Fas-induced Apoptosis in Human Malignant Melanoma Cell Lines Is Associated with the Activation of the p34<sup>cdc2</sup>-related PITSLRE Protein Kinases*

Maria E. Ariza†‡, Marianne Broome-Powell‡, Jill M. Lahti¶§, Vincent J. Kidd¶, and Mark A. Nelson‡§

From the †Arizona Cancer Center, Tucson, Arizona 85724, the ‡Pathology Department, University of Arizona, Tucson, Arizona 85724, and the §Department of Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38101

The Cdc2L locus encoding the PITSLRE protein kinases maps to chromosome band 1p36 and consists of two duplicated and tandemly linked genes. The purpose of the present study was to determine whether diminution of PITSLRE kinases leads to deregulation of apoptosis. The human melanoma cell lines A375 (Cdc2L wild-type alleles) and UACC 1227 (mutant Cdc2L alleles) were tested with agonist anti-Fas monoclonal antibody. We found that exposure of these cells to anti-Fas for 24, 48, or 72 h resulted in differential sensitivity to Fas-induced apoptosis. In A375, cell death started at 24–48 h post-treatment, and it was maximal by 72 h. Conversely, UACC 1227 cells were resistant to Fas-mediated apoptosis. Induction of PITSLRE histone H1 kinase activity was observed in A375 anti-Fas treated but not in UACC 1227 cells. Also, the PITSLRE protein kinase activity in A375 anti-Fas-treated cells preceded maximal levels of apoptosis. Finally, fluorescence confocal microscopy revealed a nuclear localization of PITSLRE proteins in normal melanocytes and A375 cells but a cytoplasmic localization in UACC 1227 cells. The differences in PITSLRE protein and cellular localization between A375 and UACC 1227 cells appear to account for the differences in sensitivity of the two cell lines to anti-Fas and staurosporine. These observations suggest that alterations in PITSLRE gene expression and protein localization may result in the loss of apoptotic signaling.

Apoptosis is a highly regulated process that plays a major role in development and homeostasis (1). The pathways of cellular proliferation and apoptosis appear to be linked to minimize the occurrence of neoplasia (2). It has also been proposed that deregulation of apoptosis is a pathogenic process in some bone marrow disorders (3) and in tumor development (4, 5). The cell surface receptor Fas/APO-1 (CD95) is a type-I transmembrane protein that belongs to the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor superfamily

(6–8). Binding of anti-Fas antibody or Fas ligand (FasL) to Fas receptor triggers apoptosis in vivo and in vitro, in sensitive cells (8, 9). There is evidence suggesting that apoptosis induced by TNF and Fas involves a common mechanism. Both Fas and TNF receptors contain dead domains (DD), which provide receptor-triggered signaling that may allow “cross-talk” between their pathways (10–12). TNF and Fas-mediated apoptosis involves a family of cysteine proteases related to the interleukin-1β-converting enzyme (ICE-like) family, which are currently considered to be the central executioners of apoptosis (13–15). Although Fas and its ligand are expressed in a variety of cells, including melanocytes, their importance in negative growth regulation has been studied primarily in the immune system (4, 16, 17). Thus, their role in apoptotic events concerning nonimmune tumor cells needs to be further investigated.

Malignant melanoma is a relatively common neoplasm and the only cutaneous malignancy that metastasizes and causes death. The incidence of melanoma is rising faster than any other cancer in the United States, and it is expected to reach an all time high rate of 1 in 75 by the year 2000. Recent evidence suggests that failure of cells to undergo apoptotic cell death might contribute to the pathogenesis of a variety of human diseases including cancer (18). Previous work in our laboratory demonstrated that deletions of chromosome region 1p36 are one of the most frequent cytogenetic abnormalities found in melanoma (19). The Cdc2L locus encoding the PITSLRE protein kinases maps to chromosome band region 1p36 (20). We have also shown that one allele of the Cdc2L locus on 1p36 was either deleted or translocated in eight of fourteen different melanoma cell lines (21). Decreased expression of the PITSLRE proteins from the remaining allele was observed in several cell lines and surgical malignant melanoma specimens (21).

The PITSLRE proteins are part of the large family of p34<sup>cdc2</sup>-related kinases whose functions appear to be linked to control of cell division and possibly programmed cell death (22–25). The PITSLRE p110 isoforms are reported to be involved in the regulation of RNA splicing/translation during the cell cycle (26). The larger p110 PITSLRE isoforms are also cleaved by multiple caspases during Fas- and TNFα-induced cell death (27). Furthermore, ectopic expression of a p50-PITSLRE construct that resembles the final caspase-modified product induces apoptosis in CHO cells (25). Finally, Fas-mediated T-cell death is correlated with PITSLRE proteolysis and increased histone H1 kinase activity (25). The purpose of the present

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‡ To whom correspondence should be addressed: Arizona Cancer Center, Rm. 3963 B, 1515 N. Campbell Ave., Tucson, AZ 85724. Tel.: 520-626-4515; E-mail: mnelson@azcc.arizona.edu.

§ The abbreviations used are: TNF, tumor necrosis factor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; 7AAD, 7-amino-actinomycin D; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DDT, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; RT, room temperature; PARP, poly ADP-ribose polymerase.
study was to determine whether alterations in the PITSRLRE isoforms could lead to a disruption in the apoptotic signaling pathway(s) in cultured melanoma cells.

MATERIALS AND METHODS

Cell Culture—A375 and UACC 1227 human melanoma cell lines were obtained from the Arizona Cancer Center Tissue Culture Shared Resource. Human melanoma cells were grown as monolayers in RPMI 1640 medium supplemented with 5% (v/v) diazyl and heat-inactivated fetal calf serum, 1% l-glutamine, and 1% penicillin-streptomycin (10,000 units/ml-10,000 μg/ml). Normal human melanocytes were isolated from newborn foreskin and cultured in modified M15 medium supplemented with 5% fetal calf serum as described previously (37). Media and reagents were purchased from Life Technologies, Inc., Grand Island, NY.

Cell Surface Expression of Fas—Cell surface expression of Fas receptor in A375 and UACC 1227 melanoma cells was detected by flow cytometry (38). Briefly, cells were harvested, washed twice with PBS and incubated for 60 min on ice with 20 μg/ml of either anti-Fas mouse monoclonal IgM antibody or a nonspecific isotype-matched monoclonal antibody in PBS containing 1% fetal calf serum, 0.02 mM NaN3, and 0.5 mM EDTA. All the chemicals and monoclonal antibodies were purchased from Sigma. Cells were washed twice with PBS and incubated for 30 min at 4 °C with affinity purified FITC-conjugated goat anti-mouse IgM (Becton Dickinson). Cells were washed twice with PBS and analyzed for Fas expression on a FACScan flow cytometer (Becton Dickinson).

RNAse Protection Assay—Total RNA isolated from HeLa cells (5 μg) and the melanoma cell lines A375, UACC 1227, and UACC 903 was analyzed for distinct mRNA species using Pharamingen’s RiboQuant™ multi-probe analysis kit. BAP0-2, BAP0-3, and BAP0-5 probe template sets. α-32P-labeled antisense RNA probes were synthesized, allowed to hybridize to target RNA, and digested with RNAse A, as described by the manufacturer. The remaining RNAse-protected probes were purified and resolved on denaturing polyacrylamide (5%) gels at 40 watts for 3 h, dried, and analyzed by autoradiography (~80 °C, overnight).

Cell Culture—On day 1, A375 and UACC 1227 cells (1 × 105) were cultured in RPMI 1640 medium supplemented with 5% (v/v) diazyl and heat-inactivated fetal calf serum, 1% l-glutamine, and 1% penicillin-streptomycin and incubated overnight at 37 °C in a humidified 5% CO2 environment. On day 0, cells were washed twice with PBS and treated with either 0.5 μg/ml anti-Fas monoclonal antibody, CH-11 (anti-Fas mAb, Upstate Biotechnology, Lake Placid, NY) or 10 ng/ml staurosporine. For staurosporine, and 72 h of incubation was allowed. Untreated control cells were harvested by low speed centrifugation, resuspended in PBS, and analyzed for apoptosis using a FACStar flow cytometer (Becton Dickinson). Unstained A375 and UACC 1227 cells were used as negative controls. Discrimination of the three populations (dead cells as the percentage of viable cells in treated samples relative to nontreated control cells.

Histone H1 Kinase Assay—A375 cells were treated with P38-6 Δ (24, 48, and 72 h. A375 cells were also treated with anti-Fas, staurosporine (10 ng/ml), or in combination with protease inhibitors of caspase 3 (DEV-FMK, 20 μM) or caspase 8 (IETD, 20 μM) for 48 h, harvested, washed twice with ice-cold PBS and resuspended in ice-cold PBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonident P-40, 5 mM EDTA, 5 mM EGTA, 15 mM MgCl2) containing 60 mM glycylglycine, 0.1 mM sodium orthovanadate, 0.1 mM sodium fluoride, 15 μM β-nitrophenylphosphate, aprotinin (10 μg/ml), leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM benzamide for 30 min. Following lysis, the samples were washed twice in lysis buffer, and the protein content was determined using the bichinchoninic acid assay (Pierce) with bovine serum albumin as the standard. Total cell lysate (200 μg) was pre-cleared with rabbit sera to mouse, and PITSRLRE proteins were immunoprecipitated with PITSRLRE GNI affinity purified polyclonal antisera directed against the first 72 amino acids of the p58 PITSRLRE kinase p1 (39) and protein A-garose. The resultant immunoprecipitates were analyzed for histone H1 kinase activity using H1 buffer (50 mM Tris-HCl (pH 7.5), 15 mM MgCl2, 1 mM DTT), 50 μM ATP, 8 μCi [32P]ATP (>3000 Ci/mmol), and histone H1 (2–5 μg/ml). Histone H1 phosphorylation was analyzed by 15% SDS-polyacrylamide gel electrophoresis and autoradiography. The gels were exposed either for 4 h or overnight at –80 °C on Kodak X-AR5 film. Quantitation of histone H1 phosphorylation was determined by phosphoimaging. The gels were visualized with a Molecular Dynamics 400A PhosphorImager™, and the relative kinase activity was estimated by quantitating the labeled histone H1 bands using the Molecular Dynamic ImageQuant software.

Western Blot Analysis—Western blot analysis of various melanoma cell lines were performed using the PITSRLRE p2N100 (1:5000) affinity purified polyclonal antisera, PARP (1:1000) and β-actin (1:8000) antibodies as described previously (21, 31). Briefly, protein extracts (either 30 or 50 μg) from control and treated samples were separated by SDS-polyacrylamide gel electrophoresis transferred to a polyvinylidine difluoride membrane and the blots probed with the different antibodies. A secondary probe with horseradish peroxidase-labeled antibodies (Amersham Pharmacia Biotech) was detected by enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech).

Microinjection and Transfection—Cells were grown on 35-mm tissue culture dishes containing sterile glass coverslips embedded in the plastic (Martek Corporation). Prior to microinjection, the plasmid DNAs (pCH110, pcDNA 3.0, and DR3/pcDNA 3.0) were diluted to 200 ng/μl in 50 mM Hepes, 100 mM NaPO4, pH 7.2, and the pCHO110 reporter plasmid was mixed with an equal volume of either control (pcDNA 3.0) or the test plasmid (DR3/pcDNA 3.0). For each experiment, identical number of cells in three different areas of the dish were injected with the automatic Eppendorf Transactor II system using a femtotip II capillary, with an injection pressure of 59.9 psi and an injection time of 0.6 s. After injection, the cells were returned to the 37 °C incubator. Sixteen hours later, the cells were stained for β-galactosidase by incubation in the X-gal staining solution (250 μg/ml of X-gal in 5% FBS, 100 mM NaPO4, pH 7.2, 150 mM NaCl, 5 mM KCl, and 1 mM MgCl2) for 18 h at 37 °C. Following incubation, the cells were transferred using the Fugene reagent (Roche Molecular Biochemicals) according to the protocol of the manufacturer. Cells were washed twice with PBS and fixed in formalin for 20 min at room temperature (RT). Cells were rinsed three times with PBS and permeabilized with 100% methanol at –20 °C for 6 min. Cells on coverslips were incubated with 5% bovine serum albumin in PBS for 10 min and removed. Goat serum (1:10 dilution in PBS) was added to all coverslips for 10 min, removed, and the coverslips incubated with the primary antibody P2N100 (1:500 dilution) directed against PITSRLRE proteins for 1 h at RT. Coverslips were washed three times with PBS for 5 min each and then incubated with streptavidin (1:100) for 30 min at RT. Following incubation, coverslips were rinsed three times with PBS for 5 min and stained with X-gal as described previously. The number of X-gal-stained cells in 20 random fields were quantitated for each dish.

Immunofluorescence Confocal Microscopy—Normal melanocytes, A375 and UACC 1227 melanoma cells, were grown on coverslips, washed twice with PBS and fixed in formalin for 20 min at room temperature (RT). Cells were rinsed three times with PBS and permeabilized with 100% methanol at –20 °C for 6 min. Cells on coverslips were incubated with 5% bovine serum albumin in PBS for 10 min and removed. Goat serum (1:10 dilution in PBS) was added to all coverslips for 10 min, removed, and then the coverslips incubated with the primary antibody P2N100 (1:500 dilution) directed against PITSRLRE proteins for 1 h at RT. Coverslips were washed three times with PBS for 5 min each and then incubated with streptavidin (1:100) for 30 min at RT. Following incubation, coverslips were rinsed three times with PBS for 5 min and incubated with 14 μg/ml FITC (1:3000) for 30 min. Coverslips were washed three times with PBS for 5 min, and biotinylated goat anti-rabbit (GAR, 1:100 dilution with 1% bovine serum albumin in PBS) was added for 1 h. Coverslips were washed again with PBS and incubated with Cy5-streptavidin for 1 h, washed three times with PBS, and incubated with RNase (100 μg/ml) for 1 h. Following incubation, coverslips were washed three times and incubated with YoYo-1 (1:50 dilution) for 15
human malignant melanoma cell lines express fas receptor—

to rule out the possibility that A375 or UACC 1227 cells are resistant to anti-Fas mAb-mediated cell death because they do not express Fas receptor, cells were analyzed for the expression of cell surface Fas receptor by immunofluorescence and flow cytometry. Immunofluorescence and flow cytometric analysis demonstrated that both cell lines, A375 and UACC 1227, express quantitatively similar levels of Fas receptor on the cell surface (Fig. 1). Furthermore, confocal microscopy revealed that the Fas receptor was localized on the cell membrane as well as, in the cytoplasm (data not shown). In addition, RNase protection analysis indicated that both cell lines express all of the components involved in the Fas/DR3/TNF signal transduction pathway(s) (Fig. 2, A and B). Although mRNA levels for Fas receptor were decreased in UACC 1227 cells when compared with A375 by RNase protection assay (Fig. 2A), UACC 1227 cells express similar or higher levels of cell surface Fas receptor than A375 by flow cytometric analysis (Fig. 1). This suggests that the efficiency of translation of CD95 mRNA in UACC 1227 cells is higher than that of A375 cells.

anti-Fas mAb treatment induces apoptosis in A375 cells but not in UACC 1227 cells—It has been reported that PITSLRE kinases might serve as effectors of an apoptotic signaling pathway(s) (25). To test this hypothesis, we used the melanoma cell lines A375, which has normal PITSLRE alleles and exhibits normal expression of PITSLRE, and UACC 1227, which has an abnormal PITSLRE allele and exhibits decreased PITSLRE expression (21). To determine the effect of PITSLRE expression on apoptotic signaling, flow cytometric analysis using 7AAD was performed. 7AAD is a fluorescent DNA-binding agent that intercalates between cytosine and guanine bases, and it is used to detect dead (7AAD-bright), apoptotic (7AAD-dim), and live (7AAD-negative) populations by fluorescence-activated cell sorting (28). Apoptosis was triggered using anti-Fas mAb CH-11. Exposure of the cells to anti-Fas mAb for 24, 48, or 72 h demonstrated that Fas-induced apoptosis begins in A375 cells 24–48 h post-treatment (5–23% apoptotic cells) and is maximal by 72 h (80% apoptotic cells) (Fig. 3, A and B). Conversely, UACC 1227 cells were resistant to Fas-induced apoptosis (Fig. 3B). The 7AAD data was validated by 1) cell sorting and morphological examination using Wright/Giemsa (DiffQuik staining method), 2) terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL), and 3) hematoxylin and eosin staining. Morphological changes consistent with apoptotic cell death including cell shrinkage, nuclear condensation, and membrane blebbing were observed in the Fas-sensitive A375 cells (Fig. 3C, a and b). In contrast, no morphological changes were seen in the UACC 1227 cells (Fig. 3C, c and d). Morphological analysis of sorted A375 cells treated with anti-Fas mAb for 72 h indicated that cells from early-late/dead apoptotic regions show nuclear condensation with marked cell shrinkage (data not shown). These data demonstrate that A375 cells are sensitive to Fas-mediated apoptosis, whereas the UACC 1227 cells are resistant to Fas-induced apoptosis. Because both cell lines have Fas receptor and all of the components of the Fas-mediated signal transduction pathway, these differences may reflect the involvement of PITSLRE kinases in the Fas-mediated signaling pathway in melanoma cells.

processing of PITSLRE proteins and activation of PITSLRE kinase activity in melanoma cell lines during anti-Fas mAb-mediated apoptosis—it is not known what substrate is responsible for the execution of the death sentence once the process of apoptotic cell death has been activated. It has been suggested that PITSLRE kinases might be potential candidates (14) because they are processed and activated following anti-Fas treatment in T cells (25). However, it is not known whether processing and activation of PITSLRE kinases take place in melanoma cells. To determine whether PITSLRE kinases are activated in melanoma cells following treatment with anti-Fas mAb, A375 and UACC 1227 cells (1 x 10⁶) were exposed to anti-Fas mAb for 24, 48, or 72 h, and PITSLRE kinase activity was measured by the histone H1 kinase assay. After 48 h of treatment with anti-Fas mAb, PITSLRE kinase activity was increased 8-fold in A375 cells, which returned to control levels by 72 h (Fig. 4A). Cleavage of PITSLRE proteins and poly (ADP-ribose) polymerase (PARP) was observed in A375 cells treated with anti-Fas mAb as early as 24 h post-treatment, and it reached maximum levels by 48 h after treatment (Fig. 5). The maximal cleavage level of PITSLRE and PARP occurred at 48 h following anti-Fas treatment and correlates with the maximum activation levels of PITSLRE kinase activity. Conversely, there was no increase in PITSLRE kinase activity in UACC 1227 Fas-treated cells at any of the time points analyzed (Fig. 4B). Furthermore, no cleavage of PITSLRE or PARP was observed in UACC 1227 cells treated with anti-Fas (data not shown). These results are consistent with the apoptosis data presented above and show that PITSLRE kinase activity precedes maximal levels of apoptosis.

nuclear microinjection of DR3 in A375 and UACC 1227 melanoma cells—Although the RNase protection experiments indicated that the downstream components of the Fas pathway...
were present in these cells, it was necessary to determine whether they were functional. One approach to this question was to examine the sensitivity of the two cell lines to signaling events mediated by other death receptors, such as DR3, or to agents that bypass the receptor pathway, such as staurosporine. We reasoned that if the two cell lines were equally sensitive to the DR3 death receptor cross-linking, the Fas receptor itself may be defective. Differences in staurosporine sensitivity would be more likely to reflect alterations in function of other downstream components of the pathway. Therefore, equal amounts of β-galactosidase plasmid, and either pcDNA 3.0 (a control plasmid) or a DR3/pcDNA 3.0 expression construct were microinjected into the nucleus of the A375 and UACC 1227 cells. Sixteen hours later, the cells were analyzed for X-gal activity. The number of X-gal positive A375 cells declined by 98% when the DR3 expression construct was co-injected. Conversely, 64% of UACC 1227 cells co-injected with the reporter, and DR3 plasmids underwent cell death. To confirm these studies, the two cell lines were also transfected with the same expression constructs. The transfection results for the A375 cells were identical to those obtained in the microinjection studies, whereas the UACC 1227 cells were slightly less sensitive (46% survival). A portion of this difference may be because of the lower expression level of the transfected DNA, as judged by the intensity of the X-gal staining. Even so, there was a significant difference in the ability of these cell lines to undergo DR3-induced apoptosis. Because DR3 expression studies suggested that there might be differences in the ability of the two cell lines to respond to death-receptor-mediated signaling events, we wanted to determine whether the two cell lines also differed in their responses to apoptosis-inducing agents, such as staurosporine, that do not require a functional death receptor pathway.

A375 and UACC 1227 Cells Differ in their Sensitivity to Staurosporine-induced Apoptosis—To determine whether alternative apoptotic pathways exist in UACC 1227 cells, staurosporine was used. Staurosporine is a death inducer known to cause apoptosis through cytochrome c release from the mitochondria and activation of caspase 9 (29, 30). A dose-response curve was performed to determine the optimal staurosporine concentration to be used for the following experiments (Fig. 6A). A staurosporine concentration of 10 ng/ml, which caused a 50% decrease in A375 cell viability, was used for subsequent experiments. Exposure of A375 and UACC 1227 cells to staurosporine (10 ng/ml) for 24, 48, or 72 h demonstrated that staurosporine-induced apoptosis begins in A375 cells at 24 h post-treatment (50% apoptotic cells), and it is maximal by 72 h (70% apoptotic cells) (Fig. 6B). Conversely, UACC 1227 cells were resistant to staurosporine-induced apoptosis at this concentration (Fig. 6B). Morphological changes consistent with apoptotic cell death were observed in the staurosporine-sensitive A375 cells (Fig. 6C, a and b). In contrast, the same concentration of staurosporine did not produce morphological changes in UACC 1227 cells (Fig. 6C, c and d). A 10-fold increase in the concentration of staurosporine (100 ng/ml) was required to induce the same percentage of apoptotic cells in UACC 1227 as that observed in A375 cells treated with 10 ng/ml (Fig. 6A). These results demonstrate that A375 cells are more sensitive to staurosporine-induced apoptosis than UACC 1227 and that an alternative apoptotic pathway is operational in UACC 1227 cells.

Caspase 3 and Caspase 8 Inhibitors Block Fas-mediated Cell Death in Melanoma Cells—To determine whether caspases 3 and/or 8 are involved in Fas-induced apoptosis in melanoma, A375 cells were pre-incubated with protease inhibitors of caspase 3 (DEV-FMK, 20 μM), caspase 8 (IETD, 20 μM), or both for 3 h, and their effect on cell survival following Fas treatment for 24, 48, or 72 h was examined. As shown in Table I, both inhibitors blocked Fas-mediated cell death, suggesting that both caspases 3 and 8 are involved in the Fas signaling pathway in melanoma cells.

Stimulation of PITSLRE Kinase Activity in A375 Cells fol-
lowing Treatment with Anti-Fas and Staurosporine Is Markedly Reduced by Protease Inhibitors of Caspases 3 and 8—To further study the involvement of PITSLRE kinases in anti-Fas- and staurosporine-mediated apoptosis, we examined whether protease inhibitors of caspases 3 and 8 had any effect on preventing the stimulation of PITSLRE kinase activity during anti-Fas- or staurosporine-induced apoptosis in melanoma cell lines. A375 and UACC 1227 cells were treated with anti-Fas mAb (0.5 μg/ml) for 24, 48, or 72 h, stained with 7AAD, and analyzed for apoptosis by flow cytometry. Results are expressed as the mean percentage of apoptotic cells ± S.D. of at least three experiments (p < 0.005). Cellular Localization of PITSLRE p110 Isoforms in Human Melanoma Cells—PITSLRE p110 isoforms are ubiquitously expressed in proliferating cells. However, the localization of p110 PITSLRE isoforms and their function(s) in normal and transformed melanoma cells are unknown. To determine whether there were differences in the cellular localization of PITSLRE isoforms in the melanoma cell lines A375 and UACC 1227 relative to normal melanocytes and whether there is a link between the localization of PITSLRE isoforms and apoptotic signaling, immunofluorescence confocal microscopy analysis was performed. The results demonstrate that p110 PITSLRE isoforms localize to the nucleus in normal melanocytes and A375 cells, as detected by using the PITSLRE specific antibody P2N100 which recognizes the p110α and β isoforms (Fig. 8, A and B). Conversely, in UACC 1227 cells, p110 PITSLRE isoforms have a cytoplasmic localization (Fig. 8C). These results clearly demonstrate that the localization of p110 isoforms in UACC 1227 cells is different from that of normal melanocytes. In addition, because UACC 1227 cells are resistant to Fas-mediated apoptosis, nuclear localization of p110 isoforms may be necessary for the complete activation of the Fas signaling pathway.

DISCUSSION

Previous studies in our laboratory using fluorescence in situ hybridization indicated that one allele of the PITSLRE gene complex on chromosome 1 was either deleted or translocated in several melanoma cell lines. Furthermore, the expression of

Fig. 3. Fas-induced apoptosis in melanoma cells. A, scattergrams of 7AAD-stained cells. Untreated control A375 cells and A375 cells treated with 0.5 μg/ml anti-Fas mAb for 24, 48, and 72 h. FSC, forward light scatter; FL3, 7AAD fluorescence; Apop, apoptotic; R1, live cells; R2, early apoptotic cells; R3, late apoptotic and dead cells. B, time course of anti-Fas mAb-induced apoptosis in melanoma cell lines. A375 and UACC 1227 cells were treated with anti-Fas mAb (0.5 μg/ml) for 24, 48, or 72 h, stained with 7AAD, and analyzed for apoptosis by flow cytometry. Results are expressed as the mean percentage of apoptotic cells ± S.D. of at least three experiments (p < 0.005). C, morphological analysis of A375 and UACC 1227 cells treated with anti-Fas mAb (0.5 μg/ml) for 72 h. Magnification ×100. a, A375 nontreated cells; b, A375 cells treated with anti-Fas; c, UACC 1227 nontreated cells; d, UACC 1227 cells treated with anti-Fas mAb.
PITSLRE proteins from the remaining allele was decreased in several melanoma cell lines and surgical melanoma specimens (21). Similar results have been observed in neuroblastoma and childhood endodermal sinus tumors (20). However, the functional consequences of genetic alterations within the Cdc2L locus encoding the PITSLRE kinases in regards to the development of melanoma are not known. Because the PITSLRE p110 isoforms may be involved in apoptosis, we wanted to evaluate the functional consequences of PITSLRE gene alterations with regard to Fas-mediated apoptotic signaling in melanoma cell lines.

In the present study, we provide evidence that the melanoma cell lines A375 and UACC 1227 express Fas receptor and that anti-Fas mAb induces apoptosis in A375, but not in UACC 1227 cells. Con. 24, 24-h nontreated control; Fas 24, 24-h anti-Fas; Con. 48, 48-h control; Fas 48, 48-h anti-Fas; Con. 72, 72-h control; Fas 72, 72-h anti-Fas. Relative kinase activity from the phosphorylated histone H1 band was determined by phosphoimaging, as described under “Materials and Methods.”

PITSLRE proteins from the remaining allele was decreased in several melanoma cell lines and surgical melanoma specimens (21). Similar results have been observed in neuroblastoma and in childhood endodermal sinus tumors (20). However, the functional consequences of genetic alterations within the Cdc2L locus encoding the PITSLRE kinases in regards to the development of melanoma are not known. Because the PITSLRE p110 isoforms may be involved in apoptosis, we wanted to evaluate the functional consequences of PITSLRE gene alterations with regard to Fas-mediated apoptotic signaling in melanoma cell lines.

In the present study, we provide evidence that the melanoma cell lines A375 and UACC 1227 express Fas receptor and that anti-Fas mAb induces apoptosis in A375, but not in UACC 1227 cells. Morphological changes consistent with apoptosis including cell shrinkage, nuclear condensation, and membrane blebbing were observed in the A375 Fas-sensitive cells, but not in the UACC 1227 Fas-resistant cells.

We demonstrate that the A375 melanoma cells, which have normal Cdc2L alleles and normal PITSLRE protein expression, are sensitive to Fas-induced apoptosis. In contrast, UACC 1227 cells, which have decreased PITSLRE expression and mutant alleles, do not undergo Fas-induced apoptosis. In addition, an increase in PITSLRE kinase activity was observed in A375 Fas-sensitive cells, but not in the UACC 1227-resistant cell line. Stimulation of PITSLRE kinase activity was also observed in A375 cells following staurosporine treatment. The stimulation of PITSLRE kinase activity was markedly reduced (50–60%) by caspase inhibitors DEV-FMK or IETD during Fas- and staurosporine-mediated cell death. We also report the caspase cleavage of PITSLRE protein and PARP during Fas-induced apoptosis. These observations demonstrate that PITSLRE kinase activation is associated with Fas- and staurosporine-mediated apoptosis in melanoma cells. Furthermore, the data presented here suggest that multiple caspases appear to be involved in the cleavage of PITSLRE during Fas-induced apoptosis in A375 cells. Recently, it has been reported that PITSLRE kinases are specifically cleaved in response to TNF by caspases 1 and 3 resulting in the activation of the PITSLRE kinase, both in vivo and in vitro (27, 31). However, the proteases responsible for the processing and activation of PITSLRE kinases in melanoma and the importance of this processing in apoptosis are unknown. We also demonstrate that caspases 3 and 8 are involved in the Fas signaling pathway in melanoma cells, and caspase 3 is involved in staurosporine-mediated cell death, which is consistent with the published reports on PITSLRE p110 isoforms being cleaved and activated by caspases during apoptosis (27, 31).

Finally, in this study we demonstrate that there is a difference in the subcellular localization of p110 PITSLRE isoforms in UACC 1227 melanoma cells relative to normal melanocytes. This result suggests that UACC 1227 cells express PITSLRE proteins that either lack the nuclear translocation signal or that contain a point mutation affecting this region of the protein. The amino-terminal domain also contains several distinct regions that may specify nuclear localization and protein stability (32, 33). Current studies in our laboratory, involving polymerase chain reaction-SSCP and direct DNA sequence analysis, suggest that UACC 1227 cells have a mutation(s) in the nuclear localization signal, which may explain the abnormal cytoplasmic localization of p110 PITSLRE isoforms. Loss or inactivation of the nuclear translocation signal in UACC 1227 cells and consequent cytoplasmic localization of p110 PITSLRE isoforms. In addition, resistance to Fas suggests a functional role of PITSLRE protein kinases in mediating Fas-
induced apoptosis. Furthermore, if we consider a role for PITSLRE p110 isoforms in Fas-mediated signal transduction processes, altered distribution of PITSLRE kinases in transformed cells may contribute to the transformed phenotype by deregulating the processes of apoptosis, spliceosome formation, or assembly/disassembly of nuclear speckles, which is involved in the regulation of RNA splicing/transcription (26).

A question that remains unknown is the identity of the substrate that executes the final death sentence following activation of caspases. One candidate (or family of candidates) is the PITSLRE kinases. PITSLRE kinase proteins have been shown to be processed and activated in cells treated with anti-Fas and TNF (25, 27, 31). Circumstantial evidence suggests that, following the processing and activation of PITSLRE kinases, they may be released from associated restraining molecules to execute the final death sentence. However, this important role for the PITSLRE kinases as final executioners of apoptosis needs to be further investigated. The mechanism that we are proposing to explain the role of PITSLRE protein kinases during apoptosis in malignant melanoma cells is shown in Fig. 9. Activation of Fas receptor by anti-Fas monoclonal antibody results in the aggregation and rapid recruitment of FADD (11). The interaction of FADD and Fas through their carboxyl-terminal death domains unmasks the amino-terminal death effector domain of FADD, allowing it to recruit and activate pro-caspase-8 to the Fas signaling complex (34), Caspase 8 activates pro-caspase 3 either directly (pathway 2) or indirectly through cytochrome c release from the mitochondria (pathway 1). Cytochrome c forms a complex with apoptotic protease activating factor 1 (Apaf-1) that binds and activates pro-caspase 9 (35). Activated caspase 9 binds to and activates pro-caspase 3. Our data indicate that PITSLRE kinase activation precedes Fas-induced apoptosis, and it is a downstream event. Because PITSLRE kinases have cleavage sites for caspases 3 and 8 and it has been shown that PITSLRE p110 isoforms are cleaved by these caspases during Fas-induced apoptosis in Jurkat cells, we are proposing that PITSLRE kinases get cleaved and activated by caspases 3 and 8 in melanoma cells. Activation of PITSLRE protein kinase results in the phosphorylation and activation of unknown downstream substrates and subsequent transcription of genes involved in

![FIG. 6. Stauroporine-induced apoptosis. A, dose-response curve of staurosporine-induced cell death in A375 and UACC 1227 cells for 72 h by standard MTT assay. B, time course of staurosporine-induced apoptosis. Cells were treated with 10 ng/ml staurosporine, incubated with 7AAD, and analyzed for apoptosis by flow cytometry. C, morphological analysis of A375 and UACC 1227 cells treated with staurosporine (10 ng/ml) for 72 h. Magnification ×100. a, A375 nontreated cells; b, A375 cells treated with staurosporine; c, UACC 1227 nontreated cells; d, UACC 1227 cells treated with staurosporine.](image)

![FIG. 7. Stimulation of PITSLRE kinase activity during anti-Fas mAb- or staurosporine-induced apoptosis is partially blocked by caspases 3 and 8 inhibitors in A375 cells. Histone H1 was used as the substrate for the assays. Autoradiograph of histone H1 kinase activity at 48 h following treatment with anti-Fas mAb, staurosporine, and/or caspase 8 and caspase 8 inhibitors. Relative kinase activity from the phosphorylated histone H1 band was determined by phosphoimaging.](image)
the final stages of apoptosis. Staurosporine-induced cell death is also another operational apoptotic pathway present in malignant melanoma cells and is shown in Fig. 9. The fact that staurosporine-induced cell death, which bypasses receptor-mediated signaling, is altered in UACC 1227 cells suggests that PITSLRE kinases are involved in the staurosporine death signaling pathway. Furthermore, these data demonstrate that PITSLRE protein kinases are involved in Fas-, staurosporine-, and DR3-mediated cell death signaling pathways. However, the function of these PITSLRE kinases during apoptosis is not known.

Finally, the data presented here suggests that alterations in Cdc2L gene expression and protein localization can result in the loss or deregulation of apoptotic signaling pathway(s). Therefore, altered PITSLRE kinases may represent a different mechanism from that reported for Fas ligand that could contribute to the immune privilege in malignant melanoma (36).

Deregulation of apoptotic signaling pathways may represent a mechanism to enhance tumorogenesis by preventing the elimination of these cells through normal checkpoint control.

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