p56lck plays a key role in transducing apoptotic signals in T cells

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Received 2 March 1995

Abstract The CD4 receptor synergizes with the T-cell antigen receptor (TCR) in helper T-cell activation. However CD4 crosslinking in the absence of simultaneous TCR engagement leaves the cells primed to activation dependent apoptosis. To assess the role of the CD4 associated protein tyrosine kinase p56lck in CD4 priming to apoptosis we have constructed Jurkat T-cell lines stably transfected with a constitutively active form of p56lck. These cells were constitutively primed to undergo apoptosis upon TCR crosslinking with specific antibodies. In addition the Jurkat JCaM1 line, which is defective for p56lck expression, was resistant to TCR induced apoptosis. These data indicate that p56lck is required for T-cell apoptosis and that CD4 priming of T-cells for antigen dependent apoptosis is due to inappropriate or partial activation of the p56lck signal transduction pathway.

Key words: Apoptosis; T-cell; T-cell receptor; CD4; Protein tyrosine kinase

1. Introduction

The autocrine growth loop initiated by the expression of interleukin-2 (IL-2) and its receptor is a key factor in the complex process of T-cell activation. IL-2 expression is activated by signals derived from the T-cell antigen receptor (TCR) after recognition of specific antigen in the context of the major histocompatibility complex (MHC) on the surface of antigen presenting cells. CD4 or CD8 molecules also play a part in this process by interacting with non-polymorphic regions of the MHC and thus modulating the TCR derived signals (reviewed in [1,2]). These signals are however not sufficient to activate IL-2 expression. Accessory signals are required, which are triggered by the interaction of other surface molecules on the antigen presenting cell with their cognate receptors on the T-cell and by lymphokine signals derived from the antigen presenting cell (reviewed in [3,4]).

In the absence of the complete set of signals required for full activation, T-cells may become committed to alternative fates. Under certain conditions, T-cell activating signals can trigger programmed cell death mediated by apoptosis, a process which involves cytoplasm condensation, membrane blebbing and the characteristic cleavage of genomic DNA to nucleosome sized multimers (reviewed in [5,6]). This process is essential for removal of potentially self-reactive cells from the immune repertoire. Subversion of this mechanism has recently been implicated in the drastic reduction of $CD4^+$ T-cells associated with AIDS (reviewed in [7]). Crosslinking of CD4 using Mabs or indirectly using HIV gp120 and gp120-specific antibodies leaves the cells primed for subsequent activation dependent apoptosis [8,9]. In addition, peripheral T-cells isolated from HIV positive patients who have high levels of circulating gp120 and gp120-specific antibodies are hypersensitive to apoptosis induced by TCR engagement [10,11]. It has been suggested that sequestration of CD4 by crosslinking such that it cannot participate to productive TCR interaction with peptide-MHC complexes is responsible for this priming for apoptotic cell death [8].

The cytoplasmic domain of CD4 interacts with the src family protein tyrosine kinase (PTK) p56lck, which plays a critical role in T-cell activation [12,13]. CD4 crosslinking with specific Mab results in the activation of p56lck [14], raising the question of the role of this activity in CD4 priming to TCR induced apoptosis. To address this question, we constructed Jurkat T-cell lines stably transfected with a constitutively active form of p56lck. These cells were hypersensitive to apoptosis induced by crosslinking with TCR specific antibodies. Conversely, the Jurkat line JCaM1, which is defective for p56lck expression [15], was resistant to TCR induced apoptosis. These data suggest that p56lck is a key signalling molecule not only in activation, but also in apoptosis of T lymphocytes.

2. Experimental

2.1. Plasmids and antibodies

NF-AT/CAT contains a trimer of the NF-AT binding site of the IL-2 promoter upstream of the CAT gene [16]. To construct stable transfectants an *Eco*RI fragment containing the cDNA encoding F505lck [17,18] was cloned into the *Xba*I site of the neo vector RcCMV (Invitrogen), after filling with Klenow polymerase. IgG from OKT3 (ATCC) hybridoma supernatants were purified on Mabtrap (Pharmacia, LKB, Uppsala, Sweden). BMA031 Mab [19] was kindly provided by R. Kurrle. α-p56lck polyclonal antibodies were a generous gift of R.M. Perlmutter. α-p59fyn polyclonal antibodies and α-phosphotyrosine Mab were purchased from Upstate Biotechnology Inc., Boston.

2.2. Transfections and CAT assays

Transient transfections, activations and CAT assays were carried out as described [20] using 10^6 cells per sample and 0.5–1 μg reporter plasmid per sample. Thin-layer chromatograms were scanned and chloramphenicol acetylation was quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). To generate stable transfectants 10^7 cells exponentially growing cells were pelleted, washed in PBS then resuspended in ice-cold cytomix as described by van den Hoff et al. [21]. 10 μg plasmid DNA were added and cells were electroporated at 300 V, 960 μF using a Bio-Rad electroporator. Cells were kept on ice for 5 min then diluted to 20 ml with RPMI 10%FCS and allowed to recover ON. They were then pelleted, resuspended at 2×10^5/ml in the same medium supplemented with supernatant from exponentially growing cells and 1 mg/ml G418 (Gibco) and plated in 96-well plates. Cells were kept under selection for 4–6 weeks till the appearance of colonics.

2.3. Immunoprecipitations, immunoblots and kinase assays

Cells were lysed and subjected to immunoprecipitation as described

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Abbreviations: CAT, chloramphenicol acetyltransferase; IL-2, interleukin-2; Mab, monoclonal antibody; MHC, major histocompatibility complex; PTK, protein tyrosine kinase; TCR, T-cell antigen receptor.

SSDI 0014-5793(95)00292-8

[22]. In vitro kinase assays using [γ-^{32}P]ATP were carried out in the absence of exogenous substrate for 4 min at RT on washed immunoprecipitates in phosphorylation buffer (25 mM HEPES pH 7.2, 100 mM NaCl, 5 mM $MgCl_2$). Alternatively, 10 μg acid-denatured enolase per sample were used as exogenous substrate in 20 mM Tris pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 μM ATP. ^{32}P-labeled proteins were resolved by SDS-PAGE and visualised and quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Immunoblots were carried out using a chemiluminescence detection kit (ECL, Amersham).

2.4. Apoptosis

Activation by cross-linking of mouse Mabs to TCR/CD3 was carried out by incubation of the cells for 30 min at 4°C with 1 μg/ml Mab followed by transfer of the cells to wells which had been precoated ON with α-mouse antibodies at 4°C, washed in PBS and blocked for 1 h with RPMI-10%FCS at 37°C. Analysis of DNA fragmentation was carried out by electrophoresis on 2% agarose gels in Loehning buffer of DNA extracted from 10^6 cells as described by Newell et al. [8]. Gels were subsequently transferred to nitrocellulose filters and hybridized to total human DNA ^{32}P-labeled by random priming. Quantitation of individual bands was performed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Apoptotic cells were identified and quantitated by flow cytometry by their subdiploid DNA content using propodium iodide staining according to the procedure described by Nicoletti et al. [23].

3. Results and discussion

Jurkat T cells were stably transfected with a gene coding for a constitutively active form of p56lck with a Tyr to Phe substitution at position 505 (F505lck). Mutation of the 505 regulatory tyrosine results in a molecule that cannot be downregulated and has a constitutively enhanced kinase activity [17,18]. Transient transfection experiments in Jurkat cells have shown that this constitutively active mutant triggers a calcium dependent gene activating signal which synergizes with phorbol ester to activate the NF-AT transcription factor [24]. Functional F505lck activity was assayed in individual G418 resistant clones by transient transfection with a NF-AT dependent reporter construct [16]. Three independent clones were found in which PMA induced NF-AT activation (Table 1).

Fig. 1 presents the characterization of clone 11, which gave the strongest NF-AT/CAT activity. Immunoblots of cell extracts with α-p56lck antiserum revealed a modest increase in the amount of total p56lck in the transfected line compared to the parental Jurkat line. Accordingly, in vitro kinase assays of p56lck immunoprecipitates showed a twofold increase in p56lck activity in the F505lck line (Fig. 1A). Immunoblots with α-phosphotyrosine antibodies revealed however a massive increase in phosphoproteins in the F505lck line (Fig. 1A). In addition, as we have recently reported [25], increased p59fyn activity in the absence of stimulation was found in this line (Fig. 1B). Thus low constitutive expression of p56lck activity results in the accumulation of significant amounts of phosphotyrosine and p59fyn activity.

Table 1
Selection of Jurkat clones expressing F505lck

Clone	CAT (fold stimulation)
F505lck(1)	0
F505lck(5)	0.6
F505lck(7)	0
F505lck(8)	3.1
F505lck(9)	2.8
F505lck(11)	10.8

[^{14}C]Chloramphenicol acetylation in CAT assays of F505lck clones after transient transfection with NF-AT/CAT and activation with PMA.

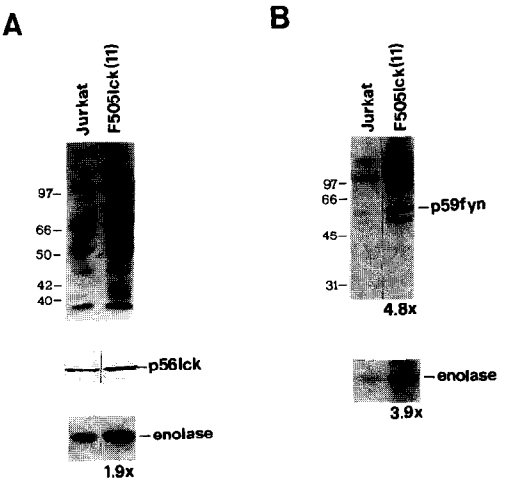

Fig. 1. Characterization of F505lck Jurkat clone 11. (A) Immunoblot of extracts of Jurkat cells and of a stable transfectant clone expressing F505lck (clone 11) resolved by SDS-PAGE and immunoblotted with an α-phosphotyrosine Mab (top) or with α-p56lck polyclonal antibodies (middle). The bottom panel shows ^{32}P incorporation into enolase in in vitro kinase assays of p56lck-specific immunoprecipitates from the same cells. (B) In vitro kinase assays of p59fyn-specific immunoprecipitates from non activated Jurkat cells and F505lck clone 11 in the absence (top) or in the presence (bottom) of enolase as substrate. ^{32}P-labeled proteins were resolved by SDS-PAGE and visualised and quantitated using a phosphorimager. The migration of molecular weight markers is indicated.

Apoptosis can be induced in T-cells by crosslinking the TCR/CD3 complex by Mabs [26]. Antibodies against different molecules in the complex have however different effects. Mabs which recognize components of the CD3 complex have significant apoptosis inducing activity whereas Mabs which recognize epitopes in the invariant region of the TCR$\alpha\beta$ chain have very little apoptosis inducing activity. Prior CD4 crosslinking has no effect on the apoptosis induced by α-CD3 Mab however this treatment primes the cells for apoptosis induced by subsequent crosslinking of the TCR with α-TCR$\alpha\beta$ Mab [8,9]. The difference between the effects of the α-CD3 and α-TCR$\alpha\beta$ Mabs is not clear. It is generally believed however that Mabs against the TCR$\alpha\beta$ chain deliver a signal qualitatively more similar to antigen.

We have tested the response of the F505lck stably transfected lines to crosslinking with α-CD3 or α-TCR$\alpha\beta$ Mabs. The results are shown in Fig. 2. As expected, TCR crosslinking on the parental Jurkat line with α-CD3 Mab resulted in massive apoptosis as evidenced by the characteristic degradation of genomic DNA to nucleosome-sized multimers, whereas crosslinking with α-TCR$\alpha\beta$ Mab caused only minor DNA degradation. Two clones transfected with the plasmid vector showed DNA fragmentation similar to the parental line. On the other hand, all three F505lck expressing clones showed substantial increases in TCR$\alpha\beta$ induced DNA fragmentation. Quantitation

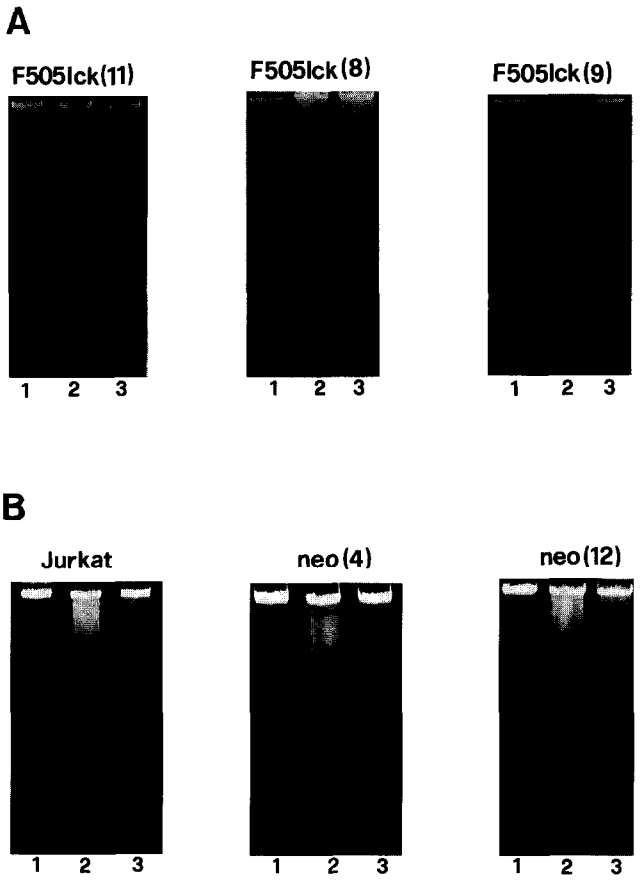

Fig. 2. F505lck Jurkat cells are hypersensitive to TCR dependent apoptosis. Ethidium bromide staining of agarose gels of DNA extracted respectively from three F505lck clones (A) and from Jurkat parental cells and two neomycin resistant clones (B). 1, non activated; 2, activated with α-CD3 Mab; 3, activated with anti-TCRαβ Mab.

of subdiploid (apoptotic) cells by flow cytometry revealed that apoptosis induced by α-TCRαβ Mab was consistently higher in F505lck transfected cells than in control cells (Table 2). The level of surface expression of the TCR complex was similar in all clones tested (data not shown) and showed no correlation with the levels of TCR-induced apoptosis. The quantitation by flow cytometry was confirmed by hybridization of blotted agarose gels similar to those shown in Fig. 2 with ^{32}P-labeled total human DNA and counting the radioactivity in individual bands

Table 2
Quantitation of apoptosis in F505lck Jurkat cells

Cell line	Apoptosis (TCR/CD3) ± S.D. (*n*)
Jurkat	0.62 ± 0.106(5)
neo (4)	0.47 ± 0.035(3)
neo (12)	0.43 ± 0.035(3)
F505lck(8)	0.99 ± 0.113(3)
F505lck(11)	1.08 ± 0.120(5)

Quantitation of apoptotic cells by flow cytometry analysis of subdiploid DNA content in Jurkat parental cells, two neomycin resistant clones and two F505lck clones. Values represent the ratio of TCRαβ-induced apoptosis to CD3-induced apoptosis, shown ± standard deviation and the number of experiments in parenthesis.

corresponding to nucleosome-sized multimers in the low molecular weight region of the gels (data not shown).

p56lck activation thus enhances the sensitivity of Jurkat cells to apoptosis induced by TCR crosslinking. We questioned whether this activity was an essential component for induction of apoptosis using a Jurkat cell variant (JCaM1) defective for functional p56lck expression [15]. As shown in Fig. 3 neither TCR nor CD3 cross-linking induced detectable DNA fragmentation in JCaM1 cells. The apoptotic mechanism was however intact since treatment with the calcium ionophore A23187 alone, which is known to induce apoptosis in most T-cells [27], resulted in DNA fragmentation to similar levels in both Jurkat and JCaM1 cells. It could be argued that α_TCR and α-CD3 Mab failed to induce apoptosis in JCaM1 cells simply because the TCR fails to signal. However, TCR signaling in JCaM1 cells is not completely defective since α-CD3 Mabs induce weak signaling [15]. More importantly, the TCR retained the ability to signal normally to the Ras regulatory pathway through phosphorylation of the Shc adaptor protein (Baldari et al., in preparation). Hence p56lck activation appears to play a key role in transducing signals involved not only in priming to apoptosis by CD4, but also in triggering of apoptosis by the TCR/CD3 complex.

These data suggest that CD4 priming of T-cells for antigen dependent apoptosis is due to the inappropriate or partial activation of the p56lck signal transduction pathway. Support for this hypothesis comes from the observations that IL-2 also primes T-cells to activation dependent apoptosis [28], and that p56lck, which is associated with the β-chain of the IL-2 receptor, is activated by IL-2/IL-2 receptor interaction [29]. Furthermore, cyclosporin A, which blocks p56lck signaling [24], also blocks TCR induced apoptosis [30]. Very little is known about signal transduction pathways leading to activation induced apoptosis in lymphocytes (reviewed in [6]). Our data suggest that, in the absence of additional survival signals required for proliferation, p56lck activation plays a key role in this process.

Acknowledgements: The authors wish to thank R. Kurrle for his generous gift of the α-TCRαβ Mab BMA031. They are also indebted to

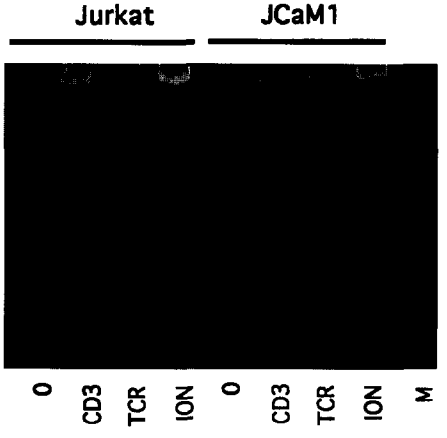

Fig. 3. JCaM1 cells are resistant to TCR/CD3 induced apoptosis. Ethidium bromide staining of an agarose gel of DNA extracted respectively from Jurkat (left) and JCaM1 (right) cells not activated (0) or activated with either α-CD3 Mab (CD3) or α-TCRαβ Mab (TCR) or A23187 (ION; 100 ng/ml). M, molecular weight marker.

A. Weiss for the kind gift of JCaM1 cells, to P. Dubois for the electroporation protocol, to I. Nicoletti for useful suggestions, to S. Grassini for technical assistance and to L.F. Falso and L. Gamberucci for participation to the artwork. This work was supported by the Istituto Superiore di Sanità, AIDS Project and the Italian Association for Cancer Research AIRC. M.M.D.S. is the recipient of a fellowship from AIRC.

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