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Activated *lck* Tyrosine Protein Kinase Stimulates Antigen-Independent Interleukin-2 Production in T Cells

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p56^{lck}, a member of the *src* family of cytoplasmic tyrosine kinases, is expressed predominantly in T cells where it associates with the T-cell surface molecules CD4 and CD8. Mutants of CD4 and CD8 that have lost the ability to associate with p56^{lck} no longer enhance antigen-induced T-cell activation. This suggests that p56^{lck} plays an important role during T-cell activation. In an effort to understand the function of p56^{lck} in T cells, a constitutively activated *lck* gene (F505*lck*) was introduced into T-helper hybridoma cell lines by retroviral infection. In four T-cell lines we examined, the activated *lck* protein stimulated interleukin-2 (IL-2) production, a hallmark of T-cell activation, in the absence of antigenic stimulation. In addition, a marked increase in antigen-independent IL-2 production was apparent when T cells infected with a temperature-sensitive F505*lck* were shifted to the permissive temperature. Only one cell line expressing F505*lck* exhibited increased sensitivity to antigenic stimulation. The SH3 domain of p56^{lck} was dispensable for the induction of antigen-independent IL-2 production. In contrast, deletion of the majority of the SH2 domain of p56^{F505lck} reduced its ability to induce spontaneous IL-2 production markedly. Activated p60$^{c\text{-}src}$ also induced antigen-independent IL-2 production, whereas two other tyrosine kinases, v-*abl* and the platelet-derived growth factor receptor, did not. Tyrosine phosphorylation of a 70-kDa cellular protein was observed after cross-linking of CD4 in T cells expressing F505*lck* but not in cells expressing F527*src*.

p56^{lck} is a member of the *src* family of cytoplasmic tyrosine kinases. It is expressed in essentially all T cells, in some B cells, and in natural killer cells (5, 24, 30, 63, 70). In T cells, p56^{lck} binds to the cytoplasmic tail of the T-cell surface molecules CD4 and CD8 (50, 55, 56, 64, 66). Amino acid residues 10 to 35 in the unique amino terminus of the *lck* protein are responsible for the binding to CD4 and CD8 (55, 64). Recently, interaction between p56^{lck} and the β subunit of the interleukin-2 (IL-2) receptor has been observed in a human lymphoid cell line that constitutively expresses a high level of the β subunit of the IL-2 receptor (14). In vitro studies have located the domain of interaction in p56^{lck} to the amino-terminal half of the catalytic domain, which includes the ATP-binding site (14). The significance of this interaction remains to be determined.

Activation of T-helper cells results from the binding of the T-cell receptor on the surface of a T cell to an antigenic peptide expressed on the surface of antigen-presenting cells as part of a complex with the class II major histocompatibility glycoproteins (MHC). Recognition of the specific antigen by the T-cell receptor initiates a set of signal transduction events including the induction of tyrosine protein phosphorylation (27, 53) and the activation of the phosphatidylinositol second messenger pathway (19, 37, 74). T-helper cells respond to such signal transducing events by secreting either IL-2 or IL-4 and by proliferation.

CD4 and CD8 enhance the response of T cells to antigen, first by increasing adhesion between T cells and antigen-presenting cells through simple binding to class I or class II MHC molecules (10, 13, 39, 47, 49), and second by delivering positive regulatory signals to the T cells (12, 34, 58, 75). However, the requirement for CD4 and CD8 during T-cell activation is not absolute. These two molecules are apparently dispensable for T cells bearing a T-cell receptor with a high affinity for specific antigen, whereas they appear to be crucial for the activation of T cells with low-affinity T-cell receptors (28).

Since p56^{lck} is in physical association with CD4 and CD8, it is likely that p56^{lck} is critical for the realization of the function of CD4 and CD8. First, cross-linking of CD4 or CD8 with anti-CD4 or anti-CD8 results in increased autophosphorylation (27) and activation of the kinase in vitro (27, 67) in some T-cell lines. Second, the ability of CD4 and CD8 to enhance the response of T cells to antigen correlates well with the ability of these molecules to complex with p56^{lck}. Mutations in the cytoplasmic domain of CD4 (12, 34) or CD8 (75) that abolish association with p56^{lck} also lead to an inability of these molecules to enhance IL-2 secretion by T cells in response to stimulation by antigen. Third, introduction of a constitutively activated mutant of p56^{lck} into a CD4$^-$ T-cell hybridoma enhances the ability of this T-cell line to produce IL-2 in response to antigen stimulation (1). Finally, *lck*-deficient mice generated by homologous recombination in embryonic stem cells display profound block in positive selection during thymocyte development and contain virtually no mature functional T cells (35).

Structurally, p56^{lck} is very similar to other members of the *src* family. The C-terminal portion of the molecule comprises a tyrosine kinase domain that is highly conserved among the *src* family members. The tyrosine kinase activity of the enzyme is regulated through the phosphorylation of two major tyrosine residues in this domain. Tyrosine 394 is the autophosphorylation site (6). Mutations removing this site reduce the kinase activity of p56^{lck} (2). Tyrosine 505 in p56^{lck} is homologous to tyrosine 527 in p60$^{c\text{-}src}$. Phosphorylation of this site inhibits the kinase activity of the protein when assayed in vivo (3, 29). The phosphorylation of this site is regulated by the expression of CD45, a T-cell-specific tyrosine phosphatase. Dephosphorylation of tyrosine 505 in-

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duced by CD45 probably leads to the activation of the *lck* kinase (41) and may be important for T-cell activation (25, 26, 45, 73).

Two other domains, the *src* homology 2 (SH2) and the *src* homology 3 (SH3) domains, located amino terminal to the catalytic domain, also regulate the activity or function of the kinase (48, 68). An SH2 domain or domains are present in a number of proteins that are involved in signal transduction, including the *abl* protein tyrosine kinase (43), the *crk* oncoprotein (32), phospholipase C-γ1 (59–61), $p120^{ras\text{-}GAP}$ (62, 69), and the p85 subunit of PI-3 kinase (11, 42, 57). Accumulating evidence suggests that the SH2 domain mediates the interaction between SH2-containing proteins and phosphotyrosine-containing molecules (33). SH3 domains are also found in a number of proteins that are involved in growth control, including $p120^{ras\text{-}GAP}$ (62, 69) and PLC-γ1 (59–61), and in the cytoskeletal protein spectrin (72). It could mediate interactions with the cytoskeleton.

Both SH2 and SH3 domains are important for regulating the transforming activity of the *src* protein (15, 23, 38, 46, 54, 71) and the *lck* kinase (48, 68). Mutations in the SH2 domain render v-*src* entirely or partially defective for transformation of fibroblasts (15, 38, 71). The SH2 domain of the F505*lck* protein is also required for full transformation of fibroblasts (48, 68). Deletion of the SH3 domain activates the c-*src* kinase (54) and the *lck* kinase (48, 68). However, the SH3 domain is not required for the transforming activity of the F505*lck* protein (48, 68).

We are interested in understanding the role of $p56^{lck}$ during T-cell activation. Specifically, we asked what effect an activated $p56^{lck}$ had on T-cell activation in four different T-helper cell lines that express different levels of CD4. Second, we examined the importance of the SH2 and SH3 domains in $p56^{lck}$ in T-cell activation. Third, we wanted to know whether other activated cytoplasmic tyrosine kinases or a growth factor receptor tyrosine kinase, when expressed in T cells, can function in a manner similar to $p56^{lck}$. The effect of an activated *src* kinase, the v-*abl* oncoprotein, and the platelet-derived growth factor (PDGF) receptor on IL-2 production was therefore examined.

MATERIALS AND METHODS

Cells. DO11.10 and 3DO18.3 are $CD4^+$ T-cell hybridomas that are specific for chicken ovalbumin and $I\text{-}A^d$ (28). DO11.10AG8.OU1 is a $CD4^-$ derivative of DO11.10 cells (28). 2B4 is a $CD4^-$, $CD3^+$, pigeon cytochrome *c*-specific T-helper hybridoma (52). The cells listed above were grown in Dulbecco-Vogt modified Eagle's medium supplemented with 10% fetal bovine serum, 5×10^{-5} M β-mercaptoethanol, 1× nonessential amino acids, and 1× sodium pyruvate. NK, an IL-2- and IL-4-dependent cell line, was grown in the above medium plus 5% conditioned medium from EL-4 cells. COS-7, a monkey kidney cell line, was grown in Dulbecco-Vogt modified Eagle's medium supplemented with 10% calf serum.

Transfection and infection. COS-7 cells were transfected with SV-ψ^--E-MLV plus the following constructs according to the protocols described previously (36): ts-F505*lck* (M439S481F505*lck*) (18), wt*lck*, F505*lck*, ΔSH2F505*lck*, ΔSH3F505*lck*, ΔSH3/2F505*lck* (48), PDGFR-β (kindly provided by Jon Cooper), all in LXSN; v-*abl* in pSRαMSVtkneo (36); and F527*src* and c-*src* in pneoMLV (22).

T cells were infected by cocultivation with the transfected COS-7 cells. Briefly, 48 h after transfection of COS-7 cells, the medium was removed, and T cells were added to the COS-7 cells in the presence of 3 μg of polybrene per ml. T cells were transferred 24 h later to a new dish and incubated for an additional 24 h. Infected cells were then selected by growth in medium containing G418 (1.3 mg/ml for DO11.10 cells, and 1.5 mg/ml for 2B4 cells). For selection, approximately 3×10^4 cells were seeded in 2 ml of medium containing G418 in each well of a 24-well cluster plate. Usually 10 days after selection, infected cells were found to be growing in all 24 wells. Giving this frequency of drug-resistant cells, each well probably contained a mixed population derived from more than one infected precursor. The cells from a single well were therefore called a pool of cells.

IL-2 secretion assays and antigen stimulation. To assay for spontaneous IL-2 release, 10^5 T cells were collected, washed, and seeded in a 96-well plate in 200 μl of growth medium. After incubation for 24 h, supernatants were removed and assayed for IL-2 content.

For antigenic stimulation, 1×10^5 T cells were incubated with 5×10^5 antigen-presenting cells (BALB/c spleen cells for DO11.10 cells, and B10.BR spleen cells for 2B4) in the presence of various amount of peptide antigen (CNBr-digested chicken ovalbumin for DO11.10 cells, and CNBr-digested pigeon cytochrome *c* for 2B4 cells) for 24 h at 37°C. Supernatants were removed and assayed for IL-2 content.

To assay for secreted IL-2, 5,000 NK cells were cultured overnight with serial dilutions of the T-cell supernatants. One microcurie of [^{3}H]thymidine was then added, and the cells were harvested 16 h later and the uptake of [^{3}H]thymidine quantified by scintillation counting. Total units of IL-2 per well was determined by comparison to a recombinant murine IL-2 standard (Biosource).

Treatment of cells and Western blotting. Cross-linking of CD4 or CD3 on T cells was carried out as described before (27). Briefly, T cells were incubated on ice for 30 min in the presence of an excess amount of rat monoclonal antibody GK1.5 (anti-CD4) or hamster monoclonal antibody 145-2C11 (anti-CD3ε). After the excess antibodies were washed away, cells were suspended in serum-free medium. Cross-linking was performed at 37°C for 1 to 2 min with rabbit anti-rat antibodies or goat anti-hamster antibodies. Cells were lysed by the addition of 2× sodium dodecyl sulfate (SDS)-gel sample buffer, and phosphotyrosine-containing proteins were assayed by Western immunoblotting as previously described (21) using a polyclonal anti-phosphotyrosine antibody (21).

For the stimulation of PDGF receptor-infected cells, PDGF-BB (Amgen) was added to the cells at a final concentration of 50 ng/ml for 10 min at 37°C.

Immunoprecipitation and in vitro kinase assays. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer as described elsewhere (17). For the immunoprecipitation of $p60^{src}$, monoclonal antibody 327 was used. An anti-peptide antibody against amino acid residues 39 to 64 of $p56^{lck}$ (kindly provided by Andrey Shaw) was used in the immunoprecipitation of various deleted forms of *lck* proteins. An anti-$p15^{gag}$ antibody was used in the immunoprecipitation of $p160^{v\text{-}abl}$.

In vitro kinase assays with immunoprecipitates were carried out as previously described (17).

RESULTS

Constitutively activated $p56^{lck}$ induces spontaneous IL-2 production. A constitutively activated mutant form of *lck* (F505*lck*) was introduced into the $CD4^+$ DO11.10 T-cell hybridoma line by retroviral infection, by using a retroviral

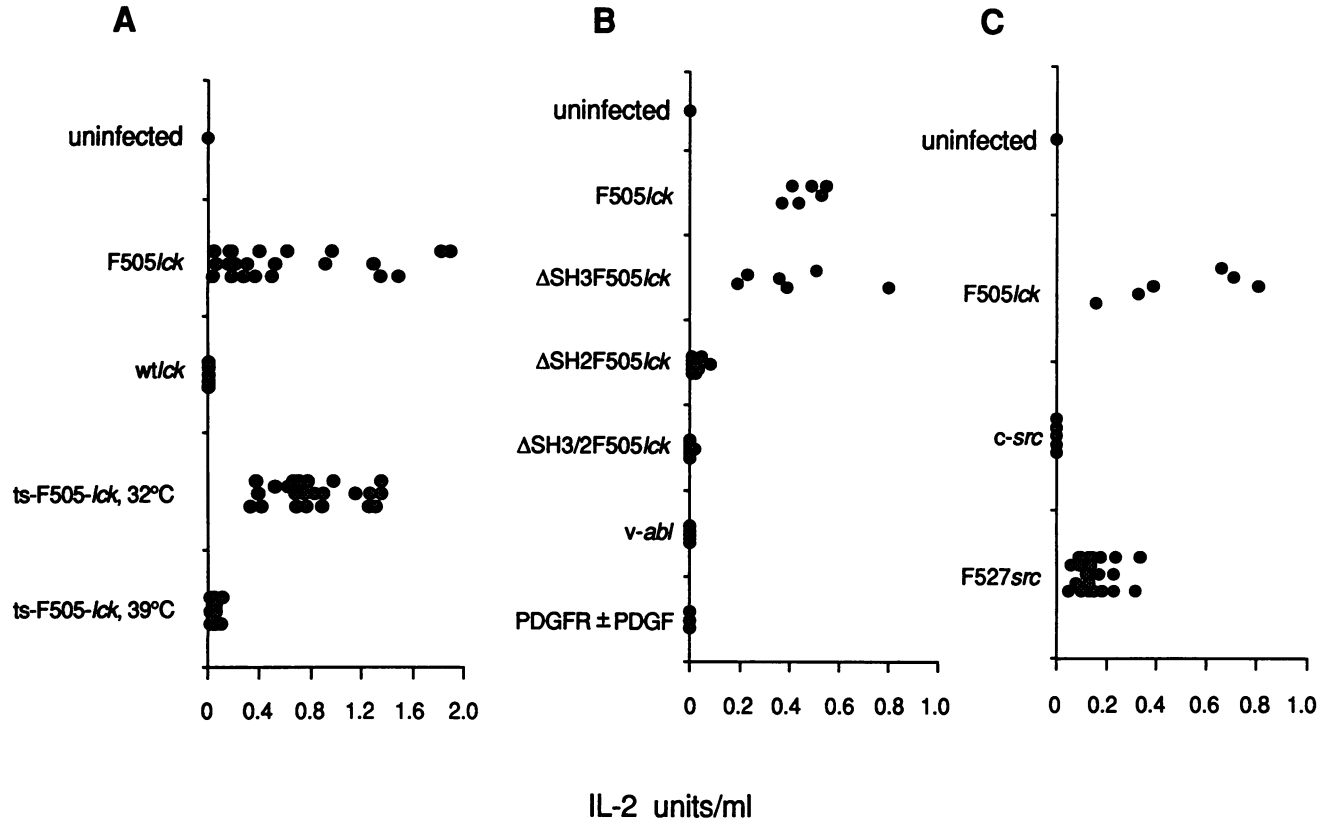

FIG. 1. Antigen-independent IL-2 production by DO11.10 cells. DO11.10 cells were infected with retroviruses encoding the constructs as indicated. G418-resistant pools were selected and assayed for spontaneous IL-2 secretion as described in Materials and Methods. Results from three sets of experiments are shown here. Each circle represents the amount of IL-2 produced by a pool of infected DO11.10 cells.

vector also expressing a neomycin resistance marker. Normally, DO11.10 cells produce IL-2 only in response to stimulation by ovalbumin/I-A^d. Pools of cells expressing $p56^{F505lck}$ were generated by selection with G418, and the effect of activated $p56^{lck}$ on IL-2 production was examined. The constitutively activated *lck* (F505*lck*) stimulated IL-2 production in the absence of antigenic stimulation in 23 of the 24 pools of infected DO11.10 cells (Fig. 1A). Anti-IL-2 antibody neutralized the growth-promoting activity produced by the cells, confirming that it was indeed IL-2 that was produced (data not shown). Uninfected DO11.10 cells, as well as 26 pools of DO11.10 cells infected with wt*lck*, did not produce detectable amounts of IL-2 in the absence of antigenic stimulation (Fig. 1A). The level of spontaneous IL-2 production by F505*lck*-infected DO11.10 cells was approximately 10% of the amount of IL-2 produced when these cells were stimulated with the optimal concentration of antigen (see below). Clones of DO11.10 cells infected with F505*lck* were also isolated from these pools by limiting dilution, and similar results were obtained with these clones (data not shown).

To confirm that the spontaneous IL-2 production is, in fact, due to the presence of the activated *lck* protein, a temperature-sensitive F505*lck* was introduced into DO11.10 cells by using the same retroviral vector. This temperature-sensitive F505*lck* (ts-F505*lck*) transforms fibroblasts at 32°C but not at 39°C (18). DO11.10 cells infected with a virus encoding ts-F505*lck* were selected at 39°C. In 21 of the 24 pools of cells infected with ts-F505*lck*, spontaneous production of a low-level IL-2 was detected at 39°C. When the cells were shifted to 32°C, antigen-independent IL-2 production increased dramatically in 23 of the 24 pools (Fig. 1A). The increase in IL-2 production produced by a shift to the permissive temperature ranged from 10- to more than 95-fold (data not shown). Only cells infected with ts-F505*lck* exhibited temperature sensitivity. Uninfected DO11.10 cells did not produce IL-2 spontaneously at either 39°C or 32°C (Fig. 1). Additionally, growth of DO11.10 cells expressing F505*lck* at 32°C did not increase the level of antigen-independent IL-2 production (data not shown).

Abraham et al. have reported that F505*lck* can render a normally unresponsive CD4-negative beef insulin-specific BI-141 helper hybridoma cell line responsive to antigenic stimulation but that it does not induce spontaneous IL-2 production (1). To examine whether the antigen-independent IL-2 secretion observed in DO11.10 cells expressing F505*lck* was unique to this cell line, similar experiments were carried out with three other T-helper hybridoma cell lines. Two of these are also ovalbumin specific: a $CD4^+$ cell line, 3DO18.3, and a $CD4^-$ cell line, DO11.10AG8.OU1. Of 12 pools of 3DO18.3 cells infected with ts-F505*lck*, 11 showed no detectable spontaneous IL-2 production at the nonpermissive temperature. However, 24 h after shift to the permissive temperature, all 12 pools showed marked spontaneous IL-2 production (Fig. 2A). Of 12 pools of $CD4^-$ DO11.10AG8.OU1 cells infected with ts-F505*lck*, 5 produced a low level of IL-2 spontaneously at the nonpermissive temperature, and all 12 pools showed spontaneous IL-2 production at the permissive temperature (Fig. 2B).

2B4 is a CD4-negative, pigeon cytochrome *c*-specific

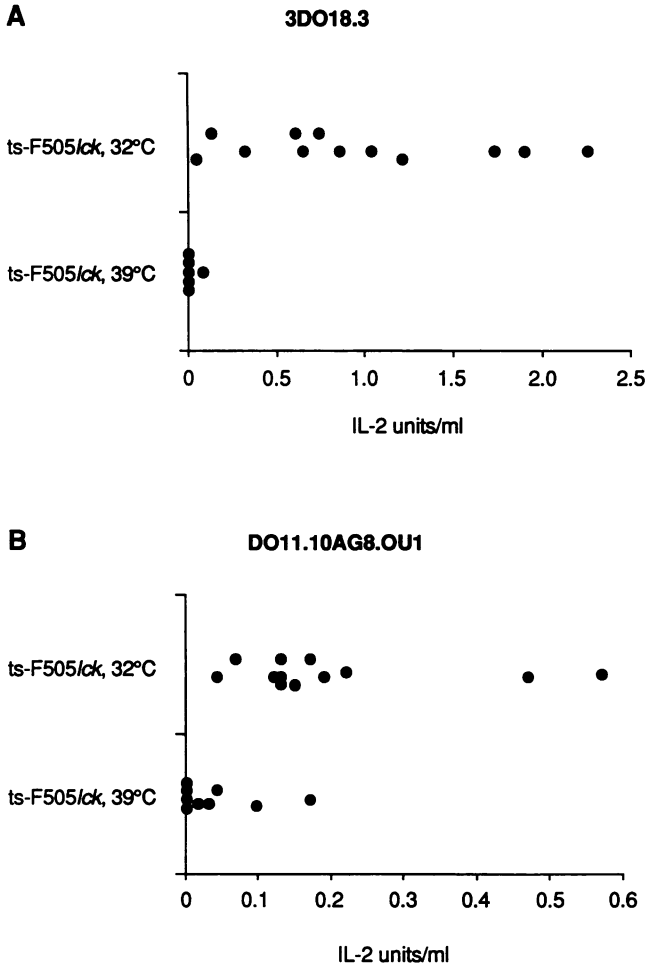

FIG. 2. Antigen-independent IL-2 production by 3DO18.3 cells and DO11.10AG8.OU1 cells. T cells were infected with retroviruses encoding the temperature-sensitive F505*lck* as described in Materials and Methods. G418-resistant pools were selected and assayed for spontaneous IL-2 secretion. Each circle represents the amount of IL-2 produced by a pool of infected cells. (A) 3DO18.3 cells; (B) DO11.10AG8.OU1.

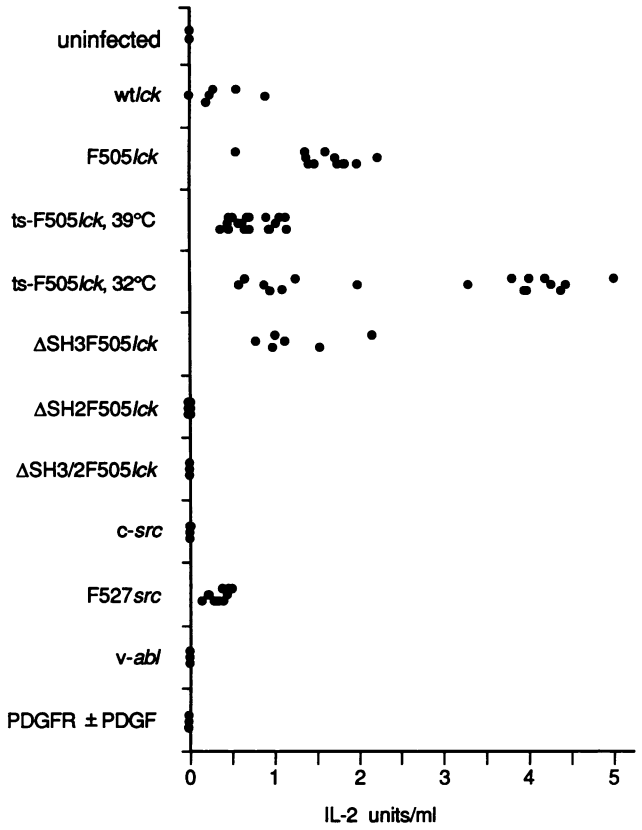

FIG. 3. Antigen-independent IL-2 production by 2B4 cells. 2B4 cells were infected with retroviruses encoding the constructs as indicated. G418-resistant pools were selected and assayed for spontaneous IL-2 secretion as described in Materials and Methods. Each circle represents the amount of IL-2 produced by a pool of infected 2B4 cells.

T-helper hybridoma. Expression of F505*lck* in 2B4 cells stimulated spontaneous IL-2 production (Fig. 3). Surprisingly, expression of wt*lck* from the retroviral vector also induced a low level of IL-2 production in all the infected pools (Fig. 3). An elevated level of the *lck* protein was detected in 2B4 cells infected with either wt*lck* or F505*lck* by Western blotting analysis (data not shown). Since wild-type $p56^{lck}$ normally is only partially phosphorylated at Tyr-505 and therefore partially active, it is possible that an elevated level of wild-type $p56^{lck}$ increased the *lck* kinase activity to a level sufficient to stimulate IL-2 production partially.

When ts-F505*lck* was introduced into 2B4 cells, a low level of spontaneous IL-2 production was apparent at the nonpermissive temperature (Fig. 3). A 3- to 10-fold induction of IL-2 production was observed when cells were shifted to the permissive temperature. The leakiness at 39°C, together with the slight effect of wt*lck* on IL-2 production, suggested that 2B4 might be very sensitive to *lck* protein kinase activity.

Together, these data suggested that the activated *lck* protein, when introduced into antigen-specific T-cell lines, is capable of inducing IL-2 production in the absence of antigenic stimulation. The effect of F505*lck* on these T cells is not due to increased expression of surface CD3 and CD4 molecules, since all infected cells expressed levels of CD3 and CD4 comparable to those in uninfected cells when analyzed by fluorescence flow cytometry (data not shown).

Role of SH2 and SH3 domains in stimulation of spontaneous IL-2 release. To assess the importance of the SH3 and SH2 domains of *lck* protein, deletion mutants of F505*lck* lacking (i) all of the SH3 domain, (ii) the majority of the SH2 domain, and (iii) both SH3 and SH2 domains (48) were introduced into both DO11.10 cells and 2B4 cells by retroviral infection. The expression of these deletion mutants in the T cells was quantified by assaying the ability of the mutant proteins to autophosphorylate in in vitro kinase assays after immunoprecipitation. This should be a gauge of protein level, since the deletion mutants have in vitro kinase activities similar to those of wild-type protein (47a). The level of expression of the SH2 deletion mutant was, in general, lower than that of the SH3 deletion mutant. There were, however, at least two pools of DO11.10 cells infected with the ΔSH2 mutant that expressed the ΔSH2 protein at the same level as pools infected with the ΔSH3 mutant (data not shown).

Deletion of the SH3 domain did not affect the ability of F505*lck* protein to induce spontaneous IL-2 production in either DO11.10 cells (Fig. 1B) or 2B4 cells (Fig. 3). In fact, the amount of IL-2 being produced by cells infected with ΔSH3F505*lck* was comparable to that produced by cells

infected with F505*lck* (Fig. 1B and Fig. 3). In contrast, deletion of majority of the SH2 domain reduced the ability of F505*lck* to induce spontaneous IL-2 production by more than 90% in both DO11.10 cells (Fig. 1B) and 2B4 cells (Fig. 3). Deletion of both the SH2 and SH3 domains completely abolished the ability of the F505*lck* protein to activate T cells (Fig. 1B and Fig. 3). However, the level of the mutant *lck* proteins in cells expressing the ΔSH3/SH2F505*lck* was considerably lower than that in cells expressing the ΔSH3F505*lck* (data not shown).

The effect of other activated tyrosine kinase on the spontaneous IL-2 production. We then asked whether spontaneous IL-2 production can also be induced by other activated tyrosine kinases, especially another member of the *src* family. The activated c-*src* protein was examined first. Retroviruses encoding either wild-type c-*src* or a c-*src* protein activated by mutation of tyrosine 527 to phenylalanine were used to infect DO11.10 cells and 2B4 cells. The c-*src* protein is normally undetectable in DO11.10 and 2B4 cells. Cells infected with either c-*src* or F527*src* showed a high level of *src* kinase activity, as assayed by autophosphorylation after immunoprecipitation (data not shown).

In both DO11.10 cells and 2B4 cells, expression of F527*src* stimulated spontaneous IL-2 production, albeit at a lower level than that stimulated by F505*lck* (Fig. 1C and Fig. 3). Expression of wild-type c-*src* had no effect in DO11.10 cells (Fig. 1C) but induced a very low level of IL-2 secretion in 7 of the 12 infected 2B4 pools (Fig. 3).

The v-*abl* gene is a mutated and constitutively activated form of the cellular proto-oncogene c-*abl*. Previous studies have shown that v-*abl* is capable of rendering lymphokine-dependent cells lymphokine independent through a nonautocrine mechanism (8, 9, 31, 44). The v-*abl* gene was introduced into DO11.10 cells and 2B4 cells by retroviral infection. By immune complex kinase assay, the $p160^{v\text{-}abl}$ protein was detected readily in immunoprecipitates prepared from infected cells (Fig. 4A). Nevertheless, the infected cells did not produce IL-2 (Fig. 1B and Fig. 3).

The PDGF receptor is a cell surface tyrosine kinase that is activated by the binding of PDGF. To examine whether activation of the PDGF receptor could stimulate the T-cell activation pathway, a retrovirus encoding PDGFR-β was used to infect DO11.10 and 2B4 cells. The expression of PDGFR-β in infected cells was confirmed by examining the tyrosine phosphorylation of PDGFR following the addition of PDGF (Fig. 4B). Uninfected 2B4 cells do not express a PDGFβ receptor (Fig. 4B, lanes 5 and 6). After the addition of 50 ng of PDGF-BB per ml, tyrosine phosphorylation of the PDGF receptor in the infected 2B4 cells was stimulated as assayed by Western blotting of whole-cell lysates by using anti-phosphotyrosine antibodies (Fig. 4B, lane 8). Both infected and uninfected DO11.10 cells displayed tyrosine phosphorylation of the PDGF receptor upon addition of PDGF-BB (Fig. 4B, lanes 2 and 4). However, IL-2 production by the DO11.10 cells or 2B4 cells expressing the PDGFR was not detected (Fig. 1B and Fig. 3) either in the presence or absence of PDGF.

Effects of activated *lck* protein on antigen-stimulated T-cell activation. We next examined the ability of the activated *lck* protein to enhance T-cell responsiveness to antigenic stimulation. DO11.10 cells and 2B4 cells expressing F505*lck* were stimulated with increasing concentrations of antigen in the presence of antigen-presenting cells. The amount of IL-2 produced by the infected cells was then quantified. No difference in the antigen-stimulated production of IL-2 between the infected and uninfected DO11.10 cells was detected (Fig. 5A). DO11.10 cells infected with the temperature-sensitive F505*lck* also did not show any difference in responsiveness to antigen between the permissive and nonpermissive temperatures (data not shown). Introduction of F505*lck* into the $CD4^-$ DO11.10AG8.OU1 cells did not induce any alteration in the antigen-dependent IL-2 production, either (data not shown). In contrast, in 2B4 cells expressing F505*lck* proteins, an approximately two- to three-fold-increased sensitivity to antigen was exhibited (Fig. 5B), whereas no difference in responsiveness to antigen was found between 2B4 cells infected with wt*lck* and uninfected cells (data not shown).

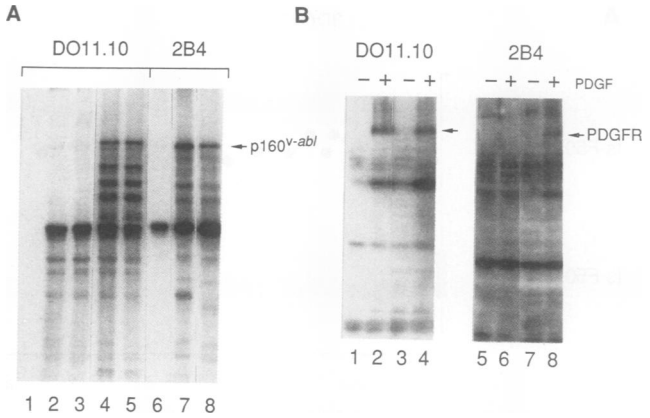

FIG. 4. Expression of $p160^{v\text{-}abl}$ and PDGF receptor in DO11.10 and 2B4 cells. (A) Immunoprecipitates were prepared from 5×10^5 DO11.10 cells or 2B4 cells with a control serum (lanes 1 and 3) or anti-$p15^{gag}$ antibodies (lanes 2 and 4 to 8) and then subjected to in vitro kinase assays as described in Materials and Methods. Lanes: 1, uninfected DO11.10 cells, control serum; 2, uninfected DO11.10 cells, anti-$p15^{gag}$; 3, DO11.10/v-*abl*-8, control serum; 4, DO11.10/v-*abl*-8, anti-$p15^{gag}$; 5, DO11.10/v-*abl*-9, anti-$p15^{gag}$; 6, uninfected 2B4 cells, anti-$p15^{gag}$; 7, 2B4/v-*abl*-5, anti-$p15^{gag}$; 8, 2B4/v-*abl*-10, anti-$p15^{gag}$. (B) DO11.10 and 2B4 cells were treated with PDGF-BB at a concentration of 50 ng/ml for 10 min at 37°C. Total cellular proteins were prepared from 3×10^5 cells before and after treatment and resolved on a 10% low-bis-SDS-polyacrylamide gel. Tyrosine protein phosphorylation was analyzed by Western blotting with anti-phosphotyrosine antibodies as described in Materials and Methods. Lanes: 1, uninfected DO11.10 cells, no PDGF-BB; 2, uninfected DO11.10 cells, 50 ng of PDGF-BB per ml; 3, DO11.10/PDGFR-3, no PDGF-BB; 4, DO11.10/PDGFR-3, 50 ng of PDGF-BB per ml; 5, uninfected 2B4 cells, no PDGF-BB; 6, uninfected 2B4 cells, 50 ng of PDGF-BB per ml; 7, 2B4/PDGFR-7, no PDGF-BB; 8, 2B4/PDGFR-7, 50 ng of PDGF-BB per ml.

Tyrosine phosphorylation of cellular proteins. The signal transduction function of tyrosine kinases is accomplished through the phosphorylation of cellular polypeptide substrates. To better understand how the F505*lck* protein triggered spontaneous IL-2 production, total cellular proteins were prepared from infected and uninfected cells, and tyrosine phosphorylation was examined by Western blotting with antibodies to phosphotyrosine.

In the absence of stimulation, T cells infected with F505*lck* did not show any detectable alteration in the tyrosine protein phosphorylation (Fig. 6A, lane 3; Fig. 6B, lane 5). A similar result was obtained by Abraham et al. (1). Tyrosine phosphorylation of a 70-kDa cellular protein (p70) occasionally was detected, especially in the clones of infected DO11.10 cells (data not shown), but not in most of the pools of infected cells. Cross-linking of CD4 stimulated the tyrosine phosphorylation of $p56^{lck}$ in uninfected cells (Fig. 6A and

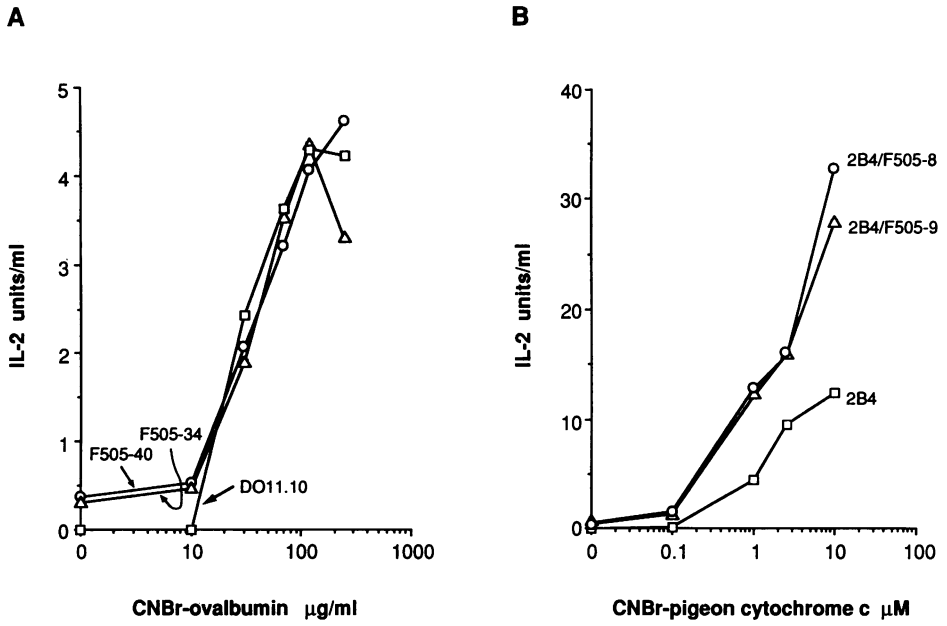

FIG. 5. Effects of an activated *lck* protein on the antigen dependence of IL-2 production. DO11.10 (A) and 2B4 (B) cells were cultured in the presence of antigen-presenting cells and increasing concentrations of CNBr-digested antigen peptides. IL-2 release was measured in the supernatant after 24 h as described in Materials and Methods.

Fig. 6B, lanes 2), as expected (27, 65). In cells expressing F505*lck*, cross-linking of CD4 stimulated the tyrosine phosphorylation of both $p56^{lck}$ and a 70-kDa protein (Fig. 6A, lanes 4; Fig. 6B, lane 6). Phosphorylation of p70 was not obvious in DO11.10 cells infected with F527*src* even after cross-linking of CD4 (Fig. 6A, lane 6). Phosphorylation of a 70-kDa protein was also not detectable in either v-*abl* or PDGF receptor-expressing cells (data not shown). This suggests that p70 might be a specific substrate for $p56^{lck}$. The fact that F527*src* was capable of activating IL-2 production without stimulating the phosphorylation of p70 suggested, however, that phosphorylation of p70 might not be critical for the activation of T cells.

Cross-linking of the T-cell receptor complex by antibody to CD3 or co-cross-linking of CD3 and CD4 led to the phosphorylation of several cellular proteins, including p120, p105, p90, p80, p75, p70, p60, and p40 on tyrosine (Fig. 6B, lanes 3 and 4) (16, 20, 51). Cells infected with F505*lck* showed a slightly increased level of phosphorylation of these cellular proteins (Fig. 6B, lanes 7 and 8), especially p60, p70, and p75, with the pattern of phosphorylation remaining the same after cross-linking of CD3 or co-cross-linking of CD3 and CD4.

DISCUSSION

We have found that at least four T-helper cell lines expressing a constitutively activated *lck* tyrosine kinase exhibited antigen-independent production of IL-2. The fact that spontaneous IL-2 production was stimulated dramatically when T cells infected with temperature-sensitive F505*lck* were shifted to the permissive temperature argues strongly that it is the activity of the *lck* kinase that is responsible for the spontaneous IL-2 production rather than a secondary change that occurred during the selection of the cells. Since F505*lck* induces antigen-independent IL-2 production in both $CD4^+$ and $CD4^-$ cells, association of $p56^{lck}$ with CD4 does not seem to be essential. The level of antigen-independent IL-2 production in the cell lines expressing the activated *lck* protein was lower than that induced by antigenic stimulation. This indicates that only a partial activation of the T cells was induced by the activated *lck* tyrosine kinase. It is likely that the stimulation of IL-2 production was induced by the phosphorylation of cellular proteins involved in T-cell activation by F505*lck* protein.

These results differ somewhat from those reported by Abraham et al. They found that a similar activated *lck* protein rendered a normally nonresponsive $CD4^-$ T-helper cell hybridoma responsive to antigenic stimulation but did not induce IL-2 production in the absence of antigen (1). Of the four cell lines we studied, including two $CD4^-$ cell lines, only the 2B4 cell line showed an enhanced antigen responsiveness after the introduction of activated $p56^{lck}$. This discrepancy between our results and those of Abraham may result from the differences in the affinities of different T-cell receptors for specific antigen in the cell lines examined. Additionally, all of the cell lines we examined express normal, readily detectable amounts of endogenous $p56^{lck}$ and are responsive to antigen without the introduction of the activated *lck* gene. In contrast, the BI-141 cell line used by Abraham et al. expresses a very low level of endogenous $p56^{lck}$ and does not respond to antigen stimulation (1).

$p56^{lck}$ contains an SH2 and an SH3 domain, both of which may regulate its function (48, 68). The SH3 domain of $p56^{F505lck}$ was found to be dispensable for stimulation of IL-2 production in both DO11.10 and 2B4 cells. This is consistent with the observation that this domain was not required for the transformation of fibroblasts by the F505*lck* protein (48, 68). However, this finding is different from that reported by Caron et al., who found that a similar, but not identical, deletion of the SH3 domain of the F505*lck* protein abolished the ability of the *lck* molecule to enhance antigen-dependent T-cell responsiveness in BI-141 cells (4). We do not know whether this is due to the difference in SH3 domain deletion

or in the cell lines examined. Deletion of the SH2 domain reduced the ability of F505*lck* to induce antigen-independent production of IL-2. The level of expression of ΔSH2F505*lck* protein in most of the infected T cells was, however, lower than that of F505*lck* or ΔSH3F505*lck*. The low level of spontaneous IL-2 production from T cells infected with ΔSH2F505*lck* could be due in these circumstances to insufficient expression of the protein. However, we isolated at least two pools of DO11.10 cells expressing ΔSH2F505*lck* that contained amounts of the *lck* protein comparable to those in pools of cells expressing ΔSH3F505*lck*. IL-2 production by the two pools of cells expressing the ΔSH2 mutant was less than 20% of that produced by the pools of cells containing the ΔSH3 mutant. This suggests that the reduced IL-2 production by ΔSH2 F505*lck* is not due solely to insufficient expression of the protein, but rather is due to the lack of the SH2 domain. Consistent with this is the finding by Caron et al. that deletion of SH2 domain abolished the ability of $p56^{lck}$ to enhance the response of BI-141 cells to antigenic stimulation (4). Since SH2 domains mediate interactions with phosphotyrosine-containing proteins, it is possible that the SH2 domain of $p56^{lck}$ is important for the interaction between the *lck* protein and specific cellular substrates or regulatory proteins.

In addition to the activated *lck* kinase, we found that activated $p60^{c\text{-}src}$ can also induce antigen-independent IL-2 production when introduced into T cells. This agrees with the result obtained by O'Shea et al., in which v-*src* was shown to induce antigen-independent IL-2 production in 2B4 cells (40). Therefore, the ability to induce IL-2 production in the absence of antigen is not an *lck*-specific phenomenon. Since $p56^{lck}$ and $p60^{c\text{-}src}$ are closely related, they may well interact with the same set of cellular substrates. Two other tyrosine kinases, v-*abl* and PDGF receptor (in the presence of PDGF), had no significant effect on IL-2 production. Although it is difficult to interpret negative results, this may suggest a functional difference between *src* and non-*src* tyrosine protein kinases.

In contrast to what is seen in fibroblasts, introduction of F505*lck*, F527*src* (Fig. 6), or v-*abl* (data not shown) genes into DO11.10, 2B4 or BI-141 (1) cells did not cause any detectable alteration in the tyrosine phosphorylation of cellular proteins in the absence of stimulation. Cells expressing F505*lck* often exhibit augmented tyrosine phosphorylation following cross-linking of the T-cell receptor (Fig. 6) (1). Phosphorylation of one cellular protein, p70, was stimulated specifically in cells infected with F505*lck* following cross-linking of CD4. This phosphorylated protein was not detected in cells expressing F527*src* nor in cells expressing v-*abl* and PDGF receptor (data not shown). This suggests that p70 might be a specific substrate of $p56^{lck}$. Phosphorylation of p70 does not seem, however, to be absolutely required for antigen-independent IL-2 production, since IL-2 was produced spontaneously in F527*src*-infected cells in which no phosphorylation of p70 was detectable. On the other hand, phosphorylation of p70 might be important for the optimal T-cell activation, since the level of IL-2 production in cells expressing F527*src* was lower than that in cells expressing F505*lck*.

The identity of p70 is currently not known. Chan et al. have reported that a 70-kDa T-cell protein, ZAP-70, is phosphorylated on tyrosine and associated with the ζ-subunit of T-cell receptor upon activation of the Jurkat T-cell line (7). Whether the p70 phosphorylated by $p56^{F505lck}$ is the ZAP-70 protein remains to be determined.

Our results show that $p56^{lck}$, when activated, can induce expression of the IL-2 gene in the absence of stimulation of the T-cell antigen receptor, albeit at a lower level than that in the presence of antigenic stimulation. This is consistent with the idea that $p56^{lck}$ plays an augmenting role in the induction of IL-2 production during antigen-dependent T-cell activation, as has been suggested previously.

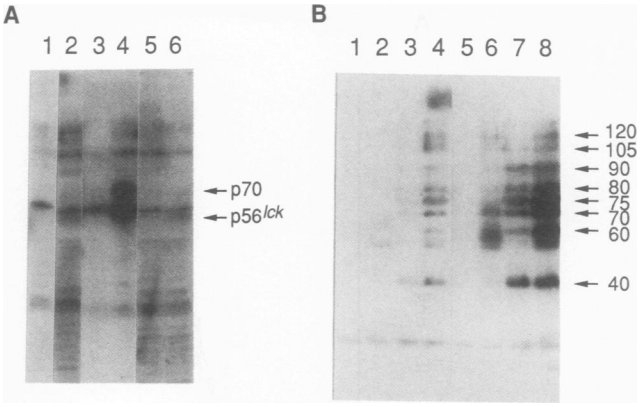

FIG. 6. Effects of an activated *lck* protein on the tyrosine protein phosphorylation. (A) Total cellular proteins were prepared from 3×10^5 uninfected (lanes 1 and 2) and infected (lanes 3 to 6) DO11.10 cells before (lanes 1, 3, and 5) and after (lanes 2, 4, and 6) cross-linking of CD4. Tyrosine phosphorylation was analyzed by Western blotting with anti-phosphotyrosine antibodies as described in Materials and Methods. Lanes: 1, uninfected DO11.10 cells, untreated; 2, uninfected DO11.10 cells, cross-linked with anti-CD4 plus RAR; 3, DO11.10/F505*lck* cells, untreated; 4, DO11.10/F505*lck* cells, with anti-CD4 plus RAR; 5, DO11.10/F527*src* cells, untreated; 6, DO11.10/F527*src* cells, with anti-CD4 plus RAR; (B) Uninfected (lanes 1 to 4) and F505*lck*-infected (lanes 5 to 8) 3DO54.8 cells were incubated with anti-CD4 (lanes 2 and 6), anti-CD3 (lanes 3 and 7), or both anti-CD3 and anti-CD4 (lanes 4 and 8) and then cross-linked with rabbit anti-rat (RAR) for CD4 or goat anti-hamster (GAH) for CD3 as described in Materials and Methods. Total cellular protein was prepared from 3×10^5 cells, and tyrosine protein phosphorylation was analyzed by Western blotting with anti-phosphotyrosine antibodies. Lanes: 1, uninfected cells, untreated; 2, uninfected cells, cross-linked with anti-CD4 plus RAR; 3, uninfected cells, cross-linked with anti-CD3 plus GAH; 4, uninfected cells, co-cross-linked with anti-CD3 and anti-CD4 plus RAR and GAH; 5, F505*lck*-infected cells, untreated; 6, F505*lck*-infected cells, cross-linked with anti-CD4 plus RAR; 7, F505*lck*-infected cells, cross-linked with anti-CD3 plus GAH; 8, F505*lck*-infected cells, co-cross-linked with anti-CD3 and anti-CD4 plus RAR and GAH.

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