p56\textsuperscript{Lck} Tyrosine Kinase Enhances the Assembly of Death-inducing Signaling Complex during Fas-mediated Apoptosis*

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Although the death-inducing signaling complex (DISC) is rapidly assembled, several lines of evidence suggest that formation of this complex is not the first consequence of cell surface CD95 (Fas) stimulation but rather a later step in this process. Activation of Fas triggers a cascade of signaling events that culminate in cellular apoptosis. Tyrosine kinases are critical effectors in T cell activation. However, their functional involvement in death receptor-mediated apoptosis is unknown. Here, we used p56\textsuperscript{Lck}-deficient cells to show that CD95-induced cell death is highly dependent on p56\textsuperscript{Lck} activity and its localization within plasma membrane. We found that p56\textsuperscript{Lck} acts upstream of the mitochondria; in the absence of p56\textsuperscript{Lck}, Bid cleavage and the release of cytochrome c were severely impaired. Moreover, p56\textsuperscript{Lck}-deficient cells or cells expressing an inactive form of p56\textsuperscript{Lck} displayed defective formation of the DISC post CD95 stimulation. \textit{In vivo} reconstitution of thymocytes from p56\textsuperscript{Lck}-deficient mice, which are resistant to apoptosis, with p56\textsuperscript{Lck} restored Fas-mediated cell death. Our results support a novel model whereby sensitivity to apoptosis is regulated through quantitative changes in the stoichiometry of DISC components triggered by p56\textsuperscript{Lck} activation and localization.

In T lymphocytes activation-induced cell death results from repeated stimulation through the T cell receptor (TCR)\textsuperscript{3} (1). Activation-induced cell death plays a crucial role in regulating peripheral homeostasis and self-tolerance by eliminating autoreactive and activated T cells. Members of the “death receptor” subgroup of the tumor necrosis factor receptor family, characterized by their intracellular “death domain” motifs, have been identified as important mediators of apoptosis (2). Like all death receptors, ligation of CD95 (Fas/APO-1) by its ligand (FasL) or agonistic antibodies triggers death-inducing signaling complex (DISC) formation (3), which typically induces caspase activation (4). DISC formation occurs through the rapid recruitment of a set of proteins including Fas-associated death domain protein (FADD), procaspase-8, procaspase-10, and the caspase 8/10 regulator c-FLIP to the cytoplasmic domain of CD95 (5, 6). This oligomerization of procaspase-8 results in its autocatalytic cleavage and release from the DISC as an active heterotetramer containing two p18 and two p10 subunits (7–10). Although the DISC is rapidly assembled, several lines of evidence suggest that formation of this complex is not the first consequence of Fas stimulation but rather a later step in the conversion of signaling “inefficient” Fas complexes into “activated” receptor complexes (11, 12). In particular, formation of Fas microclusters (12) and high stability supramolecular clusters via receptor palmitoylation (13, 14), actin reorganization (12), inducible or constitutive association with detergent-resistant microdomains known as lipid rafts (15–22), and the production of acid sphingomyelinase-mediated ceramide (11) have been proposed as important intermediate steps preceding robust DISC formation.

These studies clearly suggest that Fas-induced apoptosis can be controlled at the level of the cytoplasmic membrane. Although specific interactions between Fas, FADD, and procaspase-8/10 via their death domains and death effector domains are well documented, little is known about the mechanism(s) that regulates the recruitment of these proteins to CD95 after anti-Fas treatment. Cross-linking of CD95 has been shown to trigger several signal transduction pathways that tightly regulate apoptosis (23). Kinases such as p38 MAPK and members of the c-Jun NH\textsubscript{2}-terminal kinase family are activated and involved in regulating Fas-mediated apoptosis (24). The serine/threonine kinase B (Akt) in the phosphatidylinositol 3-kinase-dependent pathway is implicated in the inhibition of Fas-induced apoptosis (25) at the receptor level by controlling the early stages of DISC assembly (26). In addition, the serine/threonine kinase protein kinase C has also been implicated as a negative regulator of Fas-mediated apoptosis in T cells (27, 28) by inhibiting FADD recruitment to CD95 (29, 30).

The role of protein-tyrosine kinases in regulating Fas-mediated apoptosis is poorly understood. Tyrosine kinase activation has been reported to provide an early and obligate signal for Fas-mediated apoptosis, and Fas activity is abrogated by tyro-
sine kinase inhibitors (31). Despite the lack of immunoreceptor tyrosine-based motifs, CD95 is tyrosine-phosphorylated both in vitro and in vivo after Fas cross-linking (32). Furthermore, expression of Fas-associated protein (FAP-1), a tyrosine phosphatase directly associated to the cytoplasmic tail of Fas, was able to protect cells against Fas cross-linking (33). Thus far, only one protein-tyrosine kinase, p59fyn, has been identified as being involved in Fas-mediated apoptosis through its direct interaction with CD95 (34). However, the mechanisms involved in p59fyn regulation of Fas-mediated apoptosis remain unknown.

The non-receptor protein-tyrosine kinase, p56Lck, is essential to T cell signaling (35). The structure of p56Lck is typical of Src family kinases and is composed of a unique domain at the NH2 terminus (Src homology 4 domain) followed by an SH2, an SH2.5, and a tyrosine kinase or SH1 domain. Upon initial TCR cross-linking, p56Lck is recruited to the immunological synapse by CD4 (36). Once recruited, p56Lck phosphorylates immunoreceptor tyrosine-based motifs within the TCR complex and activates downstream tyrosine kinases and adaptor proteins to initiate TCR signaling (35). The importance of p56Lck in TCR signaling was clearly demonstrated in p56Lck-deficient mice that exhibited severe defects in thymocyte maturation and significant depletion of peripheral T lymphocytes (37, 38). The role of p56Lck activation in death receptor-induced cell death has been a matter of debate since conflicting and ambiguous results have been published that most probably can be attributed to the heterogeneity of experimental systems used (39–43).

Here we provide both direct and genetic evidence that p56Lck plays a primary role in Fas-mediated apoptosis in vitro and in vivo. Our data demonstrate that p56Lck activity and localization within plasma membrane are required for proper DISC formation after CD95 triggering.

**EXPERIMENTAL PROCEDURES**

**Plasmid**—Human wild type p56Lck was cloned from freshly purified and activated T-lymphocytes by reverse transcription-PCR. Kinase-active p56Lck (Tyr-505 to Phe-505, (AKp56Lck)), acyl-negative (Cys-3 and Cys-5 to Ala-3 and Ala-5) WT p56Lck (WT acyl-negp56Lck), or acyl-negative active p56Lck (AK acyl-negp56Lck) mutants were generated by PCR mutagenesis. Kinase-defective p56Lck (Lys-273 to Ala-273, (DKp56Lck)) was generated by a two-step overlap PCR (44). The authenticity of constructs was confirmed by dyeoxy sequencing. CDNAs were subsequently cloned in the SRα-Neo vector (45).

**Reagents, Cell Lines, and Apoptosis Induction**—Jurkat and JCam-1.6 cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at 37 °C, 5% CO2. Cell lines were used for more than 5 passages. To generate stable transfectant clones expressing different p56Lck mutants, JCam-1.6 cells were transfected by electroporation (240 V, 290 microfArads) with 10 μg of plasmid DNA, selected in 1 mg/ml G418 (Invitrogen), and cloned by limiting dilution. Cell death was induced with monoclonal anti-human Fas (Jo2, BD Pharmingen, 20 μg/ml), or recombinant human tumor necrosis factor-related apoptosis inducing ligand (TRAIL; R&D Systems, 2.5 ng/ml) for 6 h. Etoposide (50 mM) was used for 12 h, and cisplatin (5 mg/ml) and doxorubicin (0.6 mg/ml) were used for 24 h. Cell death and loss of mitochondrial membrane potentials were assessed using annexin V (Bio Source)/propidium iodide (PL Sigma) and JC-1 (Sigma), respectively. Cell surface CD95 expression on human cell lines and murine thymocytes were analyzed on a BD Biosciences LSR II flow cytometry (10,000 events/sample) using fluorescein isothiocyanate-conjugated anti-human (R&D Systems) or anti-murine Fas (BD Pharmingen) antibodies. Mouse thymocytes subpopulations were identified using phosphatidylethanolamine (PE)-Cy5-conjugated anti-CD4 and PE-conjugated anti-CD8a antibodies (BD Pharmingen).

**Subcellular Fractionation**—To determine the release of mitochondrial proteins, a digitonin-based subcellular fractionation technique was used as previously described (46).

**Mice**—Six- to eight-week-old C57BL/6 and p56Lck-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male and female mice were maintained in our animal facility, and all experiments were performed in compliance with the University of Montreal guidelines. p56Lck+/− and C57BL/6 were crossed to obtain first generation p56Lck−/− mice. All mice were used between the ages of 6 and 8 weeks. Thymocytes from each type of mice were isolated and homogenized in complete medium, and red blood cells were removed using Ack lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 M Na2EDTA). Dead cells and debris were eliminated using a dead cell removal kit (Miltenyi Biotech). Thymocytes were incubated at 1 × 106 cells/ml in 24-well plates precoated with murine anti-Fas (20 μg/ml) for the indicated time points. Cell death was assessed as described above.

**Immunoprecipitation and Western Blotting**—Whole cell lysates (10 μg total protein) were resolved on 10–14% SDS-PAGE and immunoblotted with anti-Fas (B-10; Santa Cruz), β-actin (Biomol), and Bid (New England Biolabs) were used as recommended by the manufacturer. The immunoprecipitation of surface CD95 was performed on 2.0 × 107 cells after incubation with anti-Fas (1 μg/ml) at 37 °C. Cells were then lysed in 600 μl of immunoprecipitation lysis buffer (0.5% Nonidet P-40, 10% glycerol, supplemented with Complete protease inhibitor mixture (Roche Applied Science) in phosphate-buffered saline) for 30 min on ice. Anti-Fas was added to untreated samples after lysis to normalize for total protein. CD95 was immunoprecipitated from the post-nuclear supernatant overnight with 40 μl of protein A-Sepharose (GE Healthcare) coupled to rabbit anti-mouse IgM (Jackson ImmunoResearch) at 4 °C on a rotator. The samples were washed 5 times and resolved on 12% SDS-PAGE and immunoblotted with anti-
RESULTS

P56Lck~ΔΔ Cells Are Impaired in Fas-mediated Apoptosis—Tyrosine kinases have been implicated in the early events of the apoptosis cascade (31). To determine the role of p56Lck in Fas-mediated cell death, we measured cell death after agonistic anti-Fas antibody treatment in the Jurkat cell line or in a Jurkat subclone, JCam-1.6, that lacks p56Lck (Fig. 1A). Despite a similar level of Fas receptor expression on both cell lines (data not shown), JCam-1.6 cells were significantly resistant (>70%) to cell death upon Fas receptor triggering when compared with Jurkat cells as determined by annexin V/PI staining (Fig. 1A, n > 5). A 10-fold increase in anti-Fas antibody concentration was not even sufficient to restore sensitivity to Fas-mediated apoptosis in JCam-1.6 cells (Fig. 1A). Although JCam-1.6 cells were resistant to Fas-mediated apoptosis, death still occurred in these cells at levels that did not exceed 25–30% that of those observed with Jurkat cells under similar conditions (Fig. 1A). We next performed experiments aimed at defining if p56Lck is also involved in other apoptosis-inducing stimuli. For this purpose, sensitivity to natural Fas ligand, TRAIL, etoposide, doxorubicin, and cisplatin at the indicated concentrations was assessed (n = 2). Results illustrated in Fig. 1B reveal a significant decrease in the relative number of annexin V+ cells in JCam-1.6 cells treated either with Fas ligand or TRAIL. However, at the concentration used other death-inducing stimuli were not affected in their capacity to induce apoptosis despite the absence of p56Lck (Fig. 1C), suggesting the unique requirement of p56Lck in Fas and TRAIL-mediated apoptotic pathways.

P56Lck Activity Is Required in Fas-mediated Apoptosis—To demonstrate that the decreased apoptotic responses of JCam-1.6 cells were caused by the specific defect in p56Lck expression, we performed “add back” experiments obtaining stable transfectants. To this end the role of p56Lck and its activity in the apoptotic pathway was investigated by reconstituting p56Lck-deficient JCam-1.6 cells with wild type (WTp56Lck), dead kinase (lysine 273 to alanine 273 (DKp56Lck)), or constitutively active kinase (AKp56Lck) proteins. Single clones were selected (inset). Jurkat and JCam-1.6 as well as JCam-1.6 transfectants expressing p56Lck mutants were treated with anti-Fas, and cell death was assessed by flow cytometry at various time points using annexin V/PI staining. B, expression of active p56Lck restores apoptotic pathway. Processing of DNA fragmentation factor-45 (DFF45) was assessed in samples from the above experiment using anti-DFF45 antibody. Experiments shown represent three independent experiments.
with DKp56Lck protein did not alter the resistance of JCam-1.6 reconstitution of JCam-1.6 cells with WTp56Lck or AKp56Lck death.

teralization but on the whole pathway of Fas-mediated cell caspase-3 activation were monitored in Jurkat and JCam-1.6 the effect of p56Lck is not only on phosphatidylserine exter-
dication loop (type II) (47). In agreement with this hypothesis, treat-
mount of Jurkat cells with anti-Fas resulted in a major loss in ΔΨm, whereas in treated JCam-1.6 cells, the ΔΨm loss was significantly decreased (4-fold) (Fig. 3B). Bid, a mediator of mitochondrial damage induced by caspase-8, relays on an apoptotic signal from the cell surface to mitochondria in Fas-mediated apoptosis. Hence, the lack of processing of this proapoptotic molecule in the absence of p56Lck (Fig. 3C) strongly indicated that the inability of Fas to induce cleavage and activation of apoptotic effector molecules in p56Lck-deficient cells stems from abnormal proximal events upstream of the mitochondrial stage. Consequently, a major disruption in the translocation of the cytochrome c from the mitochondria to the cytosol (48, 49), occurred in p56Lck-deficient but not in Jurkat cells (Fig. 3D).

ng/ml) for different time points (Fig. 2A). Results revealed that reconstitution of JCam-1.6 cells with WTp56Lck or AKp56Lck was sufficient to restore sensitivity to CD95-induced apoptosis to levels similar to or higher to those observed with parental Jurkat cells, respectively (Fig. 2A). In contrast, reconstitution with DKp56Lck protein did not alter the resistance of JCam-1.6 cells to CD95 cross-linking. Similar results were gathered from five different clones of each mutant (data not shown). Consistent with the levels of cell death measured by annexin V, faster and more efficient processing of the DFF45 was observed in JCam-1.6/AKp56Lck as compared with cells lacking p56Lck or expressing its DK-p56Lck variant (Fig. 2B), suggesting that the effect of p56Lck is not only on phosphatidylinerine externalization but on the whole pathway of Fas-mediated cell death.

Absence of p56Lck Leads to Substantial Defect in Mitochondrial Pathway—We next performed experiments aimed at defining the apoptotic pathways associated with p56Lck-dependent Fas signaling. For this purpose, caspase-8 and caspase-3 activation were monitored in Jurkat and JCam-1.6 cells. Results showed that, whereas Jurkat cells exhibited significant activation of both caspase-8 and -3, resulting in the cleavage of DFF45 (Fig. 3A, right panel), there was a substantial reduction in caspase activation and DFF45 processing in JCam-1.6 cells (Fig. 3A, left panel). The lack of caspase activation and subsequent substrate processing in the absence of p56Lck hinted at a defect in the mitochondrial amplification loop (type II) (47). Removal of p56Lck from the Plasma Membrane Abrogates Fas-mediated Apoptosis—The role of p56Lck localization within plasma membrane on Fas-induced cell death was investigated using different approaches. A point mutation in the SH2 domain has been shown previously to preferentially localize outside lipid raft compartment fractionated over sucrose gradient (50). Taking advantage of this information, we first used the SH2 domain-deleted and inactive form of p56Lck (51) to study the effect of p56Lck localization within plasma membrane on Fas-induced apoptosis. To this end, JCam-1.6 cells were transfected with a plasmid construct expressing AKp56Lck kinase deficient in the SH2 domain (JCam-1.6/AKp56Lck ΔSH2, Fig. 4A), and single clones (n = 3) were treated with anti-Fas antibody. As shown in Fig. 4A, expression of AKp56Lck ΔSH2 in JCam-1.6 cells did not restore Fas-mediated apoptosis in these cells, suggesting the importance of p56Lck localization within plasma membrane and its activity in this process. The specific involvement of p56Lck within plasma membrane was further examined by utilizing acylation-deficient mutant tyrosine kinase unable to translocate
from the cytosol to plasma membrane (52). Three clones of JCam-1.6 cells transfected with acyl-negative (acyl\(^{-}\)) p56\(^{Lck}\) or wild type p56\(^{Lck}\) (JCam-1.6/WT acyl\(^{-}\)p56\(^{Lck}\)) mutants were selected (data not shown). The disruption of p56\(^{Lck}\) localization had no effect on the cell surface expression of CD95 (Fig. 4B). Results revealed that treatment of three different clones of JCam-1.6/WT acyl\(^{-}\)p56\(^{Lck}\) or JCam-1.6/AK acyl\(^{-}\)p56\(^{Lck}\) with anti-Fas antibody for 6 h showed substantial resistance to Fas-mediated apoptosis to a level similar to that observed in JCam-1.6 cells (Fig. 4B, n = 3). Together these results provide strong evidence for the mandatory compartmentalization of p56\(^{Lck}\) to plasma membrane to initiate Fas-mediated apoptosis.

**Impaired DISC Assembly in the Absence of Active p56\(^{Lck}\)** — CD95 cross-linking with anti-Fas antibody rapidly leads to formation of the DISC. We next examined the assembly of DISC post CD95 triggering aiming to differentiate the stoichiometric difference in the level of DISC components in the presence or absence of p56\(^{Lck}\) activity. To address this issue, we treated JCam-1.6, JCam-1.6/AKP56\(^{Lck}\), JCam-1.6/AK acyl\(^{-}\)p56\(^{Lck}\), and JCam-1.6/AKP56\(^{Lck}\) \(\Delta SH2\) transfectants with anti-Fas antibody for 15 min, and Fas receptor was then immunoprecipitated as indicated under "Experimental Procedures." A, aliquots from the same reaction were incubated for 6 h to quantify levels of apoptotic induction by annexin V/PI staining. B, cell lysates from immunoprecipitation (IP) reactions were immunoblotted (IB) against different DISC components. Relative position of molecular mass is shown in kilodaltons. Results shown are representative of three independent experiments.
DISC assembly, whereas the absence of proper raft localization and activity of the p56\textsuperscript{Lck} in JCam-1.6/AK acyl-\textsuperscript{neg}p56\textsuperscript{Lck} or in the JCam-1.6/AK \DeltaSH2p56\textsuperscript{Lck} cells resulted in a significant decrease in the recruitment of caspase-8, FADD, and FLIP-L, as compared with JCam-1.6 cells. Altogether, these data confirmed that DISC assembly during Fas-mediated apoptosis is strongly dependent on the presence of p56\textsuperscript{Lck}, its activity, and on its capacity to translocate to plasma membrane.

\textbf{p56\textsuperscript{Lck} Restores Sensitivity to Fas-mediated Apoptosis in Thymocytes from p56\textsuperscript{Lck}-deficient Mice}—Our next objective was to demonstrate the requirement of p56\textsuperscript{Lck} in the induction of Fas-mediated apoptosis in primary T cells. We examined the sensitivity to Fas-mediated cell death in thymocytes isolated from p56\textsuperscript{Lck-deficient (p56\textsuperscript{Lck}-/-) mice (37) to that of heterozygotes (p56\textsuperscript{Lck}+/--) or wild type mice (p56\textsuperscript{Lck}+++) of the corresponding background (Fig. 6A). Treatment of p56\textsuperscript{Lck}+/+ thymocytes with agonistic anti-mouse Fas monoclonal antibody (Jo2, 20 \mu g/ml) induced time-de-}

\textbf{p56\textsuperscript{Lck} Enhances Fas-mediated Apoptosis}

\textbf{DISCUSSION}

In this report we have unraveled a novel role for the p56\textsuperscript{Lck} tyrosine kinase in the regulation of T cell apoptosis. We used a genetic approach to reconstitute p56\textsuperscript{Lck}-deficient cells in vitro.
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and in vivo to demonstrate that CD95-induced cell death is strongly dependent on the presence of an active form of p56\(^{Lck}\) that properly localizes within plasma membrane. Analysis of p56\(^{Lck}\)-deficient thymocytes further confirmed the functional involvement of this tyrosine kinase in Fas-mediated cell death in primary thymocytes. Inefficient CD95-induced apoptosis was not due to alterations in the expression of the CD95 receptor or of other proteins involved in the regulation of this process. Rather, defective signaling events upstream of mitochondria resulted in incomplete recruitment of DISC components post Fas cross-linking. The lack of Bid cleavage after Fas triggering further emphasized the critical role of p56\(^{Lck}\) upstream of the mitochondrial pathway and downstream of CD95. Our results establish a new paradigm for understanding the initial steps in Fas-mediated apoptotic pathway as well as the signaling required for recruitment of DISC components and DISC assembly within plasma membrane before cell death.

Several kinases, such as protein kinase C and Akt, have been shown to interfere with early stages of DISC assembly leading to the inhibition of Fas-mediated apoptosis (26, 53). The members of the Src kinase family are expressed in a wide variety of tissues, but some of them such as Lyn, Fyn, and Lck are found primarily in hematopoietic cells. It has been previously demonstrated that stimulation of several human and murine hematopoietic-derived cell lines with anti-Fas antibodies induced increased tyrosine phosphorylation of a panel of proteins observed in whole-cell lysates (39, 54). In addition, immunoprecipitation studies of anti-Fas-stimulated human Jurkat and murine 2B4.11 T cells revealed activation of the Src-family tyrosine kinases Lck and Fyn. The evidence presented in this study suggests the requirement for an active p56\(^{Lck}\) within lipid raft microdomains for proper subcellular compartmentalization of DISC molecules. Muppadi and Siegel (17) have demonstrated previously that TCR re-stimulation of activated human CD4 T cells resulted in Fas receptor translocation into lipid raft microdomains, rendering these cells sensitive to Fas-mediated apoptosis. In line with our results, it is more likely that enhanced sensitivity to cell death of activated CD4 T cell is a direct consequence of increased levels of activated p56\(^{Lck}\) upon TCR cross-linking by antigen. It is also important to examine that interleukin-2, which also induces p56\(^{Lck}\) activation, can sensitize mature T cells to apoptosis as suggested by Lenardo (55).

The apoptotic effect of anti-Fas on T cells, which we observed in our study, is clearly p56\(^{Lck}\)-dependent. This conclusion is based on three complementary sets of results. First, J.Cam1.6, a p56\(^{Lck}\)-negative mutant of Jurkat cells, does not show a significant increase of caspase-activation, whereas Jurkat cells demonstrate higher levels of apoptotic activity after a short period of anti-Fas stimulation. Second, cell lines expressing inactive p56\(^{Lck}\) show no significant level of apoptotic activity. Third, p56\(^{Lck}\)-reconstituted J.Cam1.6 cells expressing the constitutively active form of p56\(^{Lck}\) demonstrate enhanced caspase activation and cell death. The fact that all forms of caspase-8, including procaspase-8 (p54/52), cleaved (p43/41), and active fragment (p18), are observed in our immunoprecipitation experiments after CD95 triggering only in the presence of activated p56\(^{Lck}\) (Fig. 5B) suggests that p56\(^{Lck}\) activation within plasma membrane is a prerequisite to the DISC assembly. Therefore, an insufficient level of procaspase-8 activation in cells deficient of p56\(^{Lck}\) may explain the severe defect in cell death post Fas receptor triggering observed in J.Cam1.6 cell line and p56\(^{Lck}\)-deficient thymocytes. This is further demonstrated by the reduction of c-FLIP processing at the DISC (Fig. 5B) and the lack of Bid cleavage (Fig. 3C) after Fas cross-linking. Although much remains to be discovered and understood about the function of p56\(^{Lck}\), these results may permit a new means of investigating “downstream” signaling effector molecules required for Fas clustering and signaling. Our results and several recent papers from other laboratories make it clear that p56\(^{Lck}\) not only regulates T cell activation and proliferation but also plays a central role in apoptosis pathways (56–58). Recent findings by Schulze-Osthoff et al. (57) delineates a hitherto unknown function of p56\(^{Lck}\) and possibly other Src family kinases in the regulation of Bcl-2 family proteins and chemoresistance. Altogether, these data suggest that different p56\(^{Lck}\)-dependent signals are involved in the T cell stimulatory and apoptosis-sensitizing functions of p56\(^{Lck}\).

The importance of p56\(^{Lck}\) in TCR-induced or Fas-induced cell death has yielded conflicting and ambiguous results, which most probably can be attributed to the heterogeneity of experimental systems used or to the interpretation of the data (39–43). However, the evident differences may be simply explained by the altered time course of Fas-induced apoptosis in p56\(^{Lck}\)-deficient cells. Whereas short-term anti-Fas stimulation (4–6 h) favored the significant effect of p56\(^{Lck}\) (39), long-term stimulation (16–24 h) favored the absence of p56\(^{Lck}\) effect during Fas-induced apoptosis (41–43). In this study we have evaluated the effect of p56\(^{Lck}\) on Fas-induced apoptosis after 6 h of anti-Fas stimulation. Furthermore, in light of the documented evidence for the induction of the p56\(^{Lck}\) tyrosine kinase activity after Fas stimulation (31, 33, 42), we analyzed the effect of this kinase on the stoichiometry of DISC components at the early stage of the initiation of Fas signaling cascade (15 min). Thus, differences with published data reflect the different periods of stimulation used, which in our system allowed us to detect the effect of p56\(^{Lck}\) in the initial window of Fas signaling cascade activation that we are targeting.

The other aspect of the conflicting data resided in the data interpretation. Although Belka (43) showed that p56\(^{Lck}\) is involved in mitochondrial apoptotic pathway of ionizing radiation but not in Fas pathway, a deeper analysis of results may extend the implication of p56\(^{Lck}\) to the Fas-induced apoptosis. The cleavage of caspase-8 (p54/52) to p43/41 is highly reduced in p56\(^{Lck}\)-deficient J.Cam1.6 cells compared with J.Cam1.6 expressing p56\(^{Lck}\)-WT after anti-Fas stimulation. In addition, the breakdown of ΔΨm upon anti-Fas stimulation was significantly slowed (83, 69, and 33% at 6, 12, and 24 h, respectively) in p56\(^{Lck}\)-deficient cells as compared with p56\(^{Lck}\)-WT expressing cells. Thus, their results can easily be integrated into our observation, demonstrating the importance of p56\(^{Lck}\) in Fas-mediated apoptotic pathway.

Based on these findings, we propose a novel cross-talk in which the presence of p56\(^{Lck}\) activity facilitates DISC assembly at the plasma membrane after recruitment of DISC components from the cytosol (Fig. 8). In this model the absence of
p56Lck leads to major qualitative and quantitative perturbation in the stoichiometry of the DISC. Despite similar levels of Fas receptor aggregation after stimulation, DISC formation would not lead to sufficient caspase-8 activity to allow proper Bid protein cleavage. Therefore, the mitochondrial pathway required for the induction of Fas-mediated cell death in these cells is significantly impaired.

The main question remains to be answered is how p56Lck regulates CD95-mediated apoptosis? Cholesterol and sphingolipid-enriched rafts, also called detergent-resistant membranes, have been proposed as platforms for compartmentalizing dynamically regulated Fas signaling complexes at the plasma membrane (16, 22, 59–61). Although detected in various cell types, the role of lipid rafts in apoptosis has, however, been mostly studied in lymphocytes where the physiological apoptotic program occurs after Fas receptor triggering using conventional Triton X-soluble and -insoluble fractions isolated by sucrose gradient (15–22). The cells stimulated to undergo apoptosis appear to use membrane rafts in the death-signaling process by mobilization of rafts to localized regions of the membrane that are now enriched with apoptotic signaling effectors. Using a similar approach of cellular fractionation, our preliminary data reveal differential regulation and localization of DISC component within detergent-resistant membrane fractions in the presence or absence of p56Lck activity.4 However, since this detergent-based experimental technique has been recently challenged (62–64), more sophisticated biophysical approaches such as fluorescence recovery after photobleaching and fluorescence resonance energy transfer would help to dissect the involvement of p56Lck in regulating the stoichiometry of DISC component within plasma membrane lipid rafts.

The unexpected observation that p56Lck, a critical effector molecule of T cell activation and growth factor-mediated proliferation, is also an essential component of the death machinery further illustrates the intertwining between pathways leading to life or death. Moreover, our observation that p56Lck regulates DISC stoichiometry demonstrates the role of this tyrosine kinase in the homeostatic cell process. The discrimination between p56Lck substrates, which regulate T cell death or T cell activation, behooves further investigation.

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