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Retinoid-Induced Apoptosis and Sp1 Cleavage Occur Independently of Transcription and Require Caspase Activation

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Vitamin A and its derivatives, the retinoids, are essential regulators of many important biological functions, including cell growth and differentiation, development, homeostasis, and carcinogenesis. Natural retinoids such as all-*trans* retinoic acid can induce cell differentiation and inhibit growth of certain cancer cells. We recently identified a novel class of synthetic retinoids with strong anti-cancer cell activities in vitro and in vivo which can induce apoptosis in several cancer cell lines. Using an electrophoretic mobility shift assay, we analyzed the DNA binding activity of several transcription factors in T cells treated with apoptotic retinoids. We found that the DNA binding activity of the general transcription factor Sp1 is lost in retinoid-treated T cells undergoing apoptosis. A truncated Sp1 protein is detected by immunoblot analysis, and cytosolic protein extracts prepared from apoptotic cells contain a protease activity which specifically cleaves purified Sp1 in vitro. This proteolysis of Sp1 can be inhibited by *N*-ethylmaleimide and iodoacetamide, indicating that a cysteine protease mediates cleavage of Sp1. Furthermore, inhibition of Sp1 cleavage by ZVAD-fmk and ZDEVD-fmk suggests that caspases are directly involved in this event. In fact, caspases 2 and 3 are activated in T cells after treatment with apoptotic retinoids. The peptide inhibitors also blocked retinoid-induced apoptosis, as well as processing of caspases and proteolysis of Sp1 and poly(ADP-ribose) polymerase in intact cells. Degradation of Sp1 occurs early during apoptosis and is therefore likely to have profound effects on the basal transcription status of the cell. Interestingly, retinoid-induced apoptosis does not require de novo mRNA and protein synthesis, suggesting that a novel mechanism of retinoid signaling is involved, triggering cell death in a transcriptional activation-independent, caspase-dependent manner.

Retinoids play a central role in cell growth and differentiation, development, and carcinogenesis (49, 55). Retinoid signaling is mediated by two types of nuclear receptors, the retinoic acid (RA) receptors (RARα, -β, and -γ) and the retinoid X receptors (RXRα, -β, and -γ). Both types belong to the steroid-RA-thyroid hormone receptor superfamily of ligand-responsive transcription factors, which regulate transcription by binding to specific DNA sequences or by interacting with other transcription factors (42, 54, 56). Because of their antiproliferative effect, RA and its natural and synthetic derivatives, the retinoids, have been considered desirable agents for the prevention and treatment of cancer. More recently, retinoids have also been postulated to have a role in apoptosis. 4-Hydroxyphenyl retinamide (4-HPR) and CD437, two RARγ-selective retinoids (4, 19), have been shown to induce apoptosis (14, 19, 61). Most recently, it has been shown that an additional class of novel RARγ-selective retinoids can induce apoptosis in cancer cell lines and is active against solid tumors in vivo, thus being candidates for novel anticancer therapeutics (41). However, the mechanism of action of apoptotic retinoids and the apoptosis pathway they activate are unknown.

Apoptosis, a type of cellular suicide, is a physiological event important for host defense, development, homeostasis, and aging (16, 65, 73). Proteolysis plays a crucial role in apoptosis, and several members of the interleukin-1β-converting enzyme (ICE) family that can induce apoptosis when activated or overexpressed in various cell types have been identified (20–22, 36, 47, 51, 66, 69, 71, 78; reviewed in reference 35). These cysteine proteases, also called caspases (1), share the unusual property that they require an Asp residue at the P1 position of the cleavage site (26, 34, 69). Additional evidence for the central role for caspases in apoptosis has come from inhibition studies (25). Several viral proteins can inhibit apoptosis through interaction with caspases when overexpressed in mammalian cells (9, 48, 67, 68, 74). Furthermore, synthetic peptides containing an Asp at position P1 which can specifically inhibit apoptosis in intact cells as well as in cell-free systems have been developed (18, 37, 51, 60). A link between members of the bcl-2 family of apoptosis inhibitors and caspases has also been observed. CED4, an activator of apoptosis in nematodes for which no mammalian homolog has yet been described (77), physically interacts with CED9 (a bcl-2 homolog) and CED3 (the homolog of caspase 3) (12, 63).

Several proteins which are cleaved during apoptosis have been shown to be specific targets for caspase 3 and other related caspases, including poly(ADP-ribose) polymerase (PARP) (33), the 70-kDa component of the U1 small nuclear ribonucleoprotein (10), lamins (38), fodrin (45), protein kinase C δ (17), Gas2 (8), topoisomerase I (70), and sterol regulatory element-binding proteins (72). More recently, DNA-dependent protein kinase and the retinoblastoma protein have also been shown to be specifically cleaved during apoptosis (29, 62). The protein degradation is enhanced through the interaction of various caspases, leading to an amplified protease cascade (44). These caspases normally exist in the cell as inactive proenzymes which are then proteolytically activated during the apoptotic process. Caspase 8 (FLICE/MACH) was recently reported to function as a potential initiator of this protease cascade in the Fas and tumor necrosis factor alpha (TNF-α) apoptosis pathways (6, 50). Activation of Fas or TNF receptor 1 (TNFR1) by their ligands recruits caspase 8 to the membrane

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through direct interaction with FADD or TRADD, which are death domain-containing proteins that interact with Fas and TNFR1, respectively (7, 11, 27). Because of this recruitment, pro-caspase 8 is activated, and it has been proposed that activated FLICE could process ICE, which then activates CPP32 (18) and other CPP32-like caspases considered to be the final executors of the protease cascade (44). In fact, caspase 8 can process several caspases in vitro, including itself (64).

In order to elucidate apoptosis pathways activated by retinoids, we analyzed their effects on several DNA-binding proteins in various cancer cell lines. We observe that the general transcription factor Sp1 is specifically cleaved during retinoid-induced apoptosis in T cells. Proteolysis of Sp1 and loss of its DNA binding activity occur in a time- and retinoid concentration-dependent fashion, which correlates well with the onset of DNA fragmentation and cleavage of PARP. A cysteine protease activity which can specifically cleave purified Sp1 in vitro is induced in retinoid-treated apoptotic cells. Studies with peptide inhibitors indicate that distinct protease activities are responsible for the cleavage of Sp1 and PARP. Apoptosis in intact cells can be blocked by selective inhibitors of caspases, indicating that activation of caspases is necessary for retinoid-induced apoptosis. In particular, caspase 3 is required for apoptosis and subsequent Sp1 cleavage in vivo, since a specific peptide inhibitor blocks retinoid-induced cleavage of PARP and Sp1 and blocks DNA fragmentation as well. Proteolysis of Sp1 is an early event in apoptosis and follows a time course similar to that of PARP. We observe that caspase 2 (Ich1L/Nedd2) is activated concurrently with Sp1 cleavage. Importantly, retinoid-induced apoptosis does not require de novo synthesis of mRNA or protein, suggesting a novel retinoid signaling mechanism.

MATERIALS AND METHODS

Cells and reagents. EL-4 and Jurkat cells were maintained in RPMI medium containing 10% heat-inactivated fetal calf serum. Retinoids were obtained from MAXIA Pharmaceuticals and Galderma Research Inc., and stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Antibodies were obtained from G. G. Poirier (PARP), Santa Cruz Biotechnology (Sp1 and Nedd2), Transduction Laboratories (Ich1L and CPP32), Upstate Biotechnology (ICE), and Sigma (β-actin). ZVAD-fmk, ZDEVD-fmk, and ZFA-fmk were obtained from Enzyme Systems Products. Ac-YVAD-cmk was purchased from Bachem. Other reagents used in this study were obtained from Sigma, Biomol, or Calbiochem.

Cell proliferation assay. To measure cell survival, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation assay was used. A total of 10,000 cells/well were seeded in 96-well plates and treated with retinoids for various periods. Medium with retinoids was changed every 2 or 3 days as needed. Alternatively, 100,000 cells/ml were grown in the presence or absence of retinoids. At different times, aliquots were used to determine the number of live cells by trypan blue staining and counting.

DNA fragmentation analysis. To determine apoptosis, the amount of cellular DNA fragmentation was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim). Cells (200,000 cells/ml) were labeled with bromodeoxyuridine (BrdU) for 4 to 14 h. The medium was then changed, and aliquots of cells (100,000 cells/ml) were seeded in 96-well plates. The cells were not preincubated or were preincubated with cycloheximide or actinomycin D and then treated with the retinoids, as indicated below. Alternatively, cells were incubated with retinoids in the absence or in the presence of peptide inhibitors for a period of 4 to 16 h. The cells were lysed, and the amount of DNA fragmentation was quantitated by following the manufacturer's instructions. In addition, DNA ladder formation was measured by using total genomic DNA isolated from cells treated with the apoptotic agents and agarose gel electrophoresis according to standard procedures (3).

Gel retardation. Gel shift experiments were performed as described previously (57, 58). Samples (5 μg) of nuclear extracts were incubated with a ^{32}P-labeled oligonucleotide containing an Sp1-specific binding site (5′-gatccGGGGCGG GGCg-3′) in the presence of 1 μg of poly(dI-dC). After 20 min at room temperature, the protein-DNA complexes were analyzed on a nondenaturing 5% polyacrylamide gel in 1× Tris-borate-EDTA buffer in the cold. Nuclear protein extracts were obtained essentially as described elsewhere (15), except the final dialysis step was omitted. Protein concentrations were determined by using a Coomassie Plus Protein Assay Reagent (Pierce).

Immunoblot analysis. For Western blots, 25 or 50 μg of total cell extracts was separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and then immunoblotted with the corresponding antibody. Total cell extracts were prepared by resuspending the cell pellet in extraction buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 6 M urea, 15% glycerol, 2.5% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and then subjecting the mixture to boiling for 10 min and centrifugation.

In vitro proteolysis assay. Between 10 and 50 μg of cytosolic extract was incubated, in the presence of 0.1% 3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)–2 mM EDTA–1 mM dithiothreitol, with 0.2 μl of purified human Sp1 (Promega) or 1 μl of in vitro-synthesized ^{35}S-PARP. After 2 h at 37°C, 10 μl of 2× loading buffer was added, and proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Sp1 cleavage was analyzed by immunoblotting, and PARP proteolysis was analyzed by autoradiography. When protease inhibitors were used, cytosolic extracts were preincubated with the inhibitors at 37°C for 30 min; Sp1 or PARP was then added, and samples were further processed as described above. Cytosolic extracts were prepared by resuspending the cells in 10 mM HEPES (pH 7.1)–10 mM KCl–1.5 mM $MgCl_2$–5 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–1 μg of leupeptin per ml–1 μg of pepstatin A per ml–1 μg of aprotinin per ml. The cells were lysed by three cycles of freeze-thawing, followed by centrifugation for 30 min at 4°C. Human PARP cDNA (kindly provided by A. Burkle) was used to generate ^{35}S-PARP protein with a TnT Quick System of coupled transcription-translation (Promega).

RESULTS

Selective retinoids induce apoptosis in T cells. A novel class of apoptosis-inducing retinoids was examined previously (41) in a series of cancer cell lines. The T-cell lines EL-4 and Jurkat are chosen here as examples for the effects of different selected retinoids on cell survival. A very potent killing effect on EL-4 cells was seen with 6 μM CD437, MX2870-1, or 4-HPR, while RA showed no significant effect, even after prolonged periods of exposure (Fig. 1A). The antiproliferation or cell-killing effect was observable after a relatively short time of treatment, especially when CD437 or MX2870-1 was used. Cell killing was dependent on the retinoid concentration (Fig. 1B). While 4-HPR showed little effect at 2 μM and lower concentrations, both CD437 and MX2870-1 were very effective at 1 μM. The cell-killing effect of these compounds was stronger on Jurkat cells, for which shorter exposure (24 h) and lower retinoid concentrations were sufficient to significantly inhibit cell proliferation as determined by an MTT assay (Fig. 1B). These data were confirmed by counting live cells after retinoid treatments for various periods (Fig. 1C and data not shown). To determine if this killing required continued exposure to retinoids, EL-4 cells were treated with different concentrations of the retinoids for various times. The medium was then removed, fresh medium without retinoids was added, and the cells were further incubated for a total of 5 days, after which the number of live cells was determined. We found that a very short treatment of only 4 h with 6 μM CD437 or MX2870-1 was sufficient to reduce the number of live cells by approximately 50% (Fig. 1D). Treatments for 16 and 24 h completely eliminated live cells. These results show that the cell death induced by retinoids such as CD437 and MX2870-1 is irreversible and can be triggered by short exposures. 4-HPR was substantially less effective under these conditions, and only a 50% reduction in cell number was observed with a 48-h treatment.

To confirm that these retinoids indeed induced apoptosis in T cells, EL-4 and Jurkat cells were treated with different concentrations of the retinoids for 24 h, after which DNA fragmentation was analyzed by agarose gel electrophoresis. A DNA ladder was clearly observed when EL-4 cells were treated with 2 μM CD437 or MX2870-1, to a degree similar to that observed with nonretinoid apoptotic agents such as etoposide, C_2-ceramide, and staurosporine (Fig. 2A). Consistent with its weaker antiproliferative effect, a higher concentration of 4-HPR (10 μM) was necessary to induce DNA fragmentation in EL-4 cells. Similar results were obtained with Jurkat cells (Fig.

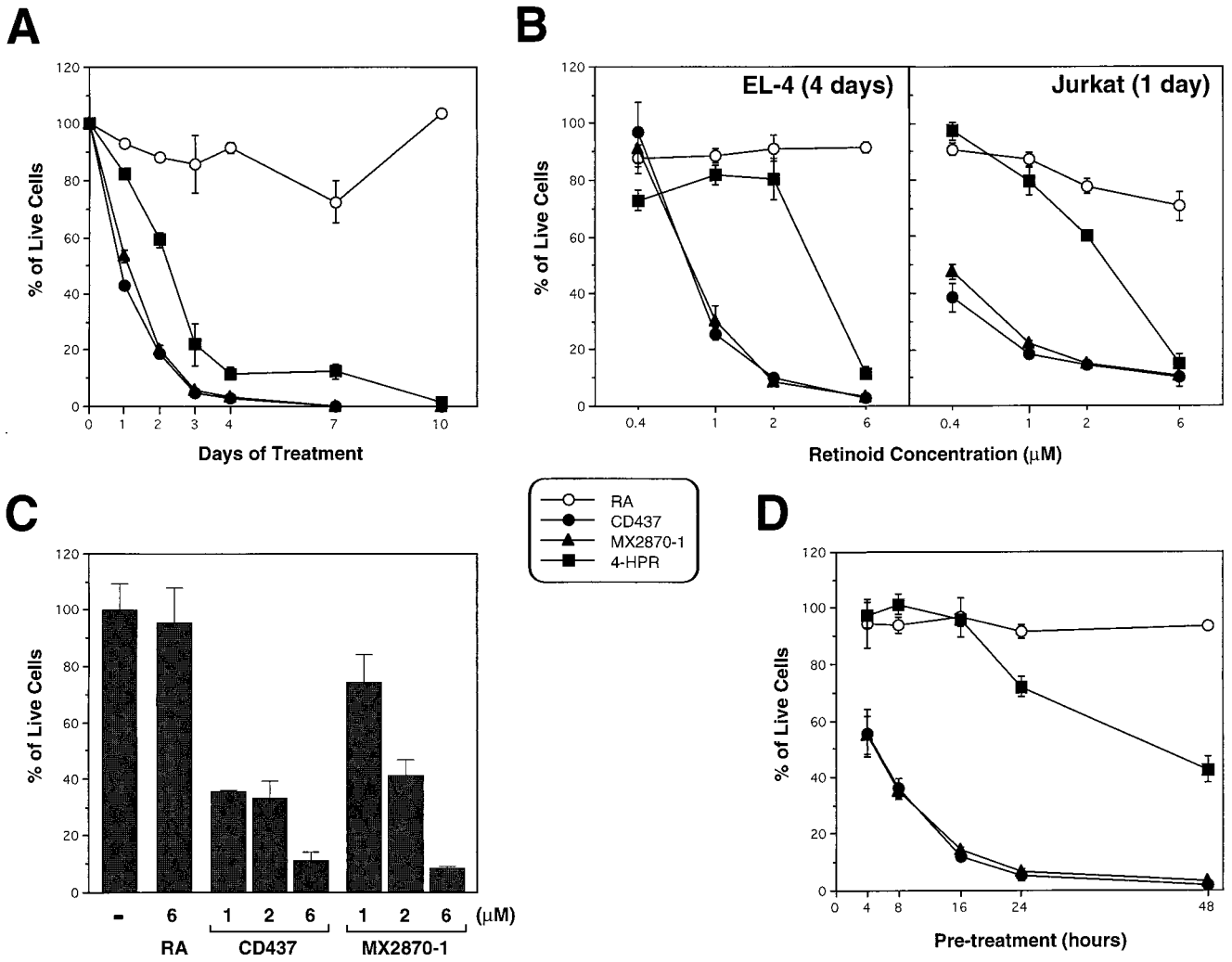

FIG. 1. Selective retinoids show strong antiproliferative effects on T cells. (A) EL-4 cells were treated with the indicated retinoids (6 μM) for several days, and cell proliferation was determined by an MTT assay as described in Materials and Methods. Control cells were grown in the presence of vehicle (DMSO). (B) EL-4 and Jurkat cells were treated with increasing concentrations of retinoids for the indicated times, after which cell survival was measured by MTT staining. (C) EL-4 cells were treated with the indicated concentrations of RA, CD437, or MX2870-1 for 24 h. Aliquots were used to measure cell survival by trypan blue staining and cell counting. (D) EL-4 cells were exposed to 6 μM retinoids (pretreatment) for 4, 8, 16, 24, or 48 h. The medium was then changed, and the cells were grown in the absence of retinoids for a total of 5 days, when the percentage of live cells was measured by using an MTT assay. All of these experiments were repeated two or three times, and results for a typical experiment performed in triplicate are shown.

2B). The induction of DNA fragmentation was time and retinoid concentration dependent (Fig. 2C). A DNA ladder was clearly detectable in EL-4 cells after 16 h of treatment with 2 μM CD437.

Sp1 DNA binding activity is lost during retinoid-induced apoptosis. To gain insights into the mechanism by which retinoids can induce apoptosis, several transcription factors that have been connected to apoptosis, including AP-1, NF-κB, c-myc, and others, were investigated. The effect of retinoid treatment on several of these nuclear proteins was analyzed by measuring their DNA binding activity in gel retardation assays. We observed some inhibition of AP-1 DNA binding and, to a lesser extent, c-myc DNA binding (data not shown). When we monitored the DNA binding activity of the general transcription factor Sp1, three specific complexes were observed in extracts from untreated cells (Fig. 3A and data not shown), as determined by antibody supershifting and competition with unlabeled oligonucleotides (58). Interestingly, a major inhibition of Sp1 DNA binding was observed in nuclear extracts prepared from EL-4 cells treated for 24 h with CD437 or MX2870-1 (Fig. 3A). 4-HPR was also able to inhibit Sp1 DNA binding activity when used at the higher concentration. In contrast, RA showed no effect. Significant inhibition of Sp1 DNA binding activity was also found in cells treated with etoposide but not in cells treated with C_2-ceramide or staurosporine. To determine whether this inhibition of Sp1 DNA binding activity correlated with decreased Sp1 protein levels, a Western blot analysis was performed. A lower level of Sp1 protein was indeed observed in extracts prepared from cells undergoing apoptosis. Importantly, a specific degradation fragment with an apparent molecular mass of 68 kDa was detected in extracts from retinoid-treated cells, indicating that specific cleavage of Sp1 occurred in EL-4 cells undergoing apoptosis (Fig. 3B). Inhibition of Sp1 DNA binding activity and proteolysis of the Sp1 transcription factor were also found in Jurkat cells (data not shown). The appearance of the truncated 68-

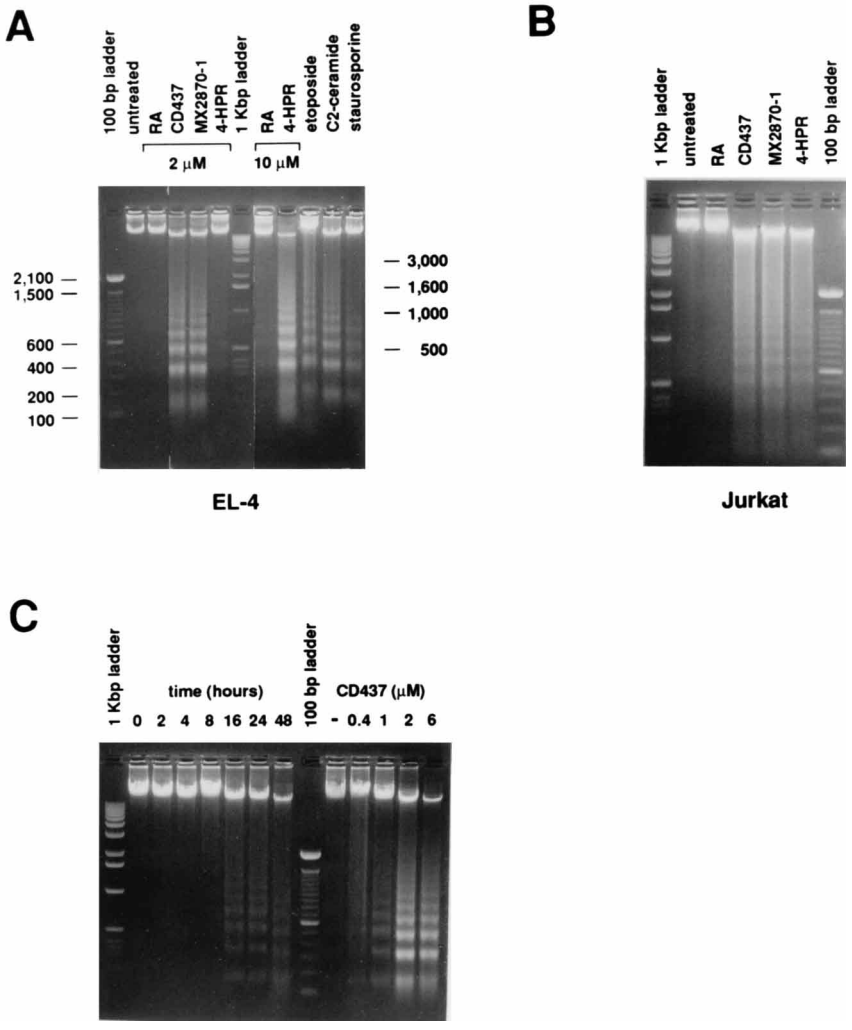

FIG. 2. Induction of DNA fragmentation in EL-4 and Jurkat cells. (A) EL-4 cells were treated for 24 h with vehicle (untreated) or 2 or 10 μM retinoids, as indicated above the lanes. As a positive control, cells were also treated for 4 h with 50 μM etoposide and for 16 h with 25 μM C_2-ceramide or 0.5 μM staurosporine. Genomic DNA was extracted, and 10 μg was analyzed in a 1.5% agarose gel. DNA molecular size markers from Gibco BRL (100-bp and 1-kbp ladders) were used. Sizes are indicated in base pairs. (B) Jurkat cells were treated with the indicated retinoids (6 μM) for 24 h, and total DNA was extracted and analyzed as described above. (C) EL-4 cells were treated with 2 μM CD437 for various periods (left lanes) or with different concentrations of the retinoid for 48 h (right lanes), as indicated above the lanes, after which DNA was extracted and analyzed. Molecular size markers are also shown.

kDa Sp1 protein correlated well with the loss of Sp1 DNA binding activity and with the induction of apoptosis in both EL-4 and Jurkat cells.

We next investigated the time course and CD437 concentration dependence of the Sp1 cleavage. Inhibition of Sp1 DNA binding activity was observed after only 8 h of retinoid exposure of EL-4 cells, and the effect was enhanced by increasing exposure times (Fig. 4A). After 24 h of retinoid treatment, Sp1 DNA binding was completely eliminated, correlating with the accumulation of the specific 68-kDa Sp1 form in Western blot analyses (Fig. 4B). Similar kinetics were observed for Jurkat cells. In this case, however, the 68-kDa Sp1 polypeptide was detectable already after 4 h of treatment, correlating with the enhanced killing effect of these retinoids on Jurkat cells (Fig. 1B). Cleavage of PARP was also analyzed and found to occur in parallel to that of Sp1 (Fig. 4C). Thus, cleavage of Sp1 is an early event during retinoid-induced apoptosis.

EL-4 cells were next treated with increasing concentrations of CD437 for 48 h. A characteristic DNA ladder was observed when increasing concentrations of CD437 were used (Fig. 2C). In parallel, we observed inhibition of Sp1 DNA binding activity and the appearance of the truncated Sp1 form in a CD437 concentration-dependent manner (Fig. 4D and E). Together, these data indicate that the induction of apoptosis in EL-4 cells by certain selective retinoids, as well as some nonretinoid compounds (etoposide), causes the activation of a particular protease which specifically cleaves the Sp1 transcription factor during the early stages of apoptosis.

In vitro cleavage of Sp1 in cytosol extracts from retinoid-treated cells. To further document that an Sp1-specific protease is indeed activated during retinoid-induced apoptosis, an in vitro proteolysis experiment was carried out. Purified human Sp1 protein was incubated with cytosolic extracts prepared from EL-4 cells exposed to either vehicle (DMSO) or 2 μM RA or CD437 for 24 h. Sp1 protein was incubated with increasing amounts of cytosolic extract for 2 h at 37°C, and the reaction products were analyzed by SDS-PAGE and Western blotting. A polypeptide of ~68 kDa was observed after incubation with extracts prepared from CD437-treated EL-4 cells but not with extracts from vehicle- or RA-treated cells (Fig. 5A

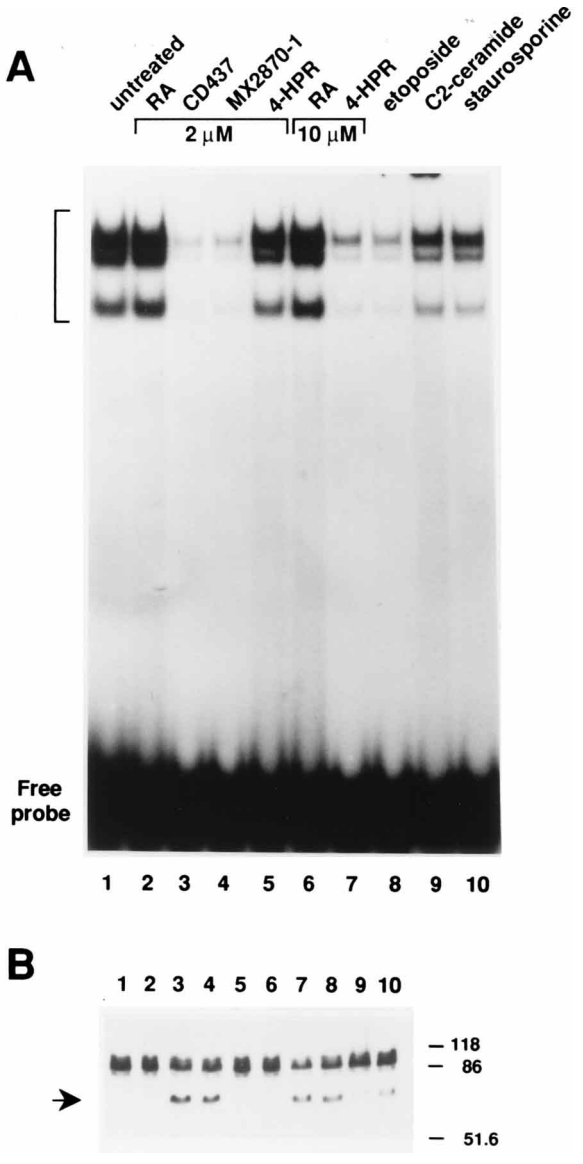

FIG. 3. Loss of Sp1 DNA binding activity during retinoid-induced apoptosis. (A) Gel shift assays were used to analyze nuclear extracts prepared from EL-4 cells treated for 24 h with retinoids (lanes 2 to 7) or other apoptosis-inducing agents indicated. Extracts were mixed with a ^{32}P-labeled Sp1 DNA binding site. Cells grown in the presence of 50 μM etoposide (lane 8), 25 μM C_2-ceramide (lane 9), or 0.5 μM staurosporine (lane 10) were treated for 4 or 16 h (as noted for Fig. 2A); after treatment, the cells were sedimented, resuspended in medium without the agent, and further incubated for a total of 24 h, after which protein extracts were prepared. Three specific Sp1 protein-DNA complexes were observed (bracket). (B) A 25-μg sample of total protein extract prepared from EL-4 cells treated as for panel A was subjected to immunoblot analysis using an anti-Sp1 antibody. Sp1 protein appeared as a double band of 95 and 105 kDa. The mobility of the truncated 68-kDa Sp1 cleavage product (arrow) is indicated. Molecular mass markers (Bio-Rad) are shown on the right.

and data not shown). This truncated Sp1 form showed mobility identical to that seen in nuclear or total cell extracts obtained from apoptotic EL-4 cells (data not shown), indicating that the same specific Sp1 cleavage occurs in vitro and in intact cells. To analyze the kinetics of the protease activation, cytosolic extracts were prepared after different times of retinoid treatment. No activity was detected after 4 h of retinoid exposure, while protein extracts prepared after 8-h or longer retinoid treatments showed specific Sp1-cleaving activity, which increased with the time of retinoid treatment and which was highest at 16 h (Fig. 5B).

To determine which protease was involved in Sp1 cleavage, several protease inhibitors were used. Experiments performed with extracts prepared from either EL-4 or Jurkat cells treated with 2 μM CD437 or MX2870-1 gave identical results (Fig. 6 and data not shown). *N*-Ethylmaleimide and iodoacetamide, two sulfhydryl-blocking reagents, inhibited cleavage of Sp1 in cytosolic extracts prepared from CD437-treated cells, indicating that a cysteine protease was involved (Fig. 6A). A specific inhibitor of caspase 1 (Ac-YVAD-cmk) partially inhibited proteolysis of Sp1. Both ZVAD-fmk, a general inhibitor of caspases with selectivity towards caspase 1 (2), and ZDEVD-fmk, a specific inhibitor of caspase 3, also prevented Sp1 cleavage in vitro, although no concentration dependence was observed. However, the peptide ZFA-fmk, an inhibitor of cathepsin B (59), had no effect on Sp1 cleavage, demonstrating that the effects of ZVAD-fmk and ZDEVD-fmk were specific and not due to the fluoromethylketone (fmk) moiety of the peptide. Other protease inhibitors were also tested. *N*α-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK), E-64, and the proteasome inhibitors LLnL, LLM, and ZLLL had no effect on Sp1 cleavage. Only *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) was able to weakly inhibit proteolysis of Sp1 (data not shown). For comparison, the effects of these inhibitors on in vitro-produced ^{35}S-PARP in cytosolic extracts from retinoid-treated cells were also tested. As expected, *N*-ethylmaleimide and iodoacetamide, as well as the caspase inhibitors ZVAD-fmk and ZDEVD-fmk, were able to completely inhibit cleavage of PARP in vitro, in a concentration-dependent maner (Fig. 6B). Ac-YVAD-cmk also significantly inhibited PARP proteolysis, as previously reported by others (37). All other protease inhibitors tested did not affect cleavage of PARP (not shown). The finding that the selective caspase inhibitors completely blocked the cleavage of PARP but not that of Sp1 suggests that the proteases responsible for the cleavage of these two proteins are different.

Caspases 2 and 3 are activated during retinoid-induced apoptosis. The more than 10 different caspases described to date fall into three groups with distinct substrate specificities (75). Representative members of these groups are caspase 1 (ICE), caspase 2 (Ich1L/Nedd2), and caspase 3 (CPP32). Caspase 1 is involved in the processing of caspase 3, which mediates proteolysis of different proteins, including PARP (18, 51). No role has been determined so far for caspase 2. To analyze whether caspase 3 was activated by retinoid exposure, cytosolic extracts from Jurkat cells treated with 2 μM CD437 for different times were prepared and analyzed by Western blotting. Figure 7A shows that caspase 3 was indeed activated, as demonstrated by the appearance of the p20 and p17 proteolytic fragments (75). The p20 polypeptide was detectable after 1 h of incubation in the presence of retinoids, and a very strong signal was detected after 4 h. At this time, the p17 polypeptide also appeared, and this correlated well with the time course of proteolysis of PARP (Fig. 4C). p17 is produced by cleavage at the N terminus of p20, forming the active p17-p12 heterodimer (51, 66). Degradation of both the p20 and the p17 fragments was observed after 16 h of treatment with retinoids. However, no induction of caspase 1 was observed by immunoblot analysis of the same extracts (data not shown). The p20 fragment of caspase 1 was detected in untreated cells and remained unchanged after retinoid treatment. Thus, retinoid treatment leads to the activation of caspase 3, which is likely to be responsible for the cleavage of PARP, but, accord-

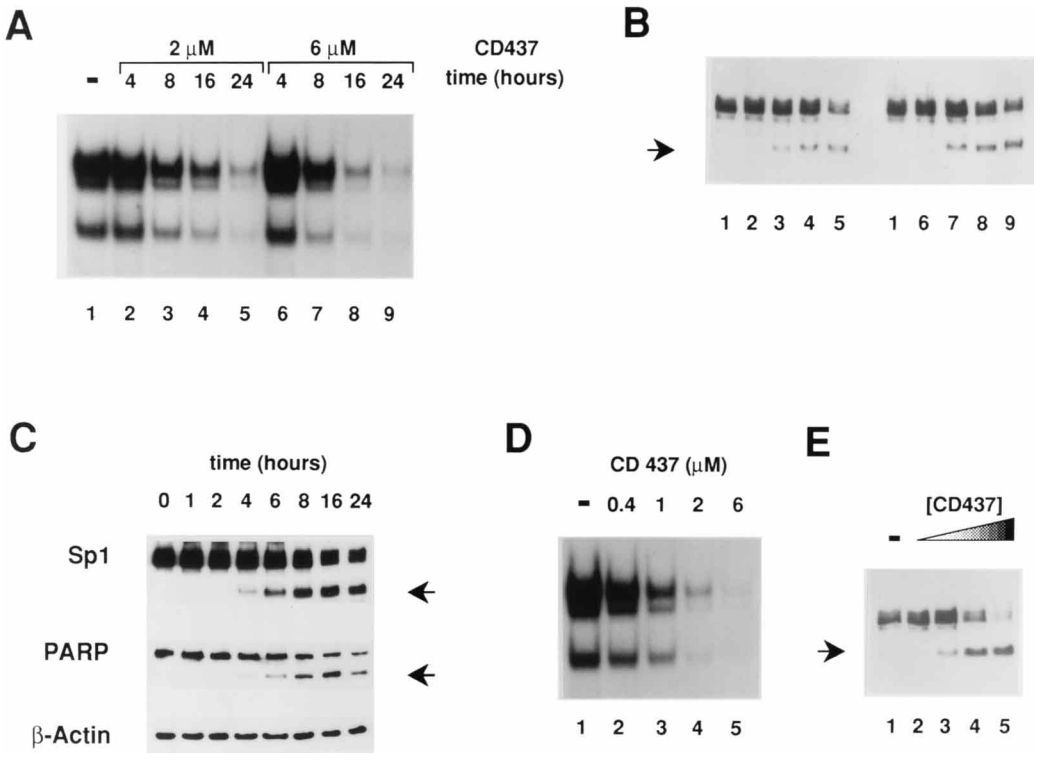

FIG. 4. Cleavage of Sp1 occurs in a time- and retinoid concentration-dependent manner. (A and B) Time course analyses of inhibition of Sp1 DNA binding and Sp1 cleavage, respectively. EL-4 cells were treated with CD437 as indicated. Nuclear extracts (A) and total cell extracts (B) were prepared after 4, 8, 16, and 24 h of exposure to retinoids and analyzed by gel retardation or immunoblotting, respectively. As a control, extracts from untreated cells were used (lanes 1). (C) Immunoblot analyses of whole-cell extracts obtained from Jurkat cells treated with 2 μM CD437 for different time periods. Specific cleavage of Sp1 and PARP was detected after 4 h of retinoid exposure (arrows). β-Actin was used to normalize for protein loading. (D and E) Gel shift and immunoblot analyses of nuclear and total protein extracts prepared from EL-4 cells treated with increasing concentrations of CD437 (as indicated in panel D). The 68-kDa Sp1 cleaved fragment observed in apoptotic cells is indicated (B and E [arrows]).

ing to our in vitro studies using peptide inhibitors (Fig. 6), it is not directly responsible for the proteolysis of Sp1.

To test the possibility that Nedd2, the murine homolog of Ich1L (caspase 2), was activated during retinoid-induced apoptosis, total cell extracts from EL-4 cells treated with retinoids and other apoptosis-inducing agents were subjected to immunoblot analysis using a polyclonal antiserum raised against the C-terminal region of Nedd2. Polypeptides p12 and p13, products of Nedd2 processing (75), were detected by Western blotting in cells undergoing apoptosis (Fig. 7B). The strongest p12 signals were found in cells treated with CD437 or MX2870-1 (Fig. 7B, lanes 3 and 4). Moreover, the same treatment also resulted in Sp1 cleavage (compare Fig. 7B with 3B), suggesting that Nedd2 might be involved in the cleavage of Sp1 in vivo. In fact, activation of the Nedd2 protease followed a time course kinetics and a CD437 concentration dependency identical to those observed for Sp1 cleavage (compare Fig. 7C with 4B and E). We also noted that the p12 signal increased with the incubation time (Fig. 7C, lanes 2 to 5 and 6 to 9) and the concentration of retinoids (lanes 10 to 14), whereas the p13 signal remained unchanged, which suggests that p12-p18 and not p13-p18 is the active heterodimer of Nedd2.

Activation of caspases is necessary for retinoid-induced apoptosis. Although caspase activation and proteolysis are general hallmarks in apoptosis, inhibition of caspases does not always prevent cells from undergoing cell death, suggesting caspase-independent pathways (25, 46). To further investigate the role of caspases in retinoid-induced apoptosis, Jurkat cells were treated with different concentrations of CD437 in the absence or presence of increasing concentrations of the peptide inhibitors ZVAD-fmk, ZDEVD-fmk, and ZFA-fmk. Apoptosis was quantitated by using an ELISA that measures the amount of cellular DNA fragmentation, allowing detection of apoptosis after only 4 h of retinoid exposure. The apoptotic effect of 2 μM CD437 on Jurkat cells was completely blocked by the caspase inhibitors ZVAD-fmk and ZDEVD-fmk in a concentration-dependent manner, but not by ZFA-fmk (Fig. 8A). These inhibitors were similarly efficient even when a

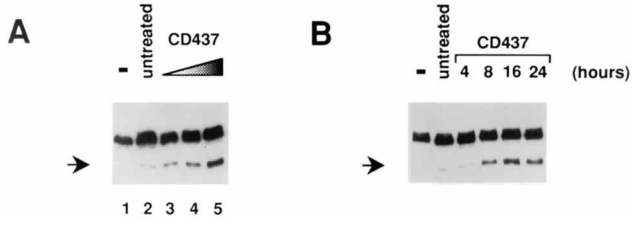

FIG. 5. Induction of a caspase activity by apoptosis-inducing retinoids. (A) Samples of 10, 20, or 50 μg (lanes 3 to 5) of cytosolic extracts obtained from EL-4 cells treated for 24 h with 2 μM CD437 were incubated for 2 h at 37°C with purified Sp1 protein. A 50-μg sample of cytosol extract from untreated cells was used as a control (lane 2). Sp1 protein was also incubated without EL-4 extract (lane 1). Proteins were analyzed by SDS–12.5% PAGE and subsequently by immunoblotting with an anti-Sp1 antibody. The 68-kDa Sp1 proteolytic product is indicated (arrow). (B) Purified Sp1 protein was incubated with 25 μg of cytosolic extracts prepared from EL-4 cells which were untreated or treated with 2 μM CD437 for the times indicated above the lanes. Purified Sp1 without EL-4 extract was also used (lane −).

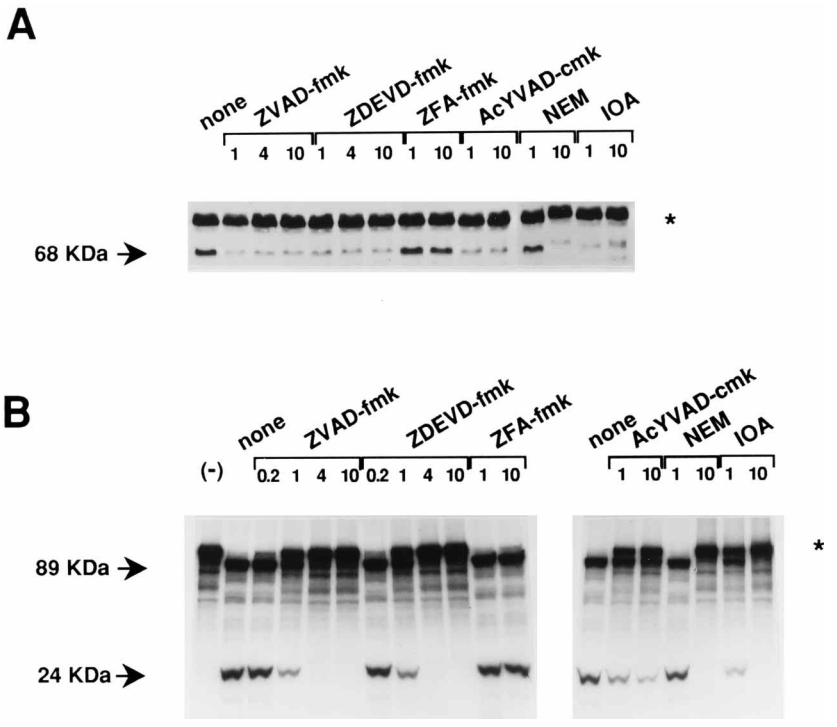

FIG. 6. Inhibition of Sp1 proteolysis by protease inhibitors in vitro. (A) Samples (25 μg) of cytosolic extracts prepared from EL-4 cells treated for 24 h with 2 μM CD437 were preincubated with the indicated protease inhibitors for 30 min at 37°C. Subsequently, 0.2 μl of Sp1 was added and proteolysis reactions were performed as described in Materials and Methods. As a control, a proteolysis reaction was also carried out in the presence of an equivalent volume (1% [vol/vol]) of vehicle (lane none), which had no effect on the cleavage of Sp1. Peptide inhibitors were used at 0.2, 1, 4, or 10 μM. *N*-Ethylmaleimide (NEM) and iodoacetamide (IOA) were used at 1 or 10 mM. The full-length protein (asterisk) and the 68-kDa proteolytic fragment are shown. (B) Effect of caspase inhibitors on the proteolysis of in vitro-translated ^{35}S-PARP. As above, protein extracts from retinoid-treated EL-4 cells were incubated with the indicated peptide inhibitors for 30 min at 37°C; ^{35}S-PARP was added, and proteolysis was performed. Samples were analyzed by SDS-PAGE and visualized by autoradiography. The positions of the cleavage products (89 and 24 kDa) and the full-length protein (asterisk) are indicated. ^{35}S-PARP alone was also used (lane −).

higher concentration of CD437 was used (6 μM) (data not shown). To determine when the caspase-dependent retinoid-induced damage became irreversible, Jurkat cells were treated with 2 μM CD437 and peptide inhibitors (50 μM) were added at different times after retinoid exposure (0, 1, 2, and 3 h). DNA fragmentation was measured after 4 h of incubation in the presence of retinoid. Figure 8B shows that apoptosis could be prevented when the peptide inhibitors were added up to 2 h after the retinoids but not when the cells were exposed to retinoids for 3 h before addition of the inhibitor. ZVAD-fmk completely blocked apoptosis when added 1 h after CD437 but caused only a partial block (60%) when added after 2 h. However, ZDEVD-fmk was equally effective between 0 and 2 h. These data indicate that the protease cascade is activated in Jurkat cells during the first 2 h of exposure to retinoids. By 3 h, the processed caspase 3 has already activated the DNA fragmentation factor (40), which induces DNA fragmentation and once activated can no longer be inhibited by the caspase inhibitors.

We next investigated whether caspases were necessary for the proteolysis of Sp1 in intact cells. Whole-cell extracts were prepared from Jurkat cells treated overnight with 2 μM CD437 alone or together with different concentrations of the peptide inhibitors. Proteolysis of Sp1 and PARP was analyzed by Western blotting. Cleavage of both Sp1 and PARP was completely prevented in the presence of ZVAD-fmk or ZDEVD-fmk, correlating with the observed inhibition of DNA fragmentation (Fig. 8C). ZVAD-fmk also inhibited the processing of caspase 3, and only low levels of p20 were found (not shown). High levels of p20 polypeptide were found in retinoid-treated cells in the presence of ZDEVD-fmk, suggesting that the initial processing of caspase 3 was mediated mainly by other proteases (probably caspase 1) but not by autoproteolysis. In agreement with other in vitro studies (43), both inhibitors blocked further processing of p20 in intact cells, so that the p17 polypeptide was not detectable (data not shown). As expected, ZFA-fmk was unable to prevent the appearance of both p20 and p17 polypeptides (not shown). Similarly, when EL-4 cells were treated with CD437 in the presence of increasing concentrations of the different peptide inhibitors, a decrease of the caspase 2 p12 signal was seen, whereas the p13 polypeptide was accumulated, correlating with a decrease in Sp1 cleavage (data not shown). Together, these data clearly show that activation of caspases is necessary for the cleavage of Sp1 and PARP, as well as for the induction of apoptosis by retinoids.

Retinoid-induced apoptosis does not require de novo synthesis of mRNA. We analyzed whether protein synthesis (cycloheximide) or RNA transcription (actinomycin D) inhibitors had any effect on CD437- or MX2870-1-induced apoptosis in Jurkat cells. When added together with the retinoids, actinomycin D was unable to inhibit apoptosis, and cycloheximide showed a partial inhibitory effect (between 30 and 40%), as determined by quantitation of DNA fragmentation (Fig. 9). However, when cells were preincubated for various periods with the protein or RNA synthesis inhibitors, the apoptotic effect of the retinoids was significantly reduced. These data clearly suggest that the protein(s) mediating retinoid-induced apoptosis is present in the untreated cells. Blocking protein

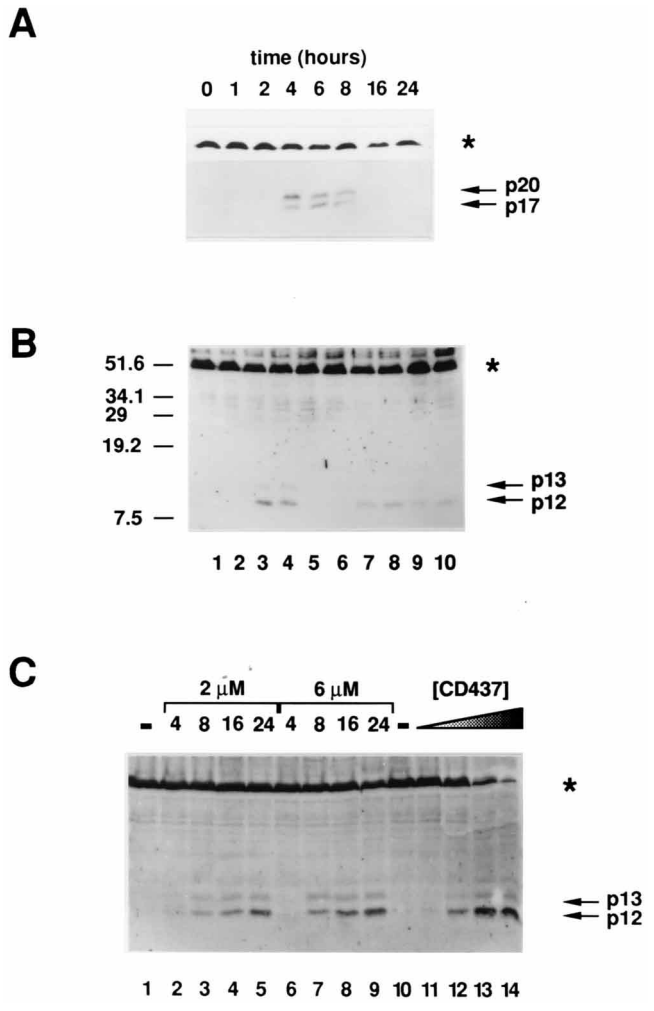

FIG. 7. Activation of caspases during retinoid-induced apoptosis. (A) Caspase 3 is activated during retinoid-induced apoptosis. Cytosolic extracts from Jurkat cells treated with 2 μM CD437 for the indicated periods were subjected to SDS-PAGE and immunoblot analysis using a monoclonal antibody against CPP32. The full-length protein is indicated (asterisk). Polypeptides p20 and p17, originating from caspase 3 processing, are detected after 4 h of retinoid treatment. The bottom part of the blot was overexposed to better visualize the p20 and p17 signals. (B) Nedd2 is activated during retinoid-induced apoptosis. Samples (50 μg) of total cell extracts from EL-4 cells were resolved by SDS–20% PAGE and analyzed by immunoblotting with anti-Nedd2 antiserum, which recognizes the full-length protein (asterisk) and polypeptides p12 and p13. Cells were treated as described for Fig. 3 (lanes 1 to 10, respectively). (C) Activation of Nedd2 follows a time course and a retinoid concentration dependence similar to those of Sp1 cleavage. Extracts prepared as for Fig. 4D and E were resolved on an SDS–20% polyacrylamide gel and analyzed by immunoblotting as described above.

synthesis or RNA transcription before retinoid treatment apparently reduces the protein necessary for the apoptotic process to be induced by retinoids. Together, these data strongly suggest that all protein(s) required for apoptosis is already present in untreated cells but has a relatively short half-life.

DISCUSSION

Retinoids regulate important events in growth and development and cell proliferation and differentiation. For several years, a great effort has been devoted to developing novel synthetic retinoids as cancer-preventive and -therapeutic agents. More recently, it has been shown that some of these retinoids can induce apoptosis in certain cancer cell lines (14, 19, 41, 61). Importantly, some of the apoptotic retinoids could be demonstrated to inhibit the growth of human lung cancer cells in vivo in the absence of major side effects (41). How these retinoids induce apoptosis is thus of considerable interest. In vitro studies have shown that the apoptosis-inducing retinoids CD437, MX2870-1, and 4-HPR selectively activate the RARγ isoform (4, 19, 41), and one possibility is that this receptor plays a key role in retinoid-induced apoptosis. It has been postulated that RARs do not mediate cell death, since RA-resistant HL-60 cells were susceptible to apoptosis after treatment with CD437 (61). However, apoptosis induction by retinoids is likely to follow a different pathway than the induction of differentiation, which might be activated by different receptor subtypes. RARγ has also been linked to apoptosis induction in F9 cells and neuroblastoma cells. The data presented here show that if RARγ mediates the apoptosis signal, transcriptional activation is not required, since actinomycin D is not able to block apoptosis when added together with the retinoids. Previous studies with 4-HPR investigated only the effects of preincubation with actinomycin D and cycloheximide (13). Those results are consistent with our data showing that inhibition of protein synthesis or RNA transcription before retinoid treatment can prevent apoptosis. However, if both retinoids and cycloheximide or actinomycin D are added together, apoptosis cannot be blocked. This situation is similar to Fas- or TNF-α-induced apoptosis, which is independent of protein synthesis and RNA transcription (28, 76). Thus, retinoid-induced apoptosis appears to involve a novel mechanism of action which may be analogous to the TNF-α-induced mechanism. Certain RARγ-selective retinoids might thus trigger apoptosis through binding to RARγ, inducing a particular conformation which allows for protein-protein interaction with the apoptotic machinery, thereby initiating the cell death pathway. However, interaction of the retinoids with an as-yet-unidentified protein, instead of RARγ, cannot be excluded at this time.

Proteolysis is a hallmark in apoptosis, and several proteins belonging to the caspase family have been cloned and shown to play a key role in apoptosis (25, 44). Our data reported here show that caspases 2 and 3 are activated in cells treated with apoptotic retinoids. Importantly, we demonstrate that caspase activation is necessary for the induction of apoptosis by our selective retinoids. A number of proteins have been shown to be cleaved during apoptosis, and it is likely that the number of both identified targets and caspases will continue to grow significantly in the near future. We report here for the first time that a general transcription factor, Sp1, is specifically cleaved during retinoid-induced apoptosis, with a concomitant loss of DNA binding activity. To our knowledge, Sp1 is the first general transcription factor found to be a target for caspases. Sp1 is a constitutive transcription factor that activates numerous genes through its interaction with specific DNA binding sites found in many promoters (30, 31). Interestingly, Sp1 has been implicated in the expression of cell cycle-regulated genes (32, 39). Besides having constitutive transcriptional activity, Sp1 has been found to synergistically cooperate with other transcription factors, such as Ets, NF-κB, E2F, and AP-1 (5, 23, 32, 39, 52, 53). Inhibition of Sp1 DNA binding through specific cleavage induced by treatment with selective, apoptosis-inducing retinoids is thus likely to have a strong impact on the overall transcription rates in retinoid-treated cells. Sp1 cleavage occurs early in apoptosis, and it can be detected in Jurkat cells after only 4 h of treatment, suggesting that it may have a potential key role in apoptosis. In fact, no Sp1 DNA binding

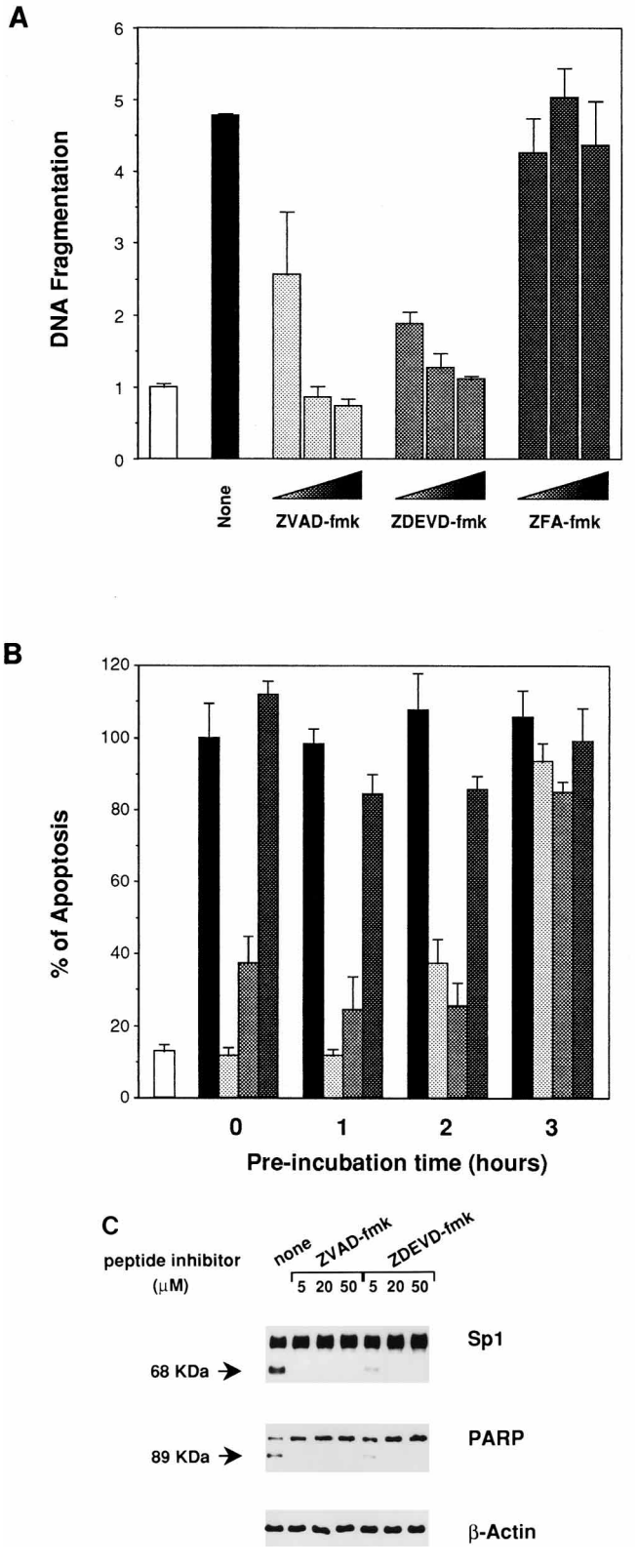

FIG. 8. Activation of ICE-like proteases is necessary for retinoid-induced apoptosis and Sp1 cleavage. (A) Caspase inhibitors block CD437-induced apoptosis in Jurkat cells in a concentration-dependent manner. Jurkat cells were labeled with BrdU for 4 h and then treated with 2 μM CD437 in the absence (black column) or presence of increasing concentrations of the indicated peptide inhibitors (5, 20, and 50 μM) for an additional 4 h. The cells were then lysed, and DNA fragmentation was measured by ELISA as described in Materials and Methods. The fold induction in DNA fragmentation is given (arbitrary units). Results with control cells (not treated with the retinoid) are also shown (white column). Results of a representative experiment performed in triplicate are shown. (B) Irreversible retinoid-induced damage occurs after 2 to 3 h of retinoid exposure. BrdU-labeled Jurkat cells were incubated with 2 μM CD437. Different peptide inhibitors (50 μM each) were added to the retinoid-treated cells at the indicated times. Cells were lysed after 4 h of retinoid exposure, and DNA fragmentation was measured by ELISA. The percentage of an apoptotic index is shown, 100% being the amount of DNA fragmentation induced by CD437 in the absence of peptide inhibitors at time zero. Symbols: ■, no inhibitor; ▨, ZVAD-fmk; ▦, ZDEVD-fmk; ▩, ZFA-fmk. (C) CD437-induced cleavage of Sp1 and PARP is prevented by peptide inhibitors in Jurkat cells. Cells were incubated with retinoids in the absence or presence of increasing concentrations of ZVAD-fmk or ZDEVD-fmk, as indicated, for 14 h. Total cell extracts were then prepared and separated by SDS–12.5% PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting using anti-Sp1, anti-PARP, and anti-β-actin antibodies, as indicated.

activity was detected after 24 h of exposure to retinoids, indicating that the Sp1 proteolytic products could have a dominant negative effect on the transcriptional activity of the wild-type protein.

Caspases are grouped into three subfamilies, as defined by their substrate specificity (75) or by phylogenetic relationships (1). Caspases exist in the cell as proenzymes, and they are proteolytically activated during apoptosis by themselves or by other caspases, originating a protease cascade that leads to the proteolysis of target proteins (44). Caspases 8 and 1 are upstream of this cascade, and they can activate caspase 3 and other related enzymes, which are the proteases responsible for the cleavage of protein targets. Our in vitro proteolysis experiments using peptide inhibitors and comparing the cleavage of PARP and Sp1 suggest that the protease activities that cleave the two substrates are different. PARP is known to be a target of caspase 3 and other, related caspases. It is also cleaved by caspase 1, although with much lower efficiency than by caspase 3. However, PARP does not appear to be a target for caspase 2 (75), although it has been shown that high concentrations of Nedd2 can cleave PARP in vitro (24). Interestingly, we find that Nedd2 (the murine caspase 2) is activated in EL-4 cells after retinoid treatment, in a time- and concentration-dependent manner that parallels the observed cleavage of Sp1. This indicates that it might be involved in the cleavage of Sp1 in vivo. Caspase 2 is highly expressed in EL-4 and Jurkat cells, as well as in other cell lines, such as Namalwa and HeLa cells, in which Sp1 cleavage was observed after retinoid-induced apoptosis. However, it is not expressed, or only at very low levels, in other cell lines in which no cleavage of Sp1 was detected (our unpublished observations). These results point towards a potential role of caspase 2 in the cleavage of Sp1 during apoptosis. Although caspase 3 is not directly responsible for the proteolysis of Sp1, inhibition of Sp1 cleavage by ZDEVD-fmk indicates that a CPP32-like activity is necessary for the Sp1 processing to occur in intact cells, probably by activating the Sp1-specific protease.

In summary, we present here data that enhance our understanding of the pathways activated during retinoid-induced apoptosis and of apoptosis in general. We have shown that the activation of caspases is required for retinoid-induced apoptosis and the specific cleavage of a novel target, the transcription factor Sp1. Our data also suggest a novel mechanism of action for these retinoids, similar to that of TNF-α, which is a transactivation-independent mode of action, since no transcriptional activity is required for the induction of apoptosis. Thus, the binding of these apoptotic retinoids to their receptor (which is probably RARγ) may induce a particular conformation that then could allow protein interaction with the

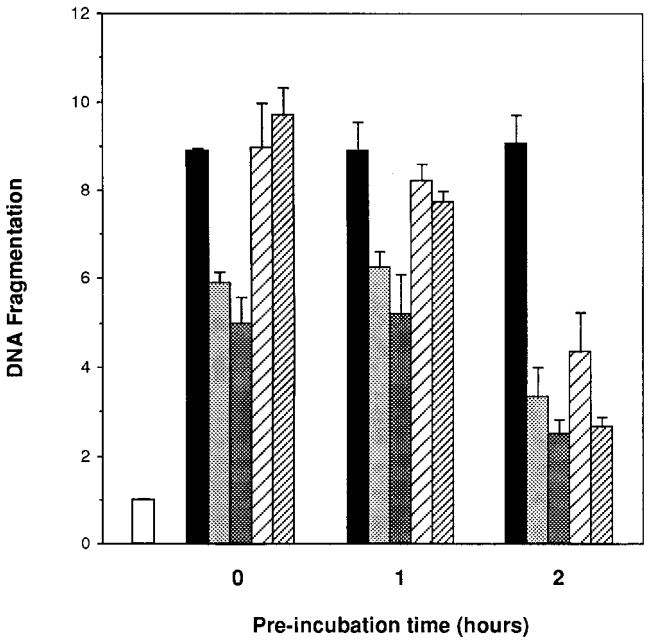

FIG. 9. Effects of cycloheximide and actinomycin D on retinoid-induced apoptosis. Jurkat cells were not preincubated or were preincubated with the indicated concentrations of cycloheximide or actinomycin D and then incubated with 2 μM CD437 for an additional 4 h. After lysis of the cells, cellular DNA fragmentation was measured by ELISA. The induction of DNA fragmentation versus untreated control cells (white column) is shown. The experiment was performed at least twice, and results of a representative experiment carried out in triplicate are shown. Symbols: ■, no inhibitor; ▨, 0.1 μg of cycloheximide per ml; ▩, 0.2 μg of cycloheximide per ml; ▨, 1 ng of actinomycin D per ml; ▨, 5 ng of actinomycin D per ml.

apoptotic machinery. This can be direct or mediated by another bridging protein(s), but it leads to the protease cascade and cell death.

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