Interaction of the IL-2 Receptor with the *src*-Family Kinase $p56^{lck}$: Identification of Novel Intermolecular Association

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In the interleukin-2 (IL-2) system, intracellular signal transduction is triggered by the β chain of the IL-2 receptor (IL-2Rβ); however, the responsible signaling mechanism remains unidentified. Evidence for the formation of a stable complex of IL-2Rβ and the lymphocyte-specific protein tyrosine kinase $p56^{lck}$ is presented. Specific association sites were identified in the tyrosine kinase catalytic domain of $p56^{lck}$ and in the cytoplasmic domain of IL-2Rβ. As a result of interaction, IL-2Rβ became phosphorylated in vitro by $p56^{lck}$. Treatment of T lymphocytes with IL-2 promotes $p56^{lck}$ kinase activity. These data suggest the participation of $p56^{lck}$ as a critical signaling molecule downstream of IL-2R via a novel interaction.

INTERLEUKIN-2 (IL-2) IS A CRITICAL REGULATOR OF LYMPHOcyte proliferation. The receptor for IL-2 (IL-2R) comprises at least two distinct polypeptides, the IL-2Rα (p55) and IL-2Rβ (p70–75) chains, that bind IL-2 with low and intermediate affinities (K_d, ~10 nM and ~1 nM), respectively. Together the two receptor chains constitute the high-affinity IL-2R (K_d, ~10 pM) by forming a noncovalently linked heterodimer (*1*). Proliferative signals are delivered by the high- and intermediate-affinity IL-2 receptors, but not by the low-affinity one (*2*).

Both human IL-2Rα and IL-2Rβ chains are typical type-I membrane proteins with a single transmembrane region (*3, 4*). Moreover, IL-2Rβ belongs to the growing family of cytokine receptors (*5*). Whereas the length of the extracellular domains of both chains is similar (219 and 214 amino acid residues for IL-2Rα and IL-2Rβ, respectively), the cytoplasmic domain of the IL-2Rβ (286 residues) is larger than that of IL-2Rα (13 residues). The signal transducing component of the IL-2R is IL-2Rβ (*2*). Although there is a high degree of sequence similarity between human and murine IL-2Rβ cytoplasmic regions, no obvious catalytic component has been discernible (*4, 6*). This may imply that interaction of the cytoplasmic region with other molecules is important in downstream signaling. Transfection of IL-2Rβ cDNA's into murine interleukin-3 (IL-3)–dependent hemopoietic cell lines permitted identification of one particular region of the IL-2Rβ chain, designated the "serine-rich" region, which is important for IL-2–induced cell growth (*7*). However, the biochemical mechanism underlying IL-2 signaling mediated through IL-2Rβ remains obscure.

Recent studies have demonstrated that IL-2 provokes a rapid increase in tyrosine phosphorylation of cellular substrates including the IL-2Rβ chain itself (*8, 9*). In addition, evidence has been provided for the coupling of a protein tyrosine kinase activity with the IL-2R (*10*). These observations suggest that activation of one or more protein tyrosine kinases occurs during the early phase of intracellular signal transduction by the interaction of IL-2 and IL-2R.

Among the protein tyrosine kinases, those of the *src*-family are particularly attractive candidates as IL-2R signal mediators. They are plasma membrane-associated proteins consisting of eight well-characterized members ($p60^{src}$, $p56^{lyn}$, $p59^{fyn}$, $p59^{yes}$ or $p62^{yes}$, $p56^{lck}$, $p59^{hck}$, $p55^{fgr}$, and $p55^{blk}$) (*11, 12*), and have been shown to participate in lymphocyte signaling in three well-defined cases: $p56^{lck}$ with CD4 and CD8 (*13*), $p59^{fyn}$ with T cell receptor (TCR)–CD3 complex (*14*), and $p56^{lyn}$ with membrane-bound immunoglobulin M (IgM) (*15*). These examples show that *src*-family kinases can couple lymphocyte surface receptors to the cell interior. Therefore, $p56^{lck}$ (*16, 17*) becomes a potentially interesting signaling molecule for IL-2 because it is predominantly expressed in those cells that physiologically respond to IL-2, such as T and NK cells (*11*). Whereas $p56^{lck}$ has been implicated in CD4- or CD8-mediated signal transduction (*18*), there is no obligate relation between the expression of $p56^{lck}$ and expression of CD4 or CD8 coreceptor molecules, suggesting that $p56^{lck}$ may have additional functions in other signal transduction systems. We now demonstrate that IL-2Rβ interacts with $p56^{lck}$, partially define the sequences responsible for this interaction, and adduce evidence supporting a physiologic role for $p56^{lck}$ in IL-2R signaling.

Association of $p56^{lck}$ with IL-2Rβ. Physical linkage between IL-2Rβ and $p56^{lck}$ was examined by sequential immunoprecipitation and immunoblotting (*19*). In normal lymphocytes, the low-level expression of IL-2Rβ (about 4000 molecules per cell) makes the direct examination of a potential association between $p56^{lck}$ and IL-2Rβ difficult. Hence, we studied the human NK-like (natural killer) cell line YT (*20*), in which IL-2Rβ is expressed at a relatively high level (about 15,000 molecules per cell) (*21, 22*). This cell line expresses functional IL-2R as monitored by IL-2–induced enhancement of cell proliferation and NK activity (*22*). Immunoblot analysis with rabbit antiserum to $p56^{lck}$ (anti-$p56^{lck}$) (serum 195.7) (*23*) revealed that expression of $p56^{lck}$ (Fig. 1A) by YT cells was slightly

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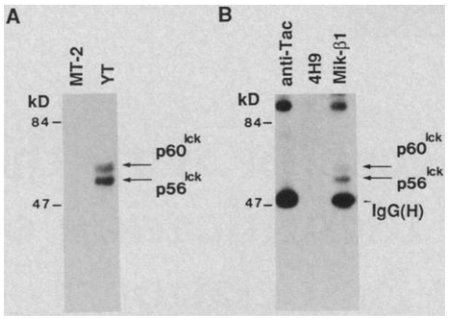

Fig. 1. Coprecipitation of $p56^{lck}$ with IL-2Rβ in the human lymphoid cell line YT. (**A**) Whole-cell lysates from YT, a human NK-like lymphoid cell line, and MT-2, an HTLV-I infected human T cell line, were fractionated on a 10 percent SDS-PAGE, transferred to a PVDF filter, and immunoblotted with rabbit antibody to $p56^{lck}$, 195.7, an antibody to residues 5 to 148 of mouse $p56^{lck}$ (*23*) that also reacts with human $p56^{lck}$. The positions of $p56^{lck}$ and $p60^{lck}$ are indicated. (**B**) Cell lysates from YT cells were immunoprecipitated with MAb anti-Tac (antibody to human IL-2Rα), 4H9 (antibody to CD7), or Mik-β1 (antibody to human IL-2Rβ). The immunoprecipitated samples were then analyzed on a 10 percent SDS-PAGE and anti-$p56^{lck}$ immunoblotting. Arrows indicate the positions of $p56^{lck}$ and $p60^{lck}$.

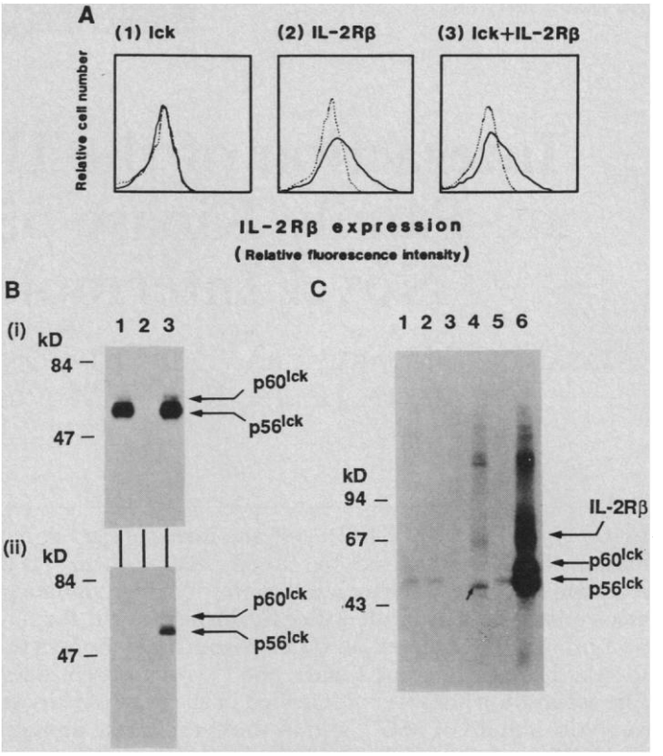

Fig. 2. Coprecipitation of $p56^{lck}$ with IL-2Rβ in transiently transfected COS cells. (**A**) Cell surface expression of human IL-2Rβ in COS cells. COS-7 cells were transfected with either the *lck* expression vector pdKCR-*lck* or the human IL-2Rβ expression vector pdKCR-β (or both); the calcium-phosphate method was used (*28*). The cells were harvested 72 hours after transfection, incubated with antibody to human IL-2Rβ MAb Mik-β1, and then stained with FITC (fluorescein isothiocyanate)-conjugated goat antibody to mouse IgG (solid lines). Dotted lines indicate the fluorescence profile of the cells stained with FITC-conjugated goat antibody to mouse IgG alone. (**B**) Analysis of $p56^{lck}$ for coprecipitation of IL-2Rβ. Cell lysates were prepared from COS cells; COS cells transfected with *lck* (pdKCR-*lck*) alone (lane 1), IL-2Rβ (pdKCR-β) alone (lane 2), or *lck* plus IL-2Rβ (pdKCR-*lck* + pdKCR-β) (lane 3). (i) Whole-cell lysates were analyzed by anti-$p56^{lck}$ immunoblotting. (ii) Cell lysates were immunoprecipitated with Mik-β1 and then analyzed by immunoblotting with anti-$p56^{lck}$. Arrows indicate the positions of $p56^{lck}$ and $p60^{lck}$. (**C**) In vitro kinase assay of immunoprecipitates from COS cells transfected with *lck* alone (lanes 1 and 2), IL-2Rβ alone (lanes 3 and 4), or *lck* plus IL-2Rβ (lanes 5 and 6). Cell lysates were immunoprecipitated with Mik-β1 (lanes 2, 4, and 6) or subclass-matched control MAb (lanes 1, 3, and 5). Immunoprecipitated samples prepared from 1×10^6 COS cells were suspended in 30 μl of buffer A (25 mM Hepes, pH 7.2, 0.1 percent NP-40, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 30 μM Na_3VO_4). After the addition of [γ-^{32}P]ATP (adenosine triphosphate) (10 μCi, Amersham), the reaction mixture was incubated for 30 minutes at 25°C, and was subjected to 10 percent SDS-PAGE under reducing conditions. Phosphorylated proteins were detected by exposure of dried gels to x-ray film for 10 to 60 minutes. The positions of $p56^{lck}$, $p60^{lck}$, and IL-2Rβ are indicated. The small arrow in lane 4 indicates an IL-2Rβ–associated putative protein kinase derived from COS cells.

less than that expression by phytohemagglutinin-activated peripheral blood lymphocytes (~70 percent) (*22*). The *lck*-encoded molecules expressed in YT cells consisted of two different species, $p56^{lck}$ and $p60^{lck}$. These two species probably reflect differences in the degree of serine-threonine phosphorylation of $p56^{lck}$ (*24*). To examine the interaction of IL-2Rβ chains with $p56^{lck}$, we prepared cell lysates from YT cells and immunoprecipitated with monoclonal antibody to human IL-2Rβ (MAb Mik-β1) (*25*). The immunoprecipitated samples were separated on 10 percent SDS-PAGE (polyacrylamide gel electrophoresis), transferred to membrane filters, and treated with anti-$p56^{lck}$. The MAb Mik-β1 specifically coimmunoprecipitated $p56^{lck}$, whereas subclass-matched immunoglobulin IgG2A monoclonal antibodies such as anti-Tac (*26*) and MAb 4H9 (*27*), which recognize IL-2Rα and CD7 molecules, respectively, did not (Fig. 1B). Both $p56^{lck}$ and $p60^{lck}$ were detected in the immunoblot, an indication that this modification does not greatly affect the association with IL-2Rβ. Based on the intensity of the immunoblot, approximately 0.5 to 1 percent of total $p56^{lck}$ protein formed a complex with IL-2Rβ. This low stoichiometry probably reflects the comparative abundance of $p56^{lck}$ relative to IL-2Rβ because the expression of $p56^{lck}$ is at least an order of magnitude higher than that of IL-2Rβ.

To gain further insight into the nature of the interaction between IL-2Rβ and $p56^{lck}$, we used a transient cDNA expression system in COS cells, similar to that used for the analysis of the interaction of CD4 or CD8 with $p56^{lck}$ (*28*). About 20 to 30 percent of COS cells that were transfected with the human IL-2Rβ cDNA expression vector pdKCR-β expressed IL-2Rβ, as indicated by our flow cytometry date (Fig. 2A). Coexpression of $p56^{lck}$ in COS cells did not affect the cell surface expression of IL-2Rβ. Expression of mouse $p56^{lck}$ protein was assessed from immunoblots of whole-cell lysates from COS cells transfected with mouse *lck* cDNA (Fig. 2B). These experiments revealed two forms of the *lck* product, the major $p56^{lck}$ and the minor $p60^{lck}$, in COS cells transfected with *lck* cDNA, presumably a reflection of variable phosphorylation of the $p56^{lck}$ product (Fig. 2B, lanes 1 and 3). Only transfected COS cells that express comparable amounts of IL-2Rβ or $p56^{lck}$ (or both) were used for further experiments.

Mik-β1 immunoprecipitates from COS cells cotransfected with IL-2Rβ and *lck* expression vectors contained $p56^{lck}$ when tested with anti-$p56^{lck}$, whereas no specific $p56^{lck}$ band was detected from COS cells expressing either IL-2Rβ or *lck* cDNA's alone (Fig. 2B). A mixing experiment with lysates containing only $p56^{lck}$ or IL-2Rβ revealed that the association of the two molecules does not occur after lysis (*22*). Specific association of $p56^{lck}$ with IL-2Rβ was also detected by in vitro kinase assay (Fig. 2C) with Mik-β1 immunoprecipitates. The immunoprecipitates from COS cells transfected with the human IL-2Rβ cDNA alone contained a weakly phosphorylated 50-kD protein (Fig. 2C, lane 4). The nature of the 50-kD protein is unclear, but it may represent a COS cell–derived endogenous protein kinase. Presumably, specifically phosphorylated 70- to 75-kD protein detected in Mik-β1 immunoprecipitates from COS

cells cotransfected with IL-2Rβ and *lck* expression vectors (Fig. 2C, lane 6) is actually IL-2Rβ itself (see below). The observation that mouse $p56^{lck}$ can interact with human IL-2Rβ in COS cells indicates that association of the two molecules occurs regardless of species differences and probably does not require other lymphoid-specific components. Essentially identical observations were made with MAb (TU-27) to human IL-2Rβ (*22*). At present MAb's for IL-2Rβ suitable for immunoblotting are not available, and therefore we could not perform the reciprocal experiments.

To examine whether phosphorylation events in the in vitro kinase assay (Fig. 2C) were mediated by *lck*-encoded protein tyrosine kinase, we performed a phosphoamino acid analysis for $p56^{lck}$, $p60^{lck}$, and the 70- to 75-kD IL-2Rβ within the Mik-β1 immunoprecipitates. All three proteins were phosphorylated only on tyrosine residues in vitro (Fig. 3A). We then examined, using a mutant IL-2Rβ molecule, the in vitro tyrosine phosphorylation sites of human IL-2Rβ. Of six tyrosine residues within the cytoplasmic domain of human IL-2Rβ, Tyr^{355}, Tyr^{358}, and Tyr^{361} are more likely to be phosphorylation sites, since these residues are surrounded by acidic amino acids and hence reside within what is believed to be a favorable sequence context (*29*). Both Tyr^{355} and Tyr^{358} are conserved between human and murine IL-2Rβ chains whereas Tyr^{361} is not (*4, 6*). Accordingly a cDNA encoding human IL-2Rβ with double point mutations (Ser^{355} and Ser^{358}) was generated by site-directed mutagenesis and expressed together with the *lck* cDNA. The mutant IL-2Rβ could form complexes with $p56^{lck}$, but phosphorylation of the p70 to 75 band was reduced, suggesting the involvement of Tyr^{355} or Tyr^{358} (or both) in the phosphorylation of IL-2Rβ by $p56^{lck}$ in vitro (Fig. 3B).

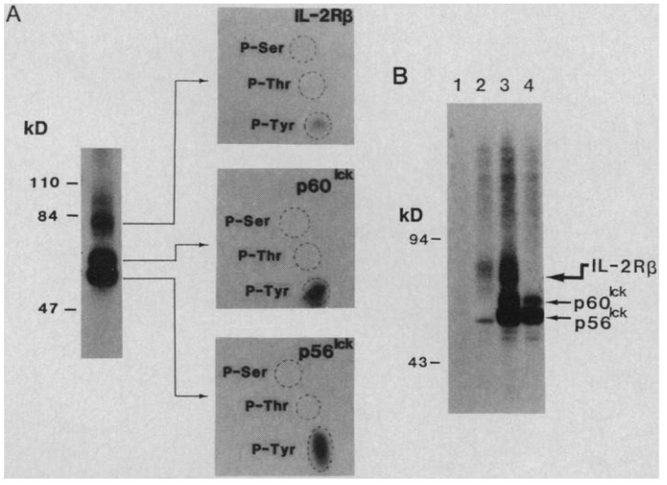

Fig. 3. Identification of in vitro IL-2Rβ phosphorylation sites by $p56^{lck}$. (**A**) Phosphoamino acid analysis of phosphorylated proteins in in vitro kinase assay. Phosphorylated proteins were eluted from dried gels and precipitated with trichloroacetic acid. The precipitates were hydrolyzed in 6 M HCl at 110°C for 2 hours. Phosphoamino acids were separated by two-dimensional electrophoresis (pH 1.9 and pH 3.5) on thin-layer cellulose plates as described (*40*). P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. (**B**) Analysis of coprecipitation of $p56^{lck}$ with IL-2Rβ mutant lacking potential tyrosine phosphorylation sites. Site-directed mutations were introduced into the human IL-2Rβ cDNA to generate a mutant receptor in which potential sites for tyrosine phosphorylation (Tyr^{355} and Tyr^{358}) were replaced with serine residues. The mutated cDNA was inserted into pdKCR vector and expressed in COS cells. Cell surface expression of the mutant IL-2Rβ was comparable to that of wild-type IL-2Rβ (*22*). Lysates prepared from COS cells transfected with *lck* alone (lane 1), IL-2Rβ alone (lane 2), *lck* plus IL-2Rβ (lane 3), or *lck* plus IL-2Rβ with Ser^{355} and Ser^{358} were immunoprecipitated with Mik-β1. The immune complexes were incubated with [γ-^{32}P]ATP as described in Fig. 2C and analyzed on a 10 percent SDS-PAGE. Positions for IL-2Rβ, $p56^{lck}$, and $p60^{lck}$ are indicated.

Delineation of association sites for IL-2Rβ–$p56^{lck}$ complex formation. To identify the molecular structures involved in $p56^{lck}$–IL-2Rβ association, we used a series of human IL-2Rβ cDNA's that encode mutant molecules lacking various portions of the cytoplasmic domain. The H mutant lacks the carboxyl-terminal 146 residues of the IL-2Rβ cytoplasmic domain, whereas the ST mutant has only the first 27 residues of the IL-2Rβ cytoplasmic domain (*7*). The S and A mutants are internal deletion mutants lacking the cytoplasmic "serine-rich" (56 amino acids) and "acidic" (70 amino acids) regions, respectively (*7*). These IL-2Rβ mutants indicated that the S and ST mutants were incapable of transducing IL-2 mitogenic signals in the IL-3–dependent pro-B cell line, BAF-B03, whereas the H and A mutants retained the signal transducing activity (*7*).

When the H- or S-mutant cDNA's were coexpressed with *lck* cDNA in COS cells, association of $p56^{lck}$ with the mutant IL-2Rβ's was still detected by anti-$p56^{lck}$ (Fig. 4A) as well as by in vitro kinase assay of the Mik-β1 immunoprecipitates (Fig. 4B). In contrast, neither the A mutant nor the ST mutant specifically associated with $p56^{lck}$ (Fig. 4, A and B). The same results were also obtained from immunoprecipitations of ^{35}S-labeled COS cell lysates in which the wild-type IL-2Rβ, H mutant, and S mutant were each coprecipitated with p56 (which is most likely to be $p56^{lck}$), whereas the ST mutant and A mutant did not, even though the amounts of immunoprecipitated IL-2Rβ in cells expressing the mutant proteins were comparable to those in cells expressing the wild-type IL-2Rβ (Fig. 4C). In fact, flow cytometric analysis revealed that the truncated forms of IL-2Rβ often exhibit higher surface expression than wild-type IL-2Rβ on COS cells (*22*). Expression levels of $p56^{lck}$ in each transfectant group did not vary significantly (Fig. 4A). Thus, while the association of $p56^{lck}$ with either H or S mutant is somewhat weaker than that observed with the wild-type IL-2Rβ

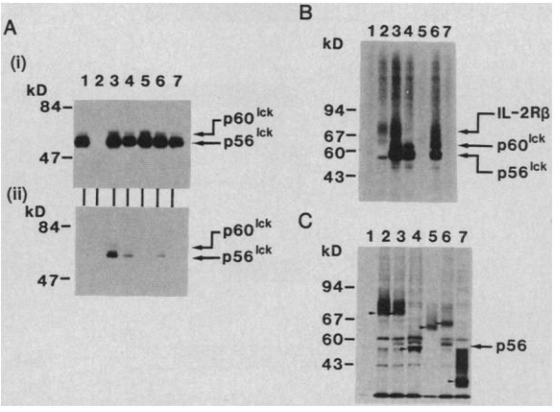

Fig. 4. Association of mutant IL-2Rβ to $p56^{lck}$. COS cells were transfected with *lck* alone (lane 1), IL-2Rβ alone (lane 2), *lck* plus IL-2Rβ (lane 3), *lck* plus H mutant (lane 4), *lck* plus A mutant (lane 5), *lck* plus S mutant (lane 6), *lck* plus ST mutant (lane 7), and harvested 72 hours after the transfection. (**A**) Coprecipitation of $p56^{lck}$ with IL-2Rβ mutants. (i) Anti-$p56^{lck}$ immunoblot of whole-cell lysates; $p56^{lck}$ and $p60^{lck}$ are indicated. (ii) Anit-$p56^{lck}$ immunoblot of Mik-β1–immunoprecipitated samples; $p56^{lck}$ and $p60^{lck}$ are indicated. (**B**) In vitro kinase assay of Mik-β1-immunoprecipitates. IL-2Rβ, $p56^{lck}$, and $p60^{lck}$ are indicated. (**C**) Metabolic labeling and immunoprecipitation of IL-2Rβ in COS cells. COS cells (4×10^6) were washed and incubated in 1.5 ml of methionine-free MEM (Eagle's minimum essential medium) supplemented with 10 percent fetal calf serum (FCS) for 15 minutes at 37°C. Then cells were resuspended in 1.0 ml of a solution of methionine-free MEM and 10 percent FCS containing [^{35}S]methionine (0.5 mCi; EXPRE^{35}S^{35}S; NEN) and incubated for 5 hours at 37°C, in 5 percent CO_2, and then lysed. Lysates were immunoprecipitated with Mik-β1 and analyzed on a 10 percent SDS-PAGE. Immunoprecipitated IL-2Rβ and its mutants are indicated by small arrows. The large arrow indicates the position of 56-kD protein coprecipitated with the wild-type IL-2Rβ (lane 3), the H mutant (lane 4), and the S mutant (lane 6).

protein (about 50 percent), these observations demonstrate that the IL-2Rβ cytoplasmic domain encompassing the "acidic" region is primarily responsible for association with $p56^{lck}$. Interestingly, this region contains Tyr^{355} and Tyr^{358}, the putative phosphorylation sites of IL-2Rβ by $p56^{lck}$ (Fig. 3B, see above). Stoichiometric analysis of the immunoprecipitation data (Fig. 4C) indicated that at least 20 to 30 percent of the IL-2Rβ is associated with $p56^{lck}$. This observation suggests that a large fraction of IL-2Rβ associates with $p56^{lck}$ in T cells, where the latter molecule is comparatively abundant.

In order to localize the IL-2Rβ interaction site on $p56^{lck}$, we first generated a series of mutant cDNA's (*30*) lacking the NH_2-terminal CD4 or CD8 binding domain (*28, 31*) or the modulatory domain containing the *src* homology sequences SH2 and SH3 (Fig. 5A). These SH2 and SH3 sequences are conserved within the *src* family protein tyrosine kinases, the c-*abl*–encoded protein tyrosine kinase, phospholipase C–γ (PLC-γ), the $p21^{ras}$ GTPase (guanosine triphosphatase)-activating protein (*ras* GAP) and the *crk*-encoded oncoprotein, among others (*32*). The $p56^{lck}$ mutants (LM-1, -2, and -3) all retained intrinsic tyrosine kinase activity. Cotransfection of the mutant *lck* cDNA's with the IL-2Rβ cDNA followed by Mik-β1 immunoprecipitation and in vitro kinase assay revealed that these $p56^{lck}$ mutants are still capable of associating with IL-2Rβ (Fig. 5B). Since rabbit anti-$p56^{lck}$, 195.7, was specific to the NH_2-terminal portion of mouse $p56^{lck}$ (residues 5 to 148), we could not perform immunoblot analysis to identify the LM-1, -2, and -3 mutant proteins. We then generated another set of mutant cDNA's for $p56^{lck}$ proteins whose kinase domains were either deleted (LM-4, LM-5) or replaced with that derived from the human insulin receptor lck–Ins-R (Fig. 5A). Both LM-4 and the lck–Ins-R chimera failed to interact with IL-2Rβ (Fig. 5, C and D). These observations indicate that the catalytic domain of $p56^{lck}$, particularly the NH_2-terminal half of the kinase domain, is involved in the association with IL-2Rβ.

The foregoing experiments show that a particular cytoplasmic segment of IL-2Rβ previously designated the "acidic" region (*4*) is primarily required for the $p56^{lck}$ association. This region is posi-

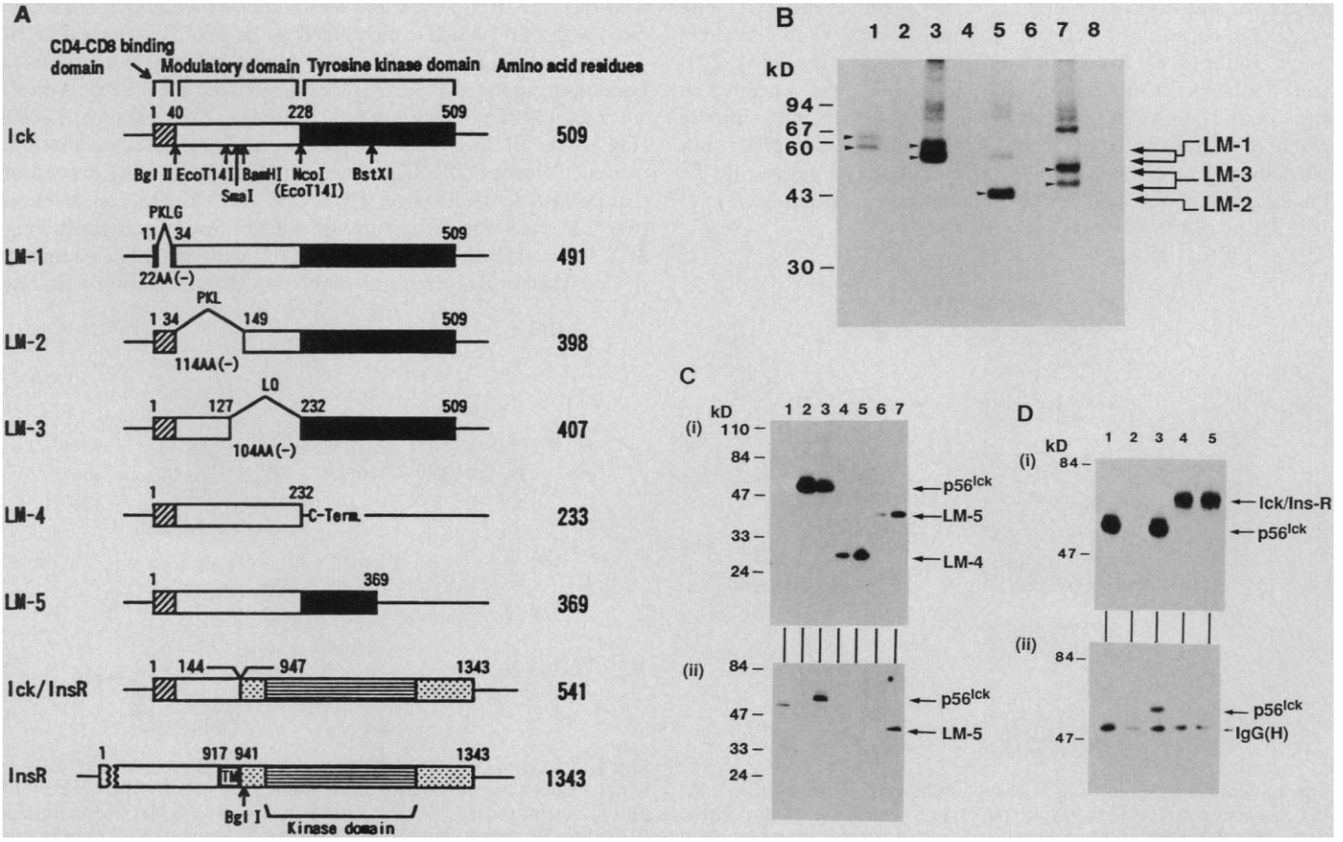

Fig. 5. Association of mutant $p56^{lck}$ to IL-2Rβ. (**A**) Schematic depiction of $p56^{lck}$ mutants. Construction of the cDNA's encoding the mutant proteins is described elsewhere (*30*). The restriction sites used to create internal deletions of *lck* sequences and to fuse the *lck* sequences to the coding region of human insulin receptor (Ins-R) cDNA are indicated. Residue numbers correspond to the amino acid sequence of mouse $p56^{lck}$ and human Ins-R. The residues generated by linker insertion are shown in the one-letter amino acid code (P, Pro; K, Lys; L, Leu; G, Gly). (**B**) Analysis of $p56^{lck}$ mutants having intact kinase domain for IL-2Rβ association. Lysates were prepared from COS cells expressing wild-type *lck* (lane 1 and 2), LM-1 (lane 3 and 4), LM-2 (lane 5 and 6) or LM-3 (lane 7 and 8) with (lanes 1, 3, 5, and 7) or without (lanes 2, 4, 6, and 8) IL-2Rβ, immunoprecipitated with Mik-β1, and analyzed by in vitro kinase assay. Samples were separated by 10 to 20 percent gradient SDS-PAGE. Arrows indicates associated $p56^{lck}$ or $p60^{lck}$ (or both) and its mutants. (**C**) Analysis of $p56^{lck}$ tyrosine kinase domain mutants for IL-2Rβ association. Cell lysates prepared from COS cells transfected with IL-2Rβalone (lane 1), *lck* alone (lane 2), IL-2Rβ plus *lck* (lane 3), LM-4 mutant with (lane 5) or without (lane 4) IL-2Rβ, LM-5 mutant with (lane 7) or without (lane 6) IL-2Rβ were immunoprecipitated with Mik-β1; the protein was fractionated by 10 to 20 percent gradient SDS-PAGE and analyzed by immunoblotting with anti-$p56^{lck}$. (i) The anti-$p56^{lck}$ immunoblot analysis of the whole-cell lysates. (ii) The anti-$p56^{lck}$ immunoblot analysis of the Mik-β1 immunoprecipitates. Arrows indicated the positions of $p56^{lck}$ and its mutants. (**D**) Analysis of lck/Ins-R chimeric kinase for coprecipitation of IL-2Rβ. Cell lysates were prepared from COS cells transfected with *lck* alone (lane 1), IL-2Rβ alone (lane 2), *lck* plus IL-2Rβ (lane 3), lck/Ins-R alone (lane 4), or lck/Ins-R plus IIL-2Rβ (lane 5). (i) Whole-cell lysates were analyzed by anti-$p56^{lck}$ immunoblotting. (ii) Cell lysates were immunoprecipitated with Mik-β1 and analyzed by immunoblotting with anti-$p56^{lck}$. Arrows indicate the positions of $p56^{lck}$, $p60^{lck}$, and lck/Ins-R chimera.

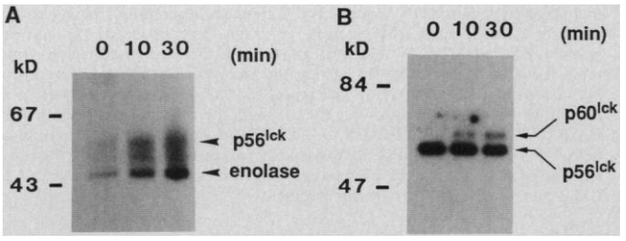

Fig. 6. Activation and modification of p56^{lck} by IL-2 stimulation. Human peripheral blood lymphocytes (PBL's) were isolated from normal adults by Ficoll-Hypaque centrifugation. Cells were cultured in RPMI 1640 supplemented with 10 percent FCS in the presence of phytohemagglutinin (PHA) at 5 μg/ml for 3 days. The PHA-activated PBL's were cultured in RPMI 1640 medium in the presence of 1 nM human recombinant IL-2 (Takeda Chemical). On day 7, cells were harvested, washed three times, and then incubated in RPMI 1640 containing 10 percent FCS without IL-2 for 20 hours. The PHA-activated, IL-2–starved human PBL's were incubated with 1 nM human recombinant IL-2 for the indicated intervals. After the incubation, cells were lysed and used for anti-p56^{lck} immunoprecipitation. (**A**) In vitro kinase assays after immunoprecipitation with anti-p56^{lck}. For kinase assays with enolase as a substrate, anti-p56^{lck} immunoprecipitates were suspended in the kinase buffer (15 μl) containing 1 μg of enolase, 30 μCi of [γ-^{32}P]ATP, and 4.5 μM unlabeled ATP and were incubated at 25°C for 30 minutes. The positions of p56^{lck} and the exogenous substrate enolase are shown. (**B**) A p56^{lck} immunoblot of PBL lysates. The arrows indicate the position of p56^{lck} and p60^{lck}.

tioned distal to the "serine-rich" region, which is important for IL-2–mediated mitogenic signal transduction in the IL-3–dependent pro-B cell line, BAF-B03 (7). To our surprise, the interaction site of p56^{lck} with IL-2Rβ resides within the tyrosine kinase catalytic domain, particularly its NH_2-terminal half. Through this interaction, IL-2Rβ becomes phosphorylated in vitro by p56^{lck}, suggesting that the reported IL-2Rβ phosphorylation in vivo (*9*) may also be mediated by p56^{lck}. The "acidic" region of IL-2Rβ contains putative tyrosine phosphorylation sites (Tyr^{355} and Tyr^{358}), and our data support the view that they are the primary targets of p56^{lck} activity. Since this interaction occurs at the catalytic site of p56^{lck} and the major phosphorylation sites of IL-2Rβ, one may envisage the requirement of the tyrosine residues for the interaction of the two molecules. However, our findings show that substitution of these two tyrosines by serine residues does not affect complex formation. This observation also argues against the possibility that the complex represents a simple enzyme-substrate type interaction. Notably, the tyrosine kinase domain derived from the human insulin receptor failed to interact with IL-2Rβ. Hence, there are structural determinants within the p56^{lck} kinase domain that permit interaction with IL-2Rβ.

Activation of p56^{lck} by IL-2 stimulation. Physical association of p56^{lck} with IL-2Rβ raises the possibility that IL-2 may normally regulate the behavior of p56^{lck}. We therefore examined the effect of IL-2 on the tyrosine kinase activity of p56^{lck} in human peripheral blood lymphocytes (PBL's). These cells were cultured for 3 days in the presence of phytohemagglutinin (PHA). The PHA-activated PBL's were then maintained in the presence of human recombinant IL-2. On day 7, cells were washed and cultured in the absence of IL-2 for an additional 20 hours. The IL-2–starved cells were incubated for various periods of time in the presence of IL-2; cell lysates were then prepared and immunoprecipitated by anti-p56^{lck}. The immunoprecipitates were tested for their ability to phosphorylate an exogenous substrate, enolase. The IL-2 treatment resulted in a five- to sixfold increase in enolase phosphorylation, indicating that IL-2 stimulation activated the p56^{lck} protein tyrosine kinase (Fig. 6A). This activation is associated with the conversion of p56^{lck} to a larger molecular species, p60^{lck} (Fig. 6B). The IL-2–induced conversion was almost completely inhibited by prior treatment of cells with Staurosporin, a potent protein serine-threonine kinase inhibitor, but not with H-7, a more selective inhibitor of protein kinase C (PKC), and hence the mobility shift may be due to increased p56^{lck} phosphorylation by protein serine-threonine kinases other than the previously characterized PKC (*22*). Activation and modification of p56^{lck} by IL-2 was also observed in the human NK-like cell line YT (*22*).

Potential role of p56^{lck} in IL-2 signaling. Three observations suggest that p56^{lck} may mediate some aspects of IL-2 signaling in lymphoid cells. (i) The IL-2Rβ chain and p56^{lck} interact to form specific complexes. (ii) Coimmunoprecipitation of IL-2Rβ and p56^{lck}, even in COS cells, results in the phosphorylation of IL-2Rβ on the tyrosine residues, analogous to what is observed in vivo after IL-2 treatment (*9*). (iii) IL-2 can directly stimulate p56^{lck} activity when added to cultured lymphocytes. Since IL-2 treatment of T cells and NK cells results in the rapid accumulation of phosphotyrosine-containing substrates (*8*), there is reason to believe that p56^{lck} participates in this signaling process. Our previous cDNA expression studies, performed in the IL-3–dependent cell line BAF-B03, identified a "serine-rich" region in the IL-2Rβ that mediates the signaling process. The "acidic" region, with which p56^{lck} interacts, is positioned adjacent to the "serine-rich" segment and has been closely conserved during evolution (*6*). Moreover, this region includes conserved tyrosine phosphorylation sites that are substrates for p56^{lck}. Hence, although the acidic region was not required for IL-2 signaling in BAF-B03 cells, we have strong reasons to believe that this part of the molecule assists in normal IL-2 signal transduction. Indeed, the BAF-B03 cell line appears to be especially permissive for growth signals from various members of the cytokine receptor family, and hence it may proliferate readily in response to suboptimal stimuli (*33, 34*). Our findings thus suggest the need for at least two (potentially more) signals, one involving the "serine-rich" region and the other the newly identified interaction site for p56^{lck} in the physiologic response to IL-2. Whether the IL-2Rβ chain itself is a critical substrate for p56^{lck}, or whether p56^{lck}, once linked to the receptor, can activate other metabolic processes awaits further investigation. Because phosphotyrosine may itself be a binding site for proteins containing SH2 domains (*32*), it is possible that p56^{lck} facilitates the interaction of the IL-2Rβ chain with physiologic regulators containing such domains (for example, PLC-γ, *ras* GAP, or phosphatidylinositol-3 kinase).

The *src*-family proteins interact with various cellular proteins by way of the NH_2-terminal unique region (*28, 31*) or the conserved SH2 region (*32*). Our findings reveal another potentially important region for intermolecular association, the NH_2-terminal half of the catalytic domain. On the basis of the structural similarity among the catalytic domains of p56^{lck} and other *src*-family proteins (*11, 12*), IL-2Rβ might also be able to interact with other protein tyrosine kinases. In fact, IL-2 is known to function in B cells (*35*) which express p56^{lyn} and p55^{blk} as well as small amounts of p56^{lck} (*11, 16*). Hence there may be some redundancy in the IL-2–mediated activation of tyrosine kinases. The same reasoning should prompt a search for other receptor structures that interact with the kinase domains of *src*-family proteins. In particular, our results suggest that other members of the newly growing cytokine receptor family may behave analogously to the IL-2Rβ chain, and may interact directly with *src*-family kinases to deliver their proliferative signals.

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19. For immunoprecipitation and immunoblotting, cells were washed in phosphate-buffered saline and lysed [20 mM tris-HCl (pH 8.0), 3 percent (v/v) Nonidet P-40, 150 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, leupeptin at 10 μg/ml, aprotinin at 10 μg/ml, pepstatin A at 1 μg/ml] for 30 minutes at 4°C (*36*). The cell lysates were centrifuged and cleared with protein A–Sepharose CL-4B (Pharmacia) that had been incubated with normal mouse serum for 1 hour at 4°C. The lysates were incubated with antibodies (1:200 dilution of ascites fluid) and 10 μl of protein A–Sepharose CL-4B at 4°C for 12 hours. The precipitates were washed seven times with the lysis buffer and subjected to 10 percent SDS-PAGE (polyacrylamide gel electrophoresis) under nonreducing condition or used for the in vitro kinase assay. For $p56^{lck}$ immunoprecipitation, cell lysates were incubated with 2 μl of 195.7 anti-$p56^{lck}$. Immunoblotting was performed with whole-cell lysates or immunoprecipitates prepared as described above. The cell lysates were fractionated by 10 percent SDS-PAGE. Gels were equilibrated in transfer buffer (125 mM tris-base, 960 mM glycine, 20 percent methanol); the separated proteins were electrophoretically transferred to PVDF membrane filter (Immobilon, Millipore) (170 mA, 90 minutes). Membranes were blocked in TBST milk [10 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.5 percent Tween 20, 5 percent nonfat dry milk] and treated with rabbit anti-$p56^{lck}$ (1:1000 dilution in TBST milk) for 2 hours at room temperature (*28*). After three washings in TBST milk, membranes were incubated with ^{125}I-labeled protein A (Amersham) (1:100 dilution in TBST milk) for 2 hours at room temperature. The membranes were washed in TBST milk once and three times in TBST, and then exposed to x-ray films.
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30. Construction of the human IL-2Rβ expression vector, pdKCR-β, has been described (*37*). In this construct, expression of the IL-2Rβ cDNA is driven by the SV40 early promoter and enhancer. For construction of pdKCR-based expression vectors, the cloning site of the pdKCR was converted from Bam HI to Sma I (blunt end) by linker insertion. The obtained plasmid pdKCRS was used for the construction. For the construction of the vector expressing H mutant of the IL-2Rβ, a 1.3-kb cDNA "insert" was excised from pIL-2Rβ9 (*4*) by Xba I, both ends were filled, and the filled fragment was inserted into the Sma I–cleaved pdKCRS. For construction of the expression vector for the ST-mutant, a 0.9-kb Xba I–digested cDNA fragment was recovered from the pIL-2Rβ30 (*7*) in which an Xba I linker had been introduced into the Pvu II site in the IL-2Rβ cDNA. The 0.9-kb Xba I cDNA fragment isolated was inserted into pdKCRS as described above. For the construction of the A- and S-mutant expression vectors, the Xba I fragments containing cDNAs for A and S mutants, respectively, were recovered from the recombinant CDM8 vectors which had been described (*7*), and then inserted into pdKCRS as described above. The mouse *lck* expression vector, pdKCR-*lck*, was constructed by ligating the Stu I–cut *lck* cDNA fragment from NT18 (*16*) to the Sma I site of the pdKCRS. Site-directed mutagenesis of the IL-2Rβ cDNA was performed with the use of synthetic oligonucleotides (5′TGC-CAGGTGTCCTTTACTTCCGACCCCTAC3′) as described (*38*). The mutated cDNA was inserted into pdKCR. Mutations were confirmed by dideoxynucleotide sequencing method. For the construction of the vector expressing the cDNA for LM-1 mutant of $p56^{lck}$, a synthetic fragment

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5'CCTTCAGGGATCATGGGCTGTGTCTGCAGCTCAAACCCTGAAGATCCCAAGC
TTGG3'
3'GGAAGTCCCTAGTACCCGACACAGACGTCGAGTTTGGGACTTCTAGGGTTCG
AACC5'
```

and NT18-derived Bgl II (blunt)–Stu I fragment of the *lck* cDNA were ligated to the Sma I site of pdKCRS. To construct the LM-2 mutant expression vector, Eco RI–cut *lck* cDNA from NT18 was first inserted into pUC19. The resultant pUC plasmid was digested with Bgl II and Bam HI and then rendered both ends blunt. During religation of the plasmid, a Hind III linker (10 bp) was introduced and Stu I fragment recovered from the resulting pUC19 plasmid was inserted into pdKCRS. For the construction of expression vectors for LM-3, NT18 was digested with Eco T14I and, after deleting a 0.3-kb Eco T14I fragment, a Pst I linker (8 bp) was introduced during religation. Then the Stu I fragment of the resulting plasmid was recovered and introduced into pdKCRS. To obtain the expression vectors for LM-4, NT18 was digested with Nco I and, after filing the ends, Xba I linker (termination linker; 14 bp) was introduced to make an in-frame nonsense codon. The resulting plasmid was digested with Stu I and the purified Stu I fragment was inserted into pdKCRS as described above. To construct the expression vector for LM-5, NT18 was digested with Bst XI. After both ends were filled, Xba I linker (termination linker) was introduced. The resulting plasmid was digested with Stu I and the Stu I fragment was inserted into pdKCRS as described. For the construction of a cDNA encoding *lck*/insulin receptor (Ins-R) chimeric molecule, a 1.45-kb human Ins-R cDNA was recovered from pSVILIR-K (*39*) by Bgl I digestion. After both ends were filled, the fragment was digested with Bam HI and the 1.3 kb fragment was purified. This 1.3-kb Ins-R cDNA fragment and a 0.9-kb Eco RI–Sma I *lck* fragment obtained from pdKCR-*lck* were ligated into Eco RI–Bam HI cleaved pdKCR vector.
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