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PITSLRE Protein Kinase Activity Is Associated with Apoptosis

JILL M. LAHTI,[1] JIALING XIANG,[1,2] LUCIE S. HEATH,[3] DARIO CAMPANA,[4] AND VINCENT J. KIDD[1]*

Departments of Tumor Cell Biology[1] and Hematology/Oncology,[4] St. Jude Children's Research Hospital, Memphis, Tennessee 38105, and Departments of Cell Biology[2] and Microbiology,[3] University of Alabama at Birmingham, Birmingham, Alabama 35294

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Minimal ectopic expression of a 58-kDa protein kinase (PITSLREβ1), distantly related to members of the *cdc2* gene family, induces telophase delay, abnormal chromosome segregation, and decreased growth rates in Chinese hamster ovary cells. Here we show that this decrease in cell growth rate is due to apoptosis. Apoptosis is also induced by ectopic expression of an amino-terminal deletion mutant containing the catalytic and C-terminal domains of PITSLREβ1 but not by other mutants lacking histone H1 kinase activity or by other members of the *cdc2* gene family. However, unlike the wild-type PITSLREβ1 overexpressors, ectopic expression of the N-terminal PITSLREβ1 mutant does not result in telophase delay or abnormal chromosome segregation. These results suggested that the function of this protein kinase could be linked to apoptotic signaling. To test this hypothesis, we examined levels of PITSLRE mRNA, steady-state protein, and enzyme activity in human T cells undergoing apoptosis after activation with the anti-Fas monoclonal antibody (MAb). All were substantially elevated shortly after Fas MAb treatment. In addition to new transcription and translation, proteolysis contributed to the increased steady-state levels of a novel 50-kDa PITSLRE protein, as suggested by the diminution of larger PITSLRE isoforms observed in the same cells. Indeed, treatment of the Fas-activated T cells with a serine protease inhibitor prevented apoptotic death and led to the accumulation of larger, less active PITSLRE kinase isoforms but not the enzymatically active 50-kDa PITSLRE isoform. Finally, induction of apoptosis by glucocorticoids in the same cell line, as well as by Fas MAb treatment of another T-cell line, led to a similar induction of 50-kDa PITSLRE protein levels over time. These findings suggest that (i) PITSLRE kinase(s) may lie within apoptotic signaling pathway(s), (ii) serine protease activation may be an early event in Fas-activated apoptosis of human T cells, and (iii) some PITSLRE kinase isoforms may be targets of apoptotic proteases.

Programmed cell death (PCD), or apoptosis, involves the activation of a specific suicide program within a cell (8, 18, 43, 44, 46, 58). Apoptosis is responsible for such diverse activities as the elimination of cells during normal embryological development and determination of the immune receptor repertoire (8, 37, 47). Apoptosis can be triggered in multiple ways, but it is not yet known whether different inducers of apoptosis have a common pathway or whether there are multiple pathways with, perhaps, some common component(s) (30, 59).

Strong similarities between some of the early events of mitosis and PCD (i.e., nuclear envelope dissolution), as well as a potential link between cell cycle checkpoint control and apoptosis, suggest possible associations between cell cycle regulators and mediators of apoptosis (26, 32, 34, 39). Indeed, the $p34^{cdc2}$ kinase has recently been shown to be associated with the initiation of PCD (49). While the function of most $p34^{cdc2}$-related cell cycle kinases appears to be prominently linked to the control of cell division, the role of others remains unclear (11, 16, 17, 22, 34, 39, 55, 57). Minimal ectopic expression of one member of the $p34^{cdc2}$ gene family, the p58 (PITSLREβ1) protein kinase, in eukaryotic cells resulted in the failure of these cells to undergo normal cytokinesis as well as an apparent mitotic delay at late telophase (7). These transformants were also found to contain approximately 20-fold more multinucleated cells, and/or micronuclei, than their parental counterparts. When the rate of cell growth was measured in the PITSLRE overexpressors, the cell doubling time was also increased three- to fourfold and DNA replication was diminished. These results suggested that PITSLRE protein kinase function was somehow linked to normal regulation of the cell cycle, possibly as a negative regulator in checkpoint control during mitosis or cytokinesis (7).

Recently, 10 isoforms of the p58 PITSLRE subfamily of protein kinases have been isolated by molecular cloning (7, 61). The discovery of multiple p58 isoforms has led to the renaming of these kinases according to an established nomenclature system which is based on the single amino acid codon designation of the conserved PSTAIRE box region of $p34^{cdc2}$ (39). Furthermore, depending on which of the three PITSLRE genes produces the protein, the cDNA and protein are designated α, β, or γ (i.e., PITSLRE A gene, α; PITSLRE B gene, β; and PITSLRE C gene, γ). Some of the isoforms, such as PITSLREα1 (T cells) and PITSLREβ1 (B cells and brain), are expressed in specific cell types, while the others are expressed ubiquitously (61).

The locus expressing these kinases consists of three duplicated and tandemly linked genes spanning approximately 90 kb (28). Two of these genes are almost complete duplications (including both exons and introns), and both express nearly identical products. The sequence of the third gene diverges completely outside of the conserved catalytic and C-terminal domains. These genes are located on human chromosome 1p36.3, a region that is deleted in neuroblastoma and many other human tumors (3, 4, 13, 14, 21, 28, 35, 50, 53). Deletion of this chromosome region occurs late in oncogenesis and is correlated with aggressive tumor growth, suggesting that one or more tumor suppressor genes may reside here. In a study of human neuroblastoma cell lines, deletion and/or translocation of one allele of the PITSLRE gene locus was observed in all cell lines with 1p36 alterations and N-*myc* gene amplification

* Corresponding author. Phone: (901) 522-0597. Fax: (901) 531-2381.

(28). Abnormalities in the expression of PITSLRE polypeptides from the remaining allele were apparent in several of these cell lines as well.

Here we show that Chinese hamster ovary (CHO) cells engineered to express the PITSLREβ1 protein kinase, as well as structural mutants, induce apoptosis when ectopically expressed. These data led to the hypothesis that some of the PITSLRE isoforms might serve as effectors of an apoptotic signaling pathway(s). This hypothesis was tested by using human T-cell lines that could be triggered to undergo apoptosis by using a Fas monoclonal antibody (MAb) (54). PITSLRE kinase mRNAs, steady-state protein levels, and enzyme activity were found to increase dramatically upon activation of Fas. In addition to induction of PITSLRE transcription and translation, proteolysis of specific, larger PITSLRE isoforms contributed significantly to increased steady-state levels of a novel, processed 50-kDa PITSLRE isoform during Fas-induced PCD. Finally, similar induction of the 50-kDa PITSLRE isoform was observed when apoptosis was induced by glucocorticoids in these cells or by the Fas MAb in another human T-cell line. These results suggest that several of the PITSLRE protein kinase isoforms may function as effectors in an apoptotic signaling pathway(s) and that this activity is mediated by this novel processed 50-kDa isoform.

MATERIALS AND METHODS

Deletion mutagenesis and antibody production. Site-directed mutagenesis of K111N and D224N was performed with oligodeoxynucleotides 5′-GTGGCTC TAAACCGGCTGAAC-3′ and 5′-AAGGTGGGTAACTTCGGGCTG-3′, respectively (64). Deletion of the N-terminal domain of PITSLREβ1 (corresponding to amino acids 1 to 74) was performed by PCR amplification using the oligodeoxynucleotides 5′-GATGCTGCAGGGCTGCCGGAGCGTC-3′ corresponding to nucleotides 204 to 224 of human PITSLREβ1 (7, 15). The testis-specific lactate dehydrogenase (LDH) tag (41) was added to the C-terminal end of PITSLREβ1, using the oligodeoxynucleotide 5′-GC TTC AGC CTC AAG TTC CCA TGG TGT GAG CAG CTC ATC GAG AAG CTG ATC GAG GAC GAG TGAAG GTCAGAGTGGAC-3′, with the underlined nucleotides corresponding to amino acids from the C-terminal end of the protein and nucleotides from the 3′ untranslated region of PITSLREβ1 (7, 15). The intervening nucleotides encode the LDH peptide sequence EQLIEKLIEDDE, which was fused, in frame, to the C-terminal end of PITSLREβ1, and to which the affinity-purified LDH antiserum was prepared as described previously (40). All mutated DNAs were sequenced for confirmation. PITSLRE antibody production and characterization have been reported previously (61). Three of these PITSLRE antibodies, GN1, GC41, and PP8, were used in these studies. The GN1 antibody corresponds to amino acid residues 1 to 72 of PITSLREβ1, the GC41 antibody corresponds to amino acid residues 232 to 249 of PITSLREβ1, and the PP8 antibody corresponds to a synthetic peptide corresponding to amino acid residues 121 to 137 of PITSLREβ1 (61).

Cell culture, expression of PITSLRE constructs, flow cytometry, and chromosome analysis. The PITSLRE constructs were cloned into the *Xba*I restriction site of pMT-*neo*, which follows the metallothionein promoter and precedes a poly(A) addition sequence from simian virus 40 (12). CHO(dhfr$^-$) cells were cultured and electroporated as previously described (7, 56). Cells were selected in 500 mg of G418 per ml for 3 weeks and then maintained in 400 mg/ml. All experiments were performed on cells grown in medium lacking heavy-metal supplements, because of the toxicity of the induced gene product. Flow cytometric analysis of the CHO cells and transfectants was performed by analyzing approximately 50,000 cells from polyclonal populations for each sample (36). The phenotype analysis of the human T-cell lines was performed by staining with commercially available antibodies for various cell surface and intracellular markers followed by fluorescence-activated cell sorting (FACS) analysis. For the growth curves, 4×10^4 cells were plated in triplicate and grown in the presence of 10% serum. Each point represents the average of three determinations ± standard error of the mean. Human CEM-C7 and Jurkat T cells were grown at a density of 2×10^6 cells per ml and treated with a Fas MAb monoclonal antibody (Upstate Biotechnology, Inc.) at 100 ng/ml. CEM-C7 cells were treated with 5×10^{-5} M dexamethasone as described by others (23). For nuclear staining, cells were fixed on coverslips, treated with Hoechst 33258, and then photographed. For chromosome analysis, cells were blocked in mitosis with 0.1 μg of colcemid per ml for 2 h. Metaphase chromosome spreads were prepared and stained with 0.05 μg of Hoechst 33258 per ml. Ploidy was determined by analysis of at least 100 chromosome spreads. For cells expressing the wild-type and ΔNH_2 PITSLREβ1 genes, chromosome numbers were determined for both single cell clones and polyclonal populations. The results from the polyclonal population are shown.

Kinase assays, Western blot (immunoblot) analysis, and Northern (RNA) blot analysis. PITSLRE protein kinase activity was measured by immunoprecipitation of 200 μg of whole cell protein extracts, using either the affinity-purified LDH antibody to detect the exogenously expressed and tagged kinase molecules or one of the three affinity-purified PITSLRE antibodies described previously (61). PITSLRE kinase activity was competed for with a 1 mM concentration of the peptide or 2 mg of fusion protein per ml. The LDH antibody was prepared as described by others (40). Kinase assays were performed as previously described (7). The CHO and CEM-C7 cell lysates were precleared with protein A beads (41), but some background kinase activity was still observed in the CHO cells because of the relatively long exposure times needed to observe kinase activity from these cells. The gels in Fig. 1b and c were exposed for 6 h at −80°C on Kodak X-AR5 film. Cerenkov counts were determined after excision of the histone H1 band from the gel to determine relative changes in kinase activity. Quantitation of PITSLRE protein kinase activities from the human T cells (assays in Fig. 3 and 4) was performed by use of phosphoimaging. The gels were visualized with a Molecular Dynamics 400A PhosphorImager, and the relative kinase activity was determined by quantitation of the labeled histone H1 bands by using the ImageQuant software. Cdk ($p34^{cdc2}$ and $p33^{cdk2}$) kinase activities were determined by binding to $p13^{suc1}$ beads as described by Arion and colleagues (1). Western blot analysis was performed with the PITSLRE GN1 antibody and a commercial $p34^{cdc2}$ polyclonal antibody (Santa Cruz, Inc.), using the ECL (enhanced chemiluminescence) reagent system and Hyperfilm (Amersham) as described elsewhere (7, 61). Equivalent numbers of cells (2×10^7) were collected and used for RNA isolations for each time point. RNA was isolated from the CEM-C7 cells as described previously (7, 61), with the exception of the addition of RNase-free DNase I (Boehringer Mannheim) to remove contaminating DNA from the later time points of Fas induction. Northern blot analysis was performed with the human p58 (PITSLREβ1) cDNA and a human β-actin cDNA (7). The probes were prepared by using a random labeling kit (Stratagene) as specified by the manufacturer. The blots were visualized by using a Molecular Dynamics 400A PhosphorImager. Exposure times were 2 h for the PITSLRE cDNA and 20 min for the β-actin cDNA.

Determination of apoptotic phenotype and treatment of cells with TPCK. For the DNA gel analysis, 5×10^5 cells were lysed and the extracted DNA (51) was electrophoresed on 1.5% agarose–1× Tris-borate-EDTA gels and stained with ethidium bromide. Fluorescence labeling of nicked DNA and analysis of cells by using in situ nick translation was performed as described by Gold et al. (24). *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) was added at a final concentration of 250 μM as described by others (45). Human CEM-C7 and Jurkat T cells were treated with TPCK either in the presence or in the absence of the Fas MAb. Equivalent numbers of cells (10^6) were collected for each time point. Protein was isolated and protein kinase assays were performed as previously described (7, 61).

RESULTS

Analysis of CHO cells ectopically expressing wild-type and mutant PITSLREβ1 kinases. Previous studies indicated that minimal ectopic expression of wild-type p58 (PITSLREβ1) protein kinase in CHO cells resulted in a dramatic phenotypic change involving late telophase delay, decreased cell growth rates, and abnormal chromosome segregation (7). PITSLREβ1 is a member of a larger subfamily of protein kinases, with at least 10 p58 (PITSLRE) isoforms generated by alternative splicing from three duplicated and tandemly arrayed human genes (28, 61).

Most of the differences between these various PITSLRE isoforms are found at the N-terminal end of the molecule. For this reason, a series of PITSLREβ1 mutants (Fig. 1a), synthesized with an LDH tag at the C terminus, were introduced into cells and assayed for kinase activity and effects on cell growth (Fig. 1). Cells transfected with wild-type PITSLREβ1 had elevated kinase activity (Fig. 1b, lane 1) which was approximately the same as the kinase activity from cells transfected with an N-terminal deletion mutant (ΔNH_2 PITSLREβ1) (Fig. 1b, lane 3). Although only low levels of protein kinase activity were observed, histone H1 was found to be a better substrate for the enzyme than casein, myelin basic protein, and several high-mobility-group proteins. Both RNAs and proteins from all of the transfected PITSLRE constructs (wild type and mutant) were expressed at equivalent low levels, as judged by reverse transcription-PCR and immunoprecipitation of labeled proteins with the same antiserum (data not shown). Two other

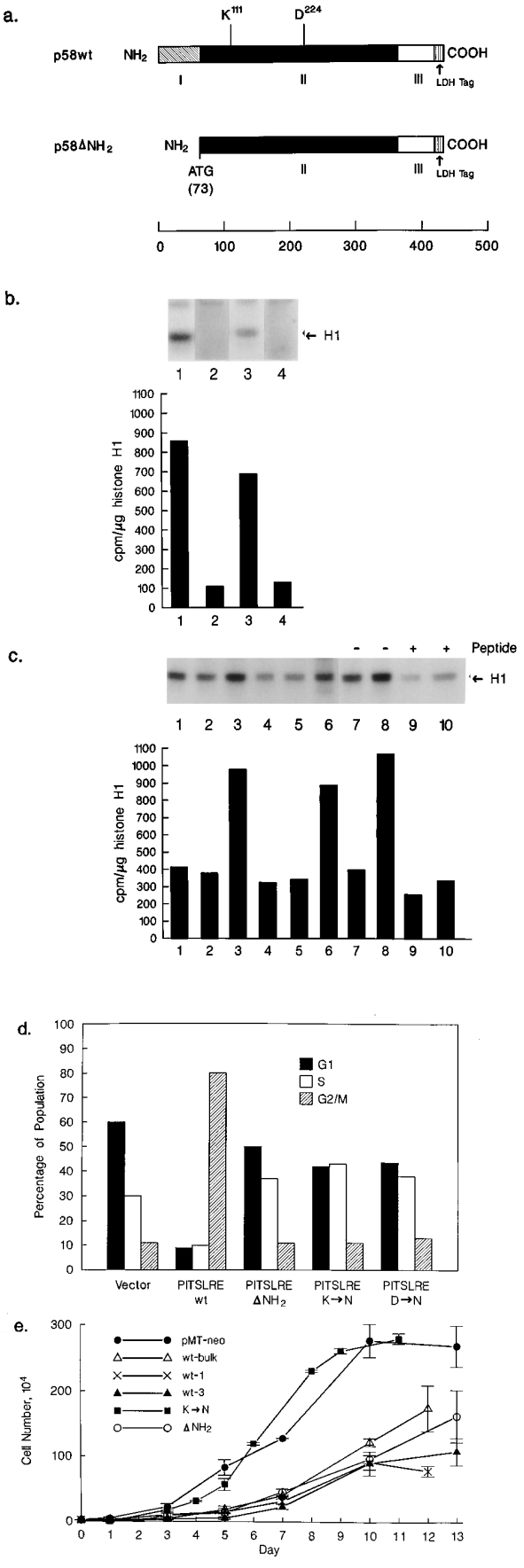

FIG. 1. Constructs expressed in CHO cells and analysis of their growth. (a) Shown are the location of the LDH tag, the positions of the mutated lysine (K^{111}) and aspartic acid (D^{224}) residues, and the location of the translation initiation codon for the ΔNH_2 construct. The scale below indicates length in amino acids. p58wt, wild-type p58. (b) Kinase activity associated with LDH-tagged PITSLRE-1 kinases. Shown are anti-LDH precipitation and kinase assay of LDH-tagged protein from cells expressing the wild-type gene (lane 1), K111N mutant (lane 2), ΔNH_2 mutant (lane 3), and pMT-*neo* (lane 4). Quantitation by Cerenkov counts of excised bands is shown below the autoradiograph. (c) PITSLRE protein kinase activity in parental CHO cells and cells expressing the wild-type gene. A peptide antibody (PP8) made to the conserved peptide surrounding the PITSLRE box was used to assay PITSLRE kinase activity from parental CHO cells (lanes 1, 7, and 9) and cells transfected with pMT-*neo* (lane 2), the wild-type gene (lanes 3, 8, and 10), the D224N mutant (lane 4), the K111N mutant (lane 5), and the ΔNH_2 mutant (lane 6). The presence (+) or absence (−) of peptide antigen is indicated. Quantitation by Cerenkov counts of excised bands is shown below the autoradiograph. (d) Cell cycle analysis of asynchronous populations of the indicated nonclonal transfected cell lines. (e) Growth curves of the cell lines used for panel d as well as two additional subclones of the wild-type kinase (wt-1 and wt-3).

PITSLREβ1 point mutants (K111N and D224N) lacked kinase activity (Fig. 1b, lane 2; Fig. 1c, lanes 4 and 5), and they did not induce the phenotypic changes observed in the cells expressing either the wild type or the ΔNH_2 mutant (data not shown).

Under all conditions tested, an antiserum to a synthetic peptide comprising the PITSLRE motif of PITSLREβ1 also precipitates a relatively low level of histone H1 kinase activity from CHO cells (Fig. 1c, lanes 1 and 7), which is increased only modestly (about twofold) in cells transfected with an expression vector encoding full-length PITSLREβ1 (Fig. 1c, lanes 3 and 8). Competition with the synthetic peptide reduced the activity to background levels (lanes 9 and 10). These results suggested that high levels of kinase activity were not tolerated by normal cells or those engineered to overproduce the enzyme. Nonetheless, overproducer cells exhibit a characteristic phenotype involving a marked telophase delay, formation of tubulin midbodies, mitotic abnormalities and aneuploidy, abnormal cytokinesis, and retarded cell growth (7) (see below).

Cells transfected with the wild-type gene collected in telophase had an abnormally high DNA content (Fig. 1d) and grew slowly (Fig. 1e). By contrast, those expressing ΔNH_2 PITSLREβ1 had a DNA content characteristic of normal CHO cells (Fig. 1d) but still proliferated at a reduced rate (Fig. 1e). The basis for these differences cannot definitely be established at this time, but one possibility is that deletion of the N-terminal end of the PITSLREβ1 kinase dissociated its effects on mitosis and cell growth. Closer examination revealed that cells expressing both the wild-type and ΔNH_2 PITSLREβ1 cDNAs undergo apoptosis, as indicated by fragmented and condensed nuclei (Fig. 2b) and DNA ladders (Fig. 2c, lanes 1 and 2). These nuclear and DNA changes were not observed in cells expressing the vector alone or other kinase-defective PITSLREβ1 mutants (Fig. 2c). Apoptosis was not induced in the CHO cells by ectopic expression of several other $p34^{cdc2}$ family members, including $p34^{cdc2}$ itself, cdk4, and PCTAIRE-3, in agreement with previous reports (20, 26). However, it has been shown that cotransfection of both $p34^{cdc2}$ and its regulatory partner, cyclin B, can lead to premature mitosis, resulting in DNA fragmentation and cell death (26). In addition, it has recently been shown that activation of the $p34^{cdc2}$ kinase is a very early event in apoptosis, induced by a lymphocyte granule protease (49). The mechanism of protease activation of $p34^{cdc2}$ in these experiments is unknown, since this kinase was not shown to be a target of specific proteolysis. Additionally, transient expression of either the PITSLREα1 or PITSLREβ2 cDNA in CHO cells does not appear to induce apoptosis (62). Also, unlike expression levels of the PITSLREβ1 and ΔNH_2

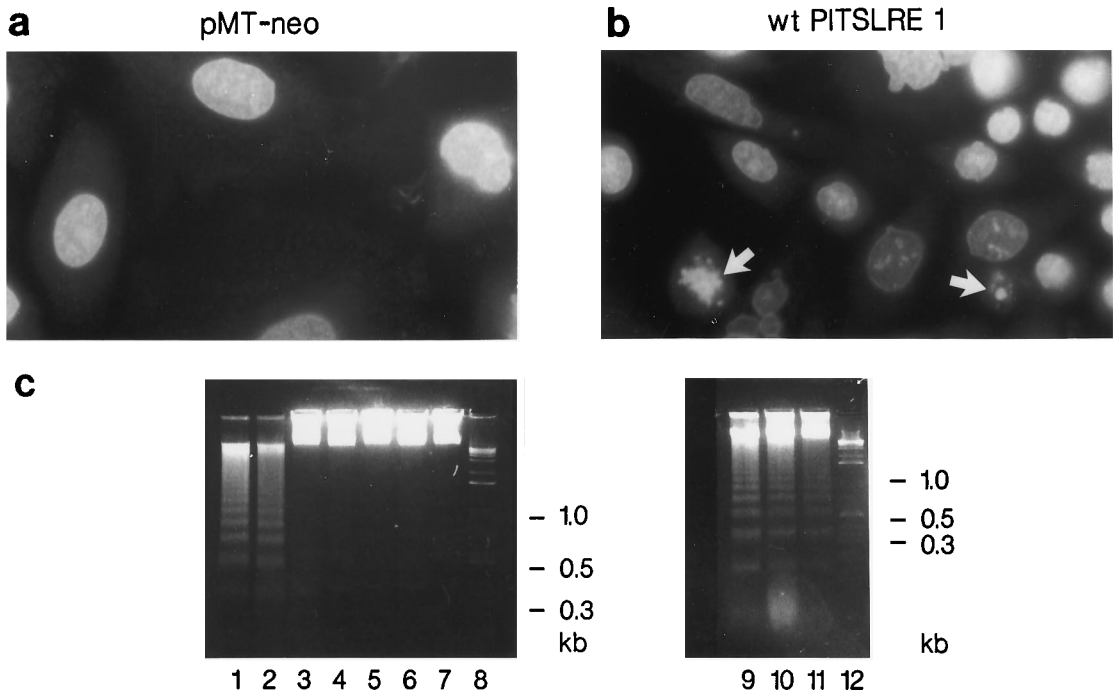

FIG. 2. Characterization of apoptosis. (a and b) Hoechst staining of nuclei from cells expressing the vector (pMT-*neo*) (a) or wild-type (wt) PITSLREβ1 gene (b). Arrows indicate apoptotic nuclei. (c) Agarose-gel electrophoresis of genomic DNA isolated from cells expressing the wild-type PITSLREβ1 gene (lane 1), the ΔNH_2 mutant (lane 2), the K111N mutant (lane 3), the D224N mutant (lane 4), PCTAIRE-3 (lane 5), $p34^{cdc2}$ (lane 6), or pMT-*neo* (lane 7). DNA marker is shown in lane 8. In the lanes at the right, cells expressing the wild-type gene were cultured in 10% serum (lane 9), 5% serum (lane 10), or 0.5% serum (lane 11). DNA markers are shown in lane 12. (d) Analysis of chromosome number of the various transfected CHO cell lines.

PITSLRE proteins, which were very low and not readily detectable by Western blotting without immunoprecipitation, both the PITSLREα1 and PITSLREβ2 proteins could be expressed, at least transiently, at very high levels by using an expression vector containing a cytomegalovirus promoter. Thus, only the shorter PITSLREβ1 and ΔNH_2 PITSLRE mutant induce apoptosis in these cells.

Continuously grown CHO cells overexpressing the wild-type PITSLREβ1 gene rapidly become aneuploid, while those overproducing the ΔNH_2 PITSLREβ1 kinase do not (Fig. 2d). Therefore, the ΔNH_2 mutant induced apoptosis in the absence of chromosomal segregation abnormalities. Apoptosis was observed in the presence of serum and was not potentiated by serum starvation (Fig. 2c, lanes 9 to 11), contrasting with data obtained from cells overexpressing c-*myc*, which die at accelerated rates in the absence of growth factors (2, 19).

Induction of PITSLRE mRNA, protein, and kinase activity during apoptosis. The results described above suggested that the PITSLRE kinase(s) might function in an apoptotic signaling pathway. To test this hypothesis, human CEM-C7 (immature T) cells, which express the Fas receptor and undergo apoptosis within 3 to 5 h of treatment with a MAb to Fas, were used to examine PITSLRE kinase regulation in response to apoptotic signals (54). Apoptotic death of the T cells treated with the Fas MAb was confirmed by using flow cytometry, which showed incorporation of fluorescein isothiocyanate (FITC)-labeled nucleotides (indicating DNA strand breaks) accompanied by the appearance of cells with subnormal DNA content, reduced volume, and increased granularity (Fig. 3A, right) (24, 51, 60). DNA ladders, also characteristic of apoptotic cells, were observed, albeit at a later time point than the DNA damage and cell size changes noted by the FACS analysis (Fig. 3A, left). Northern blotting of RNA from anti-Fas-treated CEM-C7 T cells revealed that PITSLRE mRNAs were induced within the first 30 min of treatment and reached maximal levels at 4 h (Fig. 3B). Because of the extensive sequence identity between PITSLRE kinase isoforms and the fact that all 10 of the known PITSLRE isoforms are encoded by two (3.5- and 3.7-kb) nearly identical mRNAs, we do not know the identity of the induced mRNA(s). However, Western blotting experiments, detailed below, using cells treated with the serine protease inhibitor TPCK address this point. Steady-state levels of a ~50-kDa PITSLRE protein species was markedly induced after 2 to 4 h of treatment, which is consistent with the earlier increases in steady-state PITSLRE mRNAs (Fig. 3B and C). This 50-kDa PITSLRE protein species migrates just below the PITSLREβ1 protein (Fig. 3C) and is also seen when a PITSLRE C-terminal antibody (GC41) is used (data not shown) (61). Additionally, as will be shown below, not all of the increase in steady-state p50 to p55 protein levels is due to new protein synthesis.

After 6 h of treatment with the Fas MAb, PITSLRE kinase activity had increased 16-fold, as measured by using the PITSLRE peptide antibody (Fig. 3D). A similar increase in PITSLRE kinase activity (~20-fold) was observed with a second PITSLRE antibody corresponding to the N-terminal domain of the kinase (GN1) but not with a PITSLRE antibody generated to much of the catalytic and C-terminal domains (GC41) (Fig. 3D). This latter result is most likely due to the potential inactivation of the protein kinase catalytic domain from which it is derived. The preimmune controls for this experiment were identical to the results obtained with the PITSLRE C-terminal antibody (Fig. 3D). Conversely, substantial increases in the steady-state level of $p34^{cdc2}$ were not observed, but the corresponding cyclin-dependent kinase activity

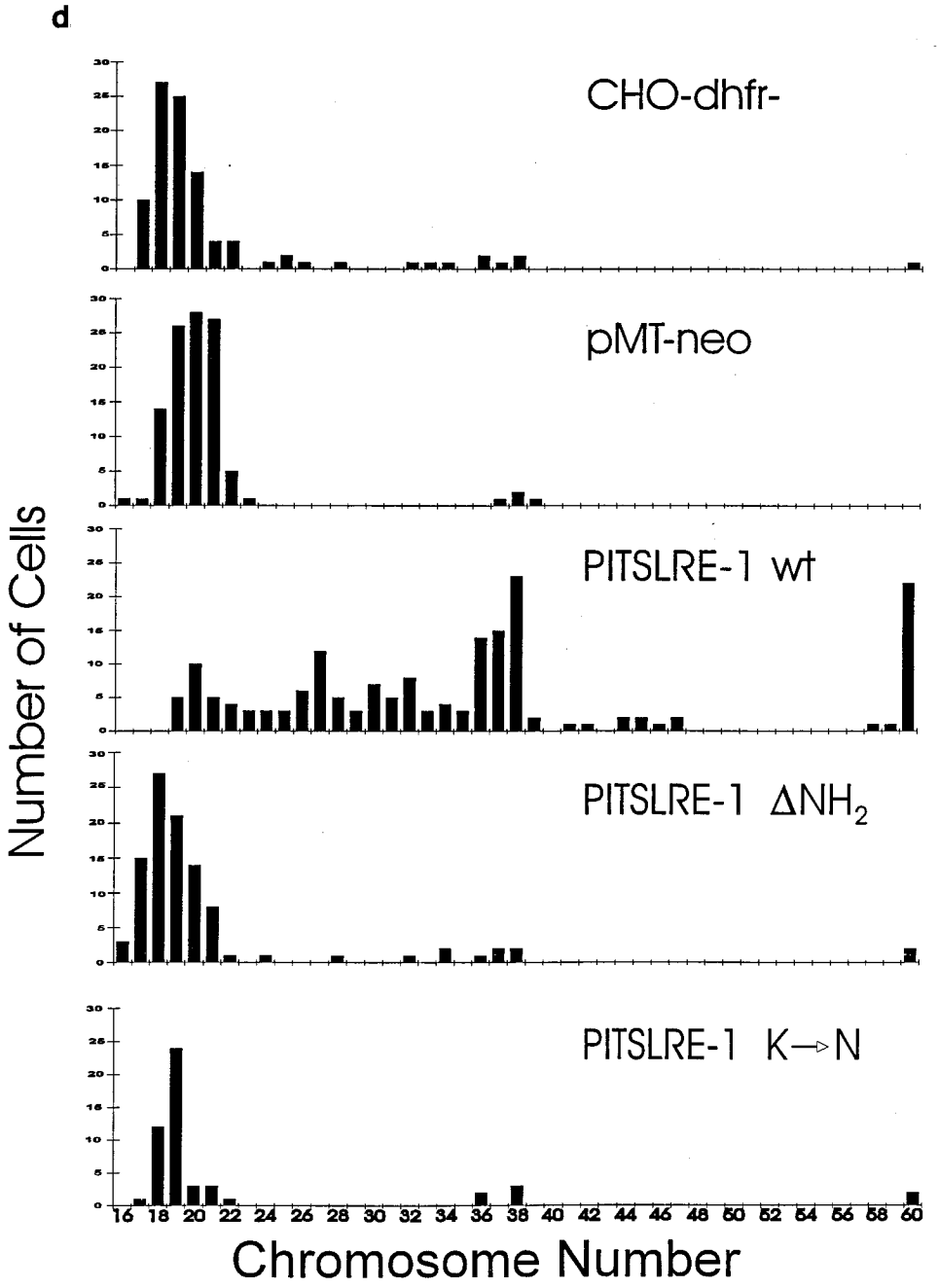

FIG. 2—*Continued.*

precipitated on p13^{suc1} beads was elevated ~2-fold (Fig. 3C and D). Studies with cytotoxic T-lymphocyte-mediated apoptosis have shown that p34^{cdc2}/cyclin activation occurs early on, apparently promoted by inappropriate Tyr-15 dephosphorylation (49). Thus, the increase that we observe in p13^{suc1}-associated kinase activity at later time points is consistent with these data and suggests that p34^{cdc2} or p34^{cdc2}-related kinase activity remains elevated for several hours in Fas-activated T cells.

The dramatic increase in steady-state PITSLRE protein levels in dying CEM-C7 cells was always accompanied by diminution of at least three additional PITSLRE isoforms, the 58-kDa (PITSLREβ1) isoform, the 65-kDa (PITSLREα1) isoform, and the 110-kDa (PITSLREα2-β2) isoforms (Fig. 3C). The PITSLREα2,β2 isoforms are ubiquitously expressed in growing cells, but the PITSLREα1 and PITSLREβ1 isoforms appear only after the CEM-C7 cells are induced to undergo apoptosis. The 65- and 58-kDa proteins can be faintly seen in Fig. 3C, but the PITSLREα1 isoform is much more pronounced in cells treated with a protease inhibitor (see below). In contrast, steady-state levels of a 90-kDa PITSLRE isoform, PITSLREα2-4, were not altered during the course of the experiment (Fig. 3C). Furthermore, when a PITSLREα2-1,β2-1-specific antibody was used, this 110-kDa protein species disap-

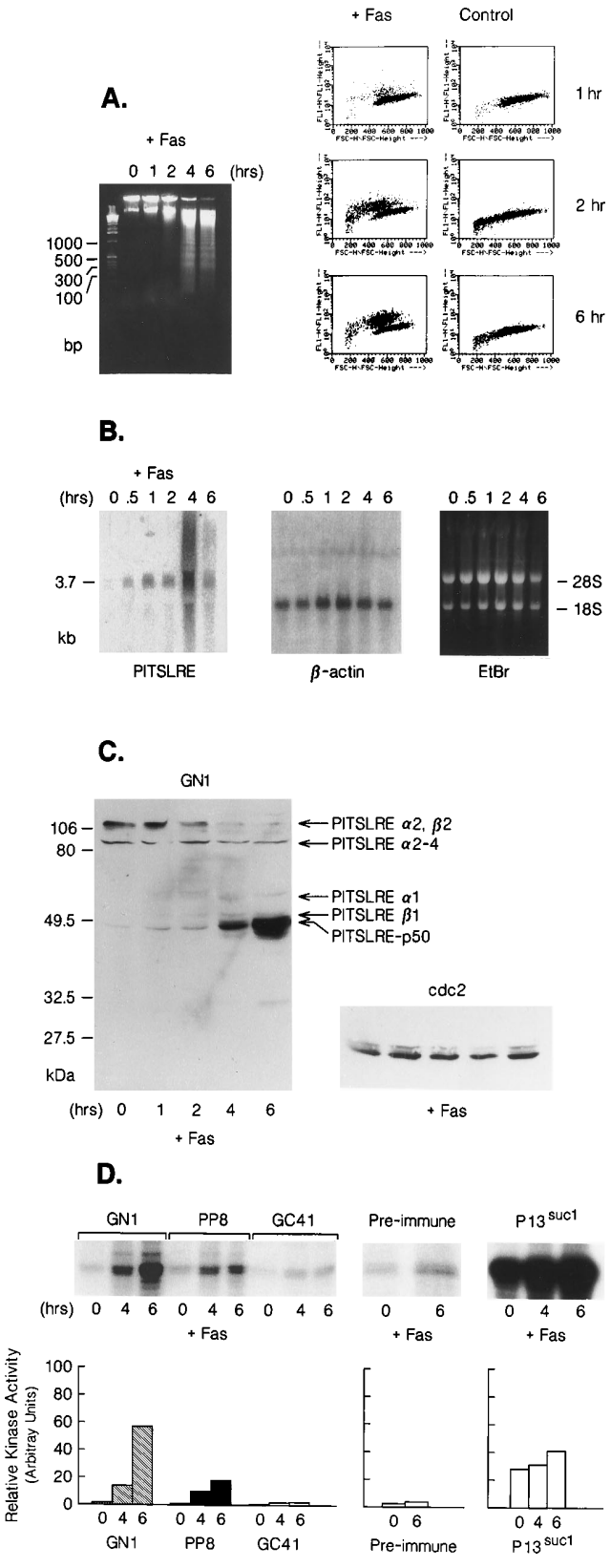

FIG. 3. Induction of PITSLRE kinase(s) during apoptosis in human CEM-C7 T cells. (A) Demonstration of apoptotic death of T cells treated with the Fas antibody. The left-hand panel shows the characteristic DNA ladder of apoptotic death from cells collected at various times following Fas antibody treatment. The right-hand panel shows flow cytometric analysis of the same cells either treated with or without Fas antibody. Forward scatter (FSC) (=cell size; *x* axis) and intensity of dUTP-FITC fluorescence (FL1; *y* axis) are illustrated. Incorporation of dUTP-FITC (indicating DNA strand breaks), seen as early as 1 h post-Fas treatment, and the decrease in cell size are typical of apoptosis. The proportion of apoptotic cells increases with time. (B) Northern blot analysis of PITLSRE kinase mRNA from Fas antibody-treated CEM-C7 cells. Thirty micrograms of total RNA isolated from control and Fas-activated T cells is shown in the right-hand panel stained with ethidium bromide (EtBr). This RNA was transferred to a Duralose membrane and sequentially hybridized with the indicated probes in the panels at the left. Time post-Fas activation is shown above each lane. The size of the PITSLRE mRNA is shown, as are the 28S and 18S rRNA species. (C) Western blot analysis of PITSLRE protein kinases and $p34^{cdc2}$ from control CEM-C7 cells and Fas-activated CEM-C7 cells. Total cellular protein (100 μg) was analyzed by Western blotting with the GN1 antibody and a commercial antibody to $p34^{cdc2}$. Identical gels were run simultaneously for this analysis. The times listed below the GN1 blot indicate cellular exposure time to the Fas antibody. These times are identical for the $p34^{cdc2}$ blot. Molecular weight markers are indicated on the left, and the positions of the various PITSLRE protein kinase isoforms detected by the GN1 antibody are shown on the right. A novel 50-kDa PITSLRE isoform was induced, just below the position indicated for p58 PITSLREβ1. (D) Kinase assays of preimmune and PITSLRE kinase (GN1, PP8, and GC41) immunoprecipitations from Fas-activated CEM-C7 cells corresponding to the indicated times. Kinase assays of $p13^{suc1}$-bound kinases were performed with the same cells as in panel A for the indicated times. Histone H1 was used as the substrate in all assays.

peared completely during Fas- and glucocorticoid-induced apoptosis (data not shown).

To determine whether proteolytic processing of any of the PITSLRE isoforms was occurring and contributing to the induction of the 50-kDa PITSLRE protein, the Fas-activated CEM-C7 cells were treated with TPCK, a serine-specific protease inhibitor (47). Cells treated with the Fas MAb in the presence of TPCK did not undergo substantial apoptosis (Fig. 4A). The use of cysteine protease inhibitors had no effect on either Fas-mediated death or processing of the PITSLRE kinases (data not shown). It is relevant that inhibitors of serine proteases prevent endonucleolysis accompanying apoptotic death in thymocytes (5). The induction of the 50-kDa PITSLRE protein and kinase activity were substantially reduced in Fas^+ $TPCK^+$ T cells, levels of the 110-kDa PITSLRE isoform were not decreased, and the induction of the 65-kDa PITSLRE kinase isoform was easily seen (Fig. 4B and C). This 65-kDa PITSLRE kinase (PITSLREα1) has been cloned and found to be virtually identical to the PITSLREβ1 protein, with the exception of 22 additional amino acids which are found at the N terminus and 4 distinct amino acid changes in the C terminus (61). When this PITSLREα1 cDNA was in vitro transcribed and translated, it produced a 65-kDa protein species when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (61). Expression of the 65-kDa PITSLREα1 isoform in these T cells is consistent with previous results demonstrating that its mRNA is expressed primarily in T cells, monocytes, and fibroblasts, whereas mRNA corresponding to the PITSLREβ1 isoform is expressed most abundantly in B cells and brain (61). Therefore, on the basis of our observations with TPCK, we designate the apoptotic PITSLRE protein species p50 PITSLRE to reflect the multiple isoforms that potentially contribute to its induction in these cells.

A smaller, but still substantial, increase in PITSLRE kinase activity was observed in CEM-C7 cells treated with TPCK (Fig. 4C). This did not substantially change when these same cells were simultaneously treated with TPCK and the Fas MAb (Fig. 4C). This TPCK-induced increase in kinase activity may be linked either directly or indirectly to posttranslational modification(s) of some or all of the PITSLRE isoforms (i.e., phosphorylation, proteolysis, etc.) which may be important for activation of these kinases. Since the larger PITSLREα2,β2

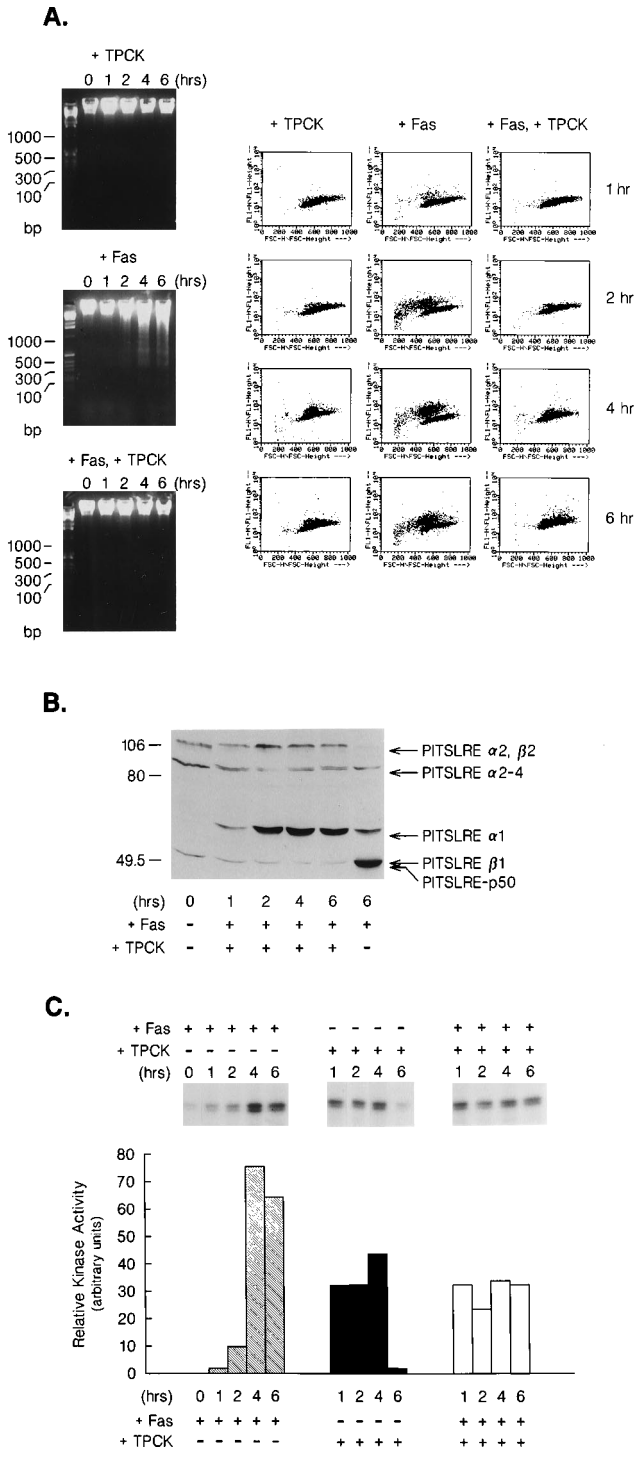

FIG. 4. Treatment of Fas-activated CEM-C7 cells with TPCK. (A) Demonstration of a block in apoptotic death of Fas-activated T cells in the presence of the serine protease inhibitor TPCK. The left-hand panel shows the characteristic DNA ladder of apoptotic death in Fas-activated T cells but not in T cells treated with TPCK alone or the Fas MAb plus TPCK. The right-hand panel shows flow cytometric analysis of the same cells either treated with TPCK only, Fas MAb only, or Fas MAb plus TPCK. Untreated control cell profiles were identical to those shown in Fig. 3. The *x* and *y* axes, as well as additional parameters, are exactly as described for Fig. 3. (B) Western blot analysis, using the GN1 antibody, of PITSLRE protein kinases from Fas-activated T cells in the presence or absence of TPCK, as indicated below the blot. Time post-Fas MAb treatment is also shown below each lane. The novel 50-kDa PITSLRE isoform does not accumulate, but the 65-kDa PITSLREα1 species that is only transiently seen in Fas-activated cells in the absence of TPCK is readily apparent. Additionally, the 110-kDa PITSLREα2 species does not decrease in intensity with Fas antibody treatment. Sizes are indicated in kilodaltons at the left. (C) Kinase assays of cell lysates from CEM-C7 cells treated with Fas MAb only, TPCK only, or Fas MAb plus TPCK. The presence or absence of each of these agents is indicated below the panel showing the phosphorylated histone H1 protein. Directly below is the quantitative analysis of this kinase activity for each treatment. The presence or absence of Fas MAb and/or TPCK is indicated.

isoforms are always present in the cell nucleus (61), it is possible that these isoforms are the source of the background kinase activity seen with TPCK. TPCK could alter the turnover rate of either the PITSLRE α2 or β2 isoform or of protein(s) responsible for posttranslational modifications that are important for their activity. At this time, we cannot definitively answer this question.

Induction of PITSLRE protein levels in glucocorticoid-treated CEM-C7 cells. One of the characteristics of the human CEM-C7 cell line is its ability to undergo apoptosis when treated with the glucocorticoid dexamethasone (6). However, apoptotic response to glucocorticoids is not nearly as rapid or complete as that seen when the same cells are treated with the Fas MAb (54). These changes are most likely due to unknown differences in the pathways or mechanisms of induction of apoptosis by these agents (9, 10, 37, 46). Such distinctions, however, provide an opportunity to examine whether PITSLRE protein kinases are altered in response to a different apoptotic signal. Because of the extended time course required for this experiment and the limited synchrony of the onset of apoptosis, only Northern blot analysis of PITSLRE RNAs and Western blot analysis of PITSLRE protein levels were performed (Fig. 5). Substantial apoptosis did not appear until 36 to 48 h after initiation of glucocorticoid treatment, as judged by the FACS-based assays and DNA ladder appearance (data not shown). No increase in the level of PITSLRE mRNAs was noted (Fig. 5A). The probe used on this Northern blot was identical to the one used on the Northern blot of the Fas MAb-treated cells. Conversely, we observed an increase in the steady-state level of the p50 PITSLRE protein by Western blotting with the GN1 antibody that was comparable to that seen in the Fas MAb-treated cells (Fig. 5 and 3). A substantial decrease in the PITSLREα2,β2 proteins was also observed in this experiment. These results are similar to the Fas-induced apoptosis and suggest that induction of the p50 PITSLRE kinase is a conserved feature of both PCD pathways.

Induction of PITSLRE protein levels and proteolytic processing in Jurkat cells. To determine whether induction of PITSLRE protein kinase(s) occurs in other T-cell lines that are responsive to Fas-mediated death, we examined apoptotic events in the human T-cell line Jurkat. A comparison of the cellular phenotype of CEM-C7 and Jurkat cells, with regard to well-characterized cell surface markers including the Fas receptor, was made (Table 1). Both cell lines express phenotypic features of human thymocytes. However, there are phenotypic differences which include the presence of cell surface CD3 T-cell receptor complex and nuclear terminal deoxynucleotidyltransferase in the Jurkat cells and their absence in CEM-C7 cells. Most importantly, the Fas receptor is present at similar levels in both cell lines. Western blot analysis of cellular proteins from Jurkat cells treated with the Fas MAb demonstrated in induction of steady-state p50 PITSLRE protein levels that is comparable to that seen in the CEM-C7 cells (Fig. 6A versus Fig. 4C). The time courses of this induction following Fas MAb treatment are also identical. In addition, the level of the PITSLREα2 protein decreased, PITSLREα1 was barely de-

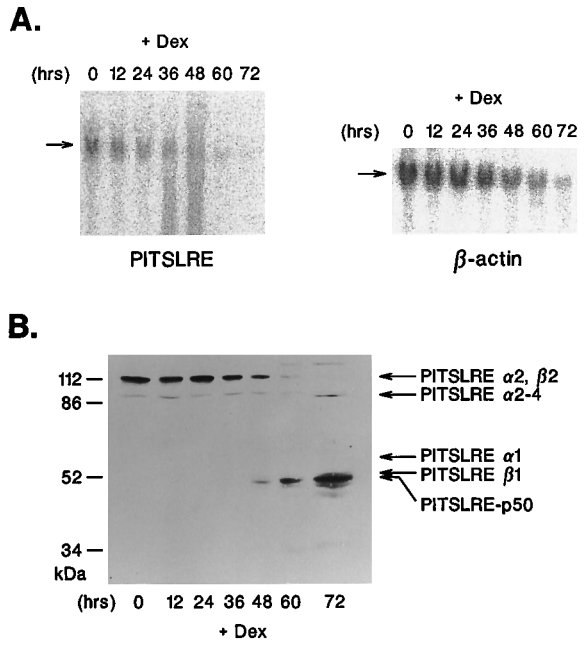

FIG. 5. PITSLRE protein expression in dexamethasone-treated CEM-C7 cells. (A) Human CEM-C7 T cells were treated with 5×10^{-5} M dexamethasone (Dex) for the time indicated above each lane. These cells were split into two tubes for either RNA or protein isolation. Total RNA (40 μg) was hybridized with the PITSLREβ1 cDNA probe. This blot was rehybridized with a β-actin control probe. (B) Total cell lysates (100 μg) were analyzed by SDS-PAGE and then Western blotted with the PITSLRE GN1 antibody. The sizes of the molecular weight standards are indicated on the left, and the positions of the various PITSLRE isoforms are indicated on the right. Induction of a novel 50-kDa PITSLRE isoform can be seen after ~48 to 60 h, and its position is indicated just below the p58 PITSLREβ1 isoform.

TABLE 1. Phenotypes of immature T-cell lines used in this study

Marker[a]	% of cells labeled[b]	
	CEM-C7	Jurkat
CD7	95	88
CD5	>99	>99
CD2	<1	>99
CD1a	23	97
CD4	>99	16
CD8	<1	<1
CD3 (surface)	5	98
TCRαβ (surface)	3	94
CD3 (cytoplasm)	>99	98
TCRβ (cytoplasm)	>99	95
TCRδ	<1	<1
TdT	<1	50
Fas	>99	83

[a] FITC-conjugated CD7 (Leu9), CD2 (Leu5b), CD5 (Leu1), CD4 (Leu3a), and isotype-matched nonreactive control antibodies were from Becton Dickinson (San Jose, Calif.); CD3 (UCHT1) and CD8 (DAKO-T8) were from Dakopatts (Carpinteria, Calif.); anti-T-cell receptor αβ (TCRαβ) (BMA031), anti-TCRβ (βF1), and anti-TCRδ (TCRδ1) were from T Cell Diagnostics (Cambridge, Mass.). Terminal deoxynucleotidyltransferase (TdT) was detected with a rabbit antiserum from Supertechs (Potomac, Md.) followed by a goat anti-rabbit immunoglobulin antiserum conjugated to tetramethyl rhodamine (Southern Biotechnology Associates, Birmingham, Ala.). CD1a (IOT2) was from AMAC (Westbrook, Maine), and anti-Fas was from Upstate Biotechnology (Lake Placid, N.Y.); their binding was visualized with an FITC-conjugated goat anti-mouse immunoglobulin antiserum (Jackson, West Grove, Pa.).

[b] Percentage of cells labeled by antibody compared with isotype-matched nonreactive control. TdT staining was examined by fluorescence microscopy; all other markers were analyzed with a FACScan flow cytometer (Becton Dickinson).

tectable, and steady-state PITSLREα2-4 protein levels were unaltered. Therefore, the overall pattern of PITSLRE protein expression was quite similar to what was observed in the apoptotic CEM-C7 cells.

When the Jurkat cells were treated with the Fas MAb in the presence of the serine protease inhibitor TPCK, the PITSLREα1 protein became readily detectable and the PITSLREα2 protein level was not affected (Fig. 6B). Furthermore, changes in the steady-state levels of the p50 PITSLRE protein species were not observed. These results are identical to what was observed when the CEM-C7 cells were treated with the Fas MAb in the presence of TPCK (Fig. 4C).

DISCUSSION

Careful examination of the phenotype of CHO cells ectopically expressing the PITSLREβ1 protein kinase revealed changes in growth parameters that were due to apoptosis. This helps to explain why stable CHO cell lines expressing either wild-type PITSLREβ1 or its N-terminal truncation mutant were difficult to establish, since even low levels of these forms of the kinase are lethal. However, we did establish stable cell lines expressing these protein kinases at very low levels. One possible explanation is the requirement for additional posttranslational modifications for kinase activation. In the wild-type PITSLREβ1 expressors, this may be due to, in part, posttranslational modifications such as proteolysis and/or phosphorylation. The N-terminal mutant may also require some of the same posttranslational modifications, such as phosphorylation, but perhaps not all. Additional removal of essential protein sequence at the N terminus may yield a kinase with reduced affinity for its substrate or substantially alter its in vivo activity. We have noticed that CHO cells expressing either of these kinases (β1 and the N-terminal mutant) do not die as readily when they come into contact with one another (27a). This finding argues that some signal for kinase activity is provided by the cellular environment and perhaps explains why these cells can grow, albeit poorly, in culture.

The ability of a PITSLREβ1 N-terminal mutant, but not kinase-inactive mutants, to induce apoptosis in the absence of DNA damage or cell cycle delay suggested that these kinases may have functional roles in an apoptotic signaling pathway. To test this possibility, human T-cell lines that express the Fas receptor, which regulates apoptosis in these cells, were examined for changes in PITSLRE mRNA, protein, and kinase activity following activation of the Fas-regulated PCD pathway. Substantial increases in PITSLRE transcription and translation, as well as significant proteolysis of specific existing and newly synthesized PITSLRE isoforms were observed. Additionally, PITSLRE protein kinase activity was elevated in these cells as a function of Fas-induced apoptosis. Glucocorticoid-induced death of the CEM-C7 cells did not induce PITSLRE mRNA expression, but it did induce the p50 PITSLRE protein species. This finding suggests that the p50 PITSLRE protein kinase can originate from other PITSLRE isoforms in the cell that may have unique functions and can circumvent any requirement for new synthesis.

Our experiments in CHO cells with the PITSLREβ1 N-terminal mutant also support the possibility that specific proteolytic cleavage in the N-terminal domain results in an active protein kinase that can induce apoptosis. Removal of this domain did not impair the ability of this kinase to induce apoptosis. In fact, expression of this N-terminal mutant resulted in apoptosis in the absence of cell cycle delays and/or apparent

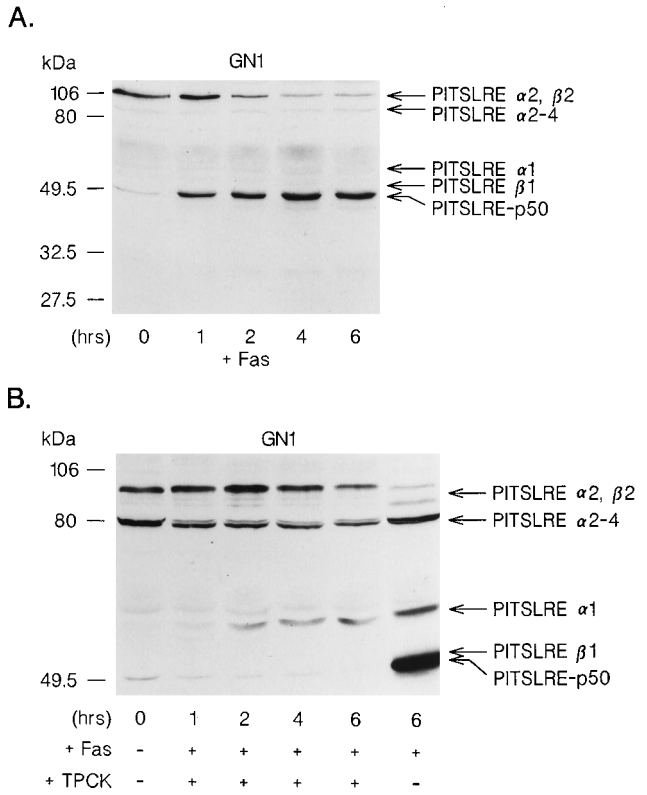

FIG. 6. PITSLRE protein expression in Jurkat cells treated with the Fas MAb in the absence (A) and presence (B) of TPCK. Cells were treated with the Fas MAb and with or without TPCK, as indicated at the bottom. Total cell lysates (100 μg) were analyzed by SDS-PAGE and then Western blotted with the PITSLRE GN1 antibody. The sizes of the molecular weight standards are indicated on the left, and the positions of the various PITSLRE isoforms are indicated on the right. Induction of a novel 50-kDa PITSLRE isoform is observed in both panels, and its position is indicated just below the p58 PITSLREβ1 isoform.

chromosomal abnormalities. These experiments imply that removal of a portion of the PITSLRE N-terminal domain does not impair either kinase activity or its ability to induce apoptosis (Fig. 2), which is consistent with the in vivo N-terminal proteolysis of these kinases to generate the p50 PITSLRE kinase that we observed in two different apoptotic pathways. It will be of interest to determine whether, and how, the p50 PITSLRE protein kinase is induced in additional apoptotic pathways. Thus, apparent differences in the mechanisms involved in apoptosis triggered by different agents may reflect subtle differences, as well as similarities, between these different pathway(s).

The increase in PITSLRE kinase activity in these cells could be due to any of several PITSLRE isoforms, eight of which contain identical catalytic and C-terminal domains (61). However, marked induction of a novel and apparently proteolytically processed 50-kDa form of this protein kinase coincides with apoptosis and increased kinase activity. Additionally, our data suggests that this processed p50 PITSLRE protein can be derived from at least four PITSLRE isoforms, α1, β1, α2, and β2. Examination of the peptide sequence for these isoforms indicates the presence of possible protease cleavage sites just upstream of the kinase domain and downstream of possible regulatory domains in the N-terminal regions of these proteins (Fig. 7) (48). It is important to also note that the PITSLRE α2-4 isoform (which does not undergo proteolysis) contains protein sequence identical to PITSLREα1, -β1, -α2-2, and -β2-2 in this region. This finding suggests that proteolysis of these latter PITSLRE isoforms is specific and may be relevant to their function. These possible proteolytic cleavage sites do not remove a significant portion of the protein which was used to generate the GN1 antibody, as indicated by the ability of the GN1 antibody to detect the p50 PITSLRE protein. Yuan and Horvitz have demonstrated that the *ced-3* death gene in *Caenorhabditis elegans* corresponds to the mammalian interleukin-1β-converting enzyme, a cysteine protease (63). In fact, ectopic expression of this protease is sufficient to induce apoptosis in fibroblasts (40a). Selective proteolytic processing of some, but not all, of the PITSLRE kinase isoforms implies that these kinases may be targets of protease action during apoptosis and is consistent with protease-directed apoptosis. Further studies are planned to examine this point more closely. The timing of proteolytic processing of the PITSLREα1, -α2, and -β2 isoforms coincides with the appearance of DNA ladders in the CEM-C7 and Jurkat T cells, which may suggest that the PITSLRE kinases are not involved in the initial signaling events associated with an activated Fas receptor. Instead, they may function downstream of other receptor-associated protein kinases.

The proteolytic processing of several PITSLRE isoforms may also be relevant to the activation of these kinases. Others have shown that removal of regulatory domains from protein kinases by proteolysis results in their activation (29, 42). A similar mechanism may be responsible for the activation of the PITSLRE kinase(s) during apoptosis. This notion is consistent with experiments in which the N-terminal truncation mutant of PITSLREβ1 induced apoptosis in the absence of cell cycle delays or abnormal chromosome segregation when ectopically expressed in CHO cells. In addition, the transient expression of full-length PITSLREα1 in CHO cells does not induce apoptosis, which is consistent with the observation that only the β1 isoform and its N-terminal truncation mutant induce apoptosis in these cells.

Our data support the role for certain PITSLRE kinase isoforms as effectors of an apoptotic signaling pathway. First, PITSLRE kinase expression and activity are induced in response to activation of a cell surface receptor involved in triggering PCD. Second, newly synthesized, as well as existing pools of PITSLRE proteins, appear to be targets of specific proteolysis following Fas-induced and glucocorticoid-induced apoptosis in human T cells. This observation agrees with experimental evidence from other laboratories that suggests a role for proteolysis in the initial onset of Fas- and glucocorticoid-mediated PCD (27, 46, 51). Finally, ectopic expression of certain PITSLRE isoforms in eukaryotic cells leads to apoptosis. This later observation qualifies the PITSLREβ1 and its N-terminal mutants as killer genes (46). Attempts to block Fas-induced apoptosis with antisense oligonucleotides to several PITSLRE isoforms have, so far, been unsuccessful. This may be due to technical complications and/or the inability to negate the effects of proteolysis of preexisting pools of PITS LRE proteins. Experiments designed to determine whether regulated expression of antisense and dominant negative forms of the PITSLREα1 and -β1 proteins can block Fas-mediated apoptosis may be informative.

Of some interest is the potential role of the PITSLRE kinases as tumor suppressors and the possible function of some of the isoforms in apoptotic signal transduction. On the basis of deletion and molecular alteration of this locus in human neuroblastoma cell lines, we have proposed that one or more of the genes encoding the PITSLRE protein kinases function as a tumor suppressor(s) (28). Others have shown that even partial abrogation of the function of certain tumor suppressor

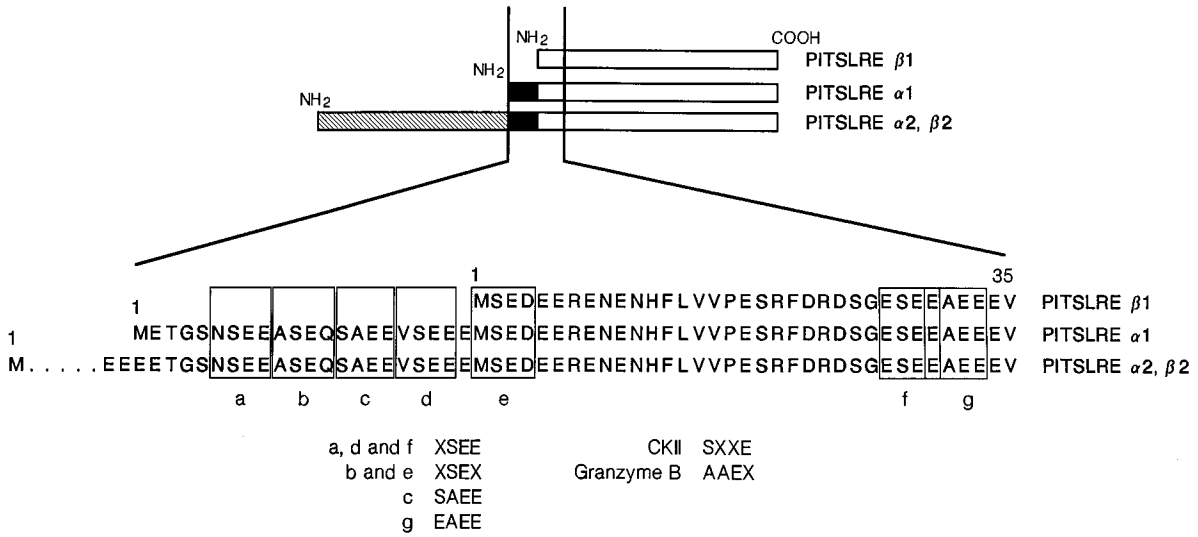

FIG. 7. Possible protease cleavage sites located in the PITSLREα1, -β1, -α2, and -β2 isoforms. A comparison of the amino acid sequences of these isoforms indicates that several potential cleavage sites (similar to that reported for granzyme B [48]) are nested within an ~30-amino-acid stretch. Additionally, a number of casein kinase II (CKII) phosphorylation sites are also clustered within this region.

genes (ca. p53) can result in a marked change in the ability of cells harboring these alterations to undergo apoptosis (31–33). This loss of function in apoptotic signaling results in substantially enhanced growth characteristics for these tumor cells. Thus, deregulation of apoptotic signaling pathway(s) may represent one way to enhance tumorigenesis by preventing the elimination of these cells through normal checkpoint control.

In conclusion, our results show that (i) ectopic expression of certain PITSLRE kinase isoforms can induce apoptosis, (ii) expression and activity of PITSLRE kinase(s) are normally induced in response to Fas activation, leading to apoptosis, (iii) activation of a serine protease(s) may be an early response to Fas activation in human T cells, and (iv) certain PITSLRE kinases are targets of proteolysis during Fas-induced apoptosis. Criteria for classification as a killer gene include induction of a gene product and its activity during apoptosis and triggering of apoptosis by the ectopic expression of such a protein (46). The results of this study suggest that certain PITSLRE kinase isoforms fit these criteria, thus qualifying them as bona fide killer genes. Results presented here also suggest that certain PITSLRE isoforms function as effectors of what may be an apoptotic signal transduction pathway(s) in immature T cells.

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The first two authors contributed equally to this work.

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