogenous marker of cytosolic caspase activation in these reconstituted models of apoptosis.

The import of proteins into the nucleus by active nuclear transport has been shown to be required for the nuclear changes observed during apoptosis.[31] Inhibition of nuclear import by a variety of techniques can block the nuclear changes observed during apoptosis. Proteins that translocate into the nucleus may be useful in studying the importance of this process in future studies. Several proteins have been shown to translocate to the nucleus in different models of apoptosis. For example, the avian Crel/NF-κB transcription factor has also been observed to translocate to the nucleus during apoptosis.[32] However, translocation of transcription factors is a normal event that is not indicative of apoptosis. The cleaved fragment of D4-GDI is the first caspase substrate reported to translocate to the nucleus, and may serve as a useful marker of nuclear import during apoptosis.

Materials and Methods

Antibody production

Lyophilized bovine DNaseII (Sigma, St. Louis, MO, USA) was dissolved in H_2O and injected onto a C18 reverse phase HPLC column (Vydac, Rainin Instrument Co., Ridgefield, NJ, USA). A 71 min linear gradient from 0–70% acetonitrile in 1% trifluoroacetic acid was used to elute the proteins. Absorbance was measured and eluted peaks were collected, lyophilized, redissolved in water, and aliquots were electrophoresed on a denaturing 10% polyacrylamide/SDS gel for size determination. The fractions were also assayed for endonuclease activity by incubation with plasmid DNA in APB buffer (10 mM sodium acetate, 10 mM sodium phosphate, 10 mM bis-trispropane, pH 5.0) for 60 min, followed by electrophoresis on a 1% agarose gel. The peak of protein that eluted at 51 min was collected and found to be the active 31 kDa endonuclease. This protein was collected in subsequent isolations, pooled, and conjugated to keyhole limpet hemocyanin with EDC using the Imject Conjugation kit (Pierce, Rockford, IL, USA). The conjugate was sent to Hazelton Research Products (Denver, PA, USA) and injected into two New Zealand rabbits. The serum was found to recognize discrete bands on Western blots of various tissue samples at 26 and 22 kDa designated p26 and p22 and rarely the expected 31 kDa (see Results).

Cell culture and drug treatments

ML-1 cells were grown in RPMI 1640+7.5% fetal bovine serum with antibiotics. Stock cultures were maintained at a density between 2×10^5 and 1×10^6 cells/ml. Apoptosis was induced by incubating 1×10^6 cells/ml with 20 μg/ml etoposide for 30 min; the drug was removed, the cells were washed twice and incubated for an additional 4 h. Various inhibitors of apoptosis were added during this subsequent incubation. Apoptosis was also induced by incubating 1×10^6 cells/ml with 800 nM staurosporine for 3 h. Chinese hamster ovary (CHO) cells were maintained in alpha-MEM+5% fetal bovine serum with antibiotics. Apoptosis was induced in CHO cells by incubation with 1 μM staurosporine for 6 h. MDA-468 and T-47D cell lines were grown in DMEM/F12 media+10% fetal bovine serum with antibiotics. L1210 cells were grown in McCoys media+15% bovine serum with antibiotics.

DNA digestion

DNA digestion was analyzed by gel electrophoresis as previously described.[12,19] Briefly, a 2% agarose gel was poured, the top part of the gel above the wells was removed and replaced with 0.9% agarose containing 16 μg/ml proteinase K and 2% SDS. 1×10^6 cells were pelleted, resuspended in 15 μl of gel loading buffer (10% glycerol, 3.3 mg/ml RNaseA), and added to each well. The gel was electrophoresed for 16 h at 30 V. The top part of the gel was removed and the remainder was stained with ethidium bromide and visualized under UV illumination.

Western analysis

1×10^6 cells were pelleted, lysed in 100 μl of 2% SDS, 50 mM Tris pH 6.8, 2 mM N-ethylmaleimide, 1 μg/ml pepstatin A, and 1 mM AEBSF. Sample volume corresponding to 10^5 cells was added to an equal volume of $2\times$ loading buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 120 mM Tris pH 6.8, and 0.001% bromophenol blue) and boiled for 5 min. The samples were electrophoresed on a 12% polyacrylamide/SDS gel, and transferred to an Immobilon P membrane (Millipore, Bedford, MA, USA). The membranes were probed with the anti-D4-GDI antibody, washed, incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody, washed, and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). The antibody was also used to probe 20 μg of lysate made from bovine spleen.

To further characterize the antiserum, triplicate Western blots of lysates from ML-1 cells, untreated or incubated with staurosporine, as well as recombinant human D4-GDI (kindly provided by Dr. Gary Bokoch, Scripps Research Institute, La Jolla, CA, USA) and bovine DNaseII were electrophoresed on a 12% polyacrylamide/SDS gel and transferred to a membrane. One membrane was probed with a

1 : 10 000 dilution of antiserum. A second membrane was probed with a 1 : 10 000 dilution of the antiserum that had been preincubated with 20 μg recombinant human D4-GDI for 2 h. A third membrane was probed with an anti D4-GDI antibody (kindly provided by Dr. Dennis Danley, Pfizer Inc., Groton, CT, USA)

Cellular fractionation

Following drug treatment, 1×10^7 cells were pelleted and the media was removed. The cells were resuspended in a hypotonic solution (50 mM HEPES pH 7, 250 mM sucrose, 2 mM N-ethylmaleimide, 1 μg/ml pepstatin A, and 1 mM AEBSF), incubated for 15 min on ice, transferred to a dounce homogenizer, and lysed with 25 strokes. The lysate was centrifuged for 10 min at 700 g and the pellet was resuspended in 1 ml of 0.5 M sucrose in HSSE (50 mM HEPES pH 7.0, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA). To further purify the nuclei, the resuspended pellet was gently layered over 1.5 M sucrose in HSSE and the nuclei pelleted in a microcentrifuge at 13 000 r.p.m. for 10 min. The supernatant from the 700 g spin was centrifuged at 100 000 g for 1 h. The pellet contained the light and heavy membrane fractions, and the supernatant contained the cytoplasmic fraction. All of the fractions were solubilized with 2% SDS buffer and subjected to Western analysis using the anti-D4-GDI antiserum. Fractions were loaded on the gel to correspond to equal cell number. Duplicate Western blots were also probed with an anti-B23 antibody as a nuclear marker (kindly provided by Dr. Scott Kaufmann, Rochester, MN, USA), or an anti-glyceraldehyde-3-phosphate dehydrogenase antibody as a cytoplasmic marker (Trevigen, Inc., Gaithersburg, MD, USA).

Purification of p26

ML-1 cells (1×10^8) were pelleted and the cytosolic fraction was isolated as described above. The cytosol from 2.5×10^7 cells was then diluted with 4 volumes of 3 M NaCl, 50 mM sodium phosphate pH 6.0, and loaded onto a 2 ml phenyl Sepharose column which had been equilibrated in the same buffer. The column was washed with 30 ml of 3 M NaCl, 50 mM sodium phosphate. The protein was eluted with 10 ml of 1 M NaCl, 50 mM sodium phosphate and 2 ml fractions were collected. The eluted fraction was loaded onto a C18 reverse phase column and eluted at 0.25 ml/min with a 100 min linear gradient from 31 – 49% acetonitrile in 1% trifluoroacetic acid. Fractions were electrophoresed on a 12% polyacrylamide/SDS gel, transferred to a Problott membrane in CAPS buffer, and stained with Coomassie blue. p26 was excised from the membrane and digested with endopeptidase Lys C. The fragments were purified on a C8 column by reverse phase HPLC. Eluted fragments were collected and sequenced on an Applied Biosystems Inc. Automated Sequencer model 473A.

Transfection experiments

Four plasmids encoding human D4-GDI in the pcDNA 3.0 vector were kindly provided by Dr. Dennis Danley (Pfizer Inc., Groton, CT, USA). The plasmids encoded wild type D4-GDI or mutants designated D4-GDI (D19N), D4-GDI (D54N), or D4-GDI (D19N,D54N). 1 μg of each plasmid was transfected into CHO cells using DOSPER Liposomal Transfection Reagent (Boehringer Mannheim). After 48 h, cells were induced to undergo apoptosis by incubation with 1 μM staurosporine for 6 h. Lysates were then analyzed by Western analysis for expression and cleavage of D4-GDI. In other experiments, cotransfections were performed using 0.5 μg of a plasmid encoding enhanced green fluorescent protein (EGFP) (Clontech, Palo Alto, CA, USA) with 1 μg of D4-GDI or D4-GDI (D19N). After 48 h, cells were induced to undergo apoptosis by incubation with 1 μM staurosporine for 6 h. Cells were incubated with 2 μg/ml Hoechst 33342 for 15 min, then scored for condensed chromatin and expression of EGFP using fluorescent microscopy.

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