
A Constitutively Active Lck Kinase Promotes Cell Proliferation and Resistance to Apoptosis through Signal Transducer and Activator of Transcription 5b Activation

Mingjian Shi, John C. Cooper, and Chao-Lan Yu

Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee

Abstract

Lck is a Src family protein tyrosine kinase and is expressed predominantly in T cells. Aberrant expression or activation of Lck kinase has been reported in both lymphoid and nonlymphoid malignancies. However, the mechanisms underlying Lck-mediated oncogenesis remain largely unclear. In this report, we establish a tetracycline-inducible system to study the biochemical and biological effects of a constitutively active Lck mutant with a point mutation at the negative regulatory tyrosine. Expression of the active Lck kinase induces both tyrosine phosphorylation and DNA-binding activity of signal transducer and activator of transcription 5b (STAT5b), a STAT family member activated in a variety of tumor cells. The active Lck kinase interacts with STAT5b in cells, suggesting that Lck may directly phosphorylate STAT5b. Expression of the constitutively active Lck mutant in interleukin-3 (IL-3)-dependent BaF3 cells promotes cell proliferation. In addition, the active Lck kinase protects BaF3 cells from IL-3 withdrawal-induced apoptotic death and leads to IL-3-independent growth. These transforming properties of the oncogenic Lck kinase can be further augmented by expression of exogenous wild-type STAT5b but attenuated by a dominant-negative form of STAT5b. All together, our results suggest the potential involvement of STAT5b in Lck-mediated cellular transformation. (Mol Cancer Res 2006;4(1):39–45)

Introduction

Lck is a member of the Src family nonreceptor protein tyrosine kinases expressed predominantly in T cells (1). Lck is essential for normal T-cell development and activation (2). The oncogenic properties of Lck *in vivo* was first shown by the development of thymic tumors in transgenic mice that

overexpress Lck (3). In humans, the *lck* gene is located at a site of frequent chromosomal abnormalities associated with lymphomas (4). Aberrant Lck expression and kinase activity have also been implicated in the pathogenesis of both lymphoid and nonlymphoid malignancies (5, 6). Like other Src family members, Lck kinase activity is negatively regulated by phosphorylation of a highly conserved tyrosine (Tyr⁵⁰⁵) located near the carboxyl terminus of the protein (7). Intramolecular interaction between phosphorylated Tyr⁵⁰⁵ and the Src homology 2 domain confers a closed conformation and excludes substrate binding to the kinase domain (8). A point mutation of Tyr⁵⁰⁵ to Phe locks Lck in an open conformation and results in a constitutively active kinase. The constitutively active Lck kinase is oncogenic and transforms fibroblasts in culture (9, 10). Nevertheless, the molecular mechanisms of Lck-mediated tumorigenesis have not been fully characterized.

Recent studies on the signal transducer and activator of transcription (STAT) have provided new insights into the mechanisms underlying oncogenesis (11, 12). STAT proteins are latent cytoplasmic transcription factors. On ligand stimulation, STAT proteins become phosphorylated by receptor or nonreceptor protein tyrosine kinases on a highly conserved tyrosine residue next to the Src homology 2 domain. Tyrosine-phosphorylated STATs dimerize, translocate to the nucleus, bind to specific DNA elements, and regulate the expression of target genes (13-15). Point mutation of the highly conserved tyrosine to phenylalanine renders the mutated STAT proteins nonfunctional and antagonizes endogenous STAT functions in a dominant-negative fashion (16, 17). Among seven known mammalian STAT family members, STAT5b is the first STAT protein confirmed to exhibit a loss-of-function in a patient with growth hormone insensitivity and immunodeficiency (18). STAT5b is not only essential in regulating important physiologic functions but also implicated in malignant transformation (19-21). Specifically, STAT5b is involved in Src-mediated oncogenesis (22, 23). Constitutive STAT5b activation is also critical for malignant transformation of both lymphoid and nonlymphoid cells (24-26).

Lck has been shown to play an important role in activating STAT5b in response to the engagement of T-cell receptors (27). STAT5b is also constitutively activated in leukemic T cells overexpressing the Lck kinase (28, 29). It remains to be determined, however, whether STAT5b activation is a direct consequence of Lck activation in tumor cells and whether STAT5b contributes to Lck-mediated oncogenesis. In this report, we establish a tetracycline-inducible system to specifically

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Requests for reprints: Chao-Lan Yu, Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615. Phone: 615-322-6036; Fax: 775-206-3170. E-mail: chao-lan.yu@vanderbilt.edu
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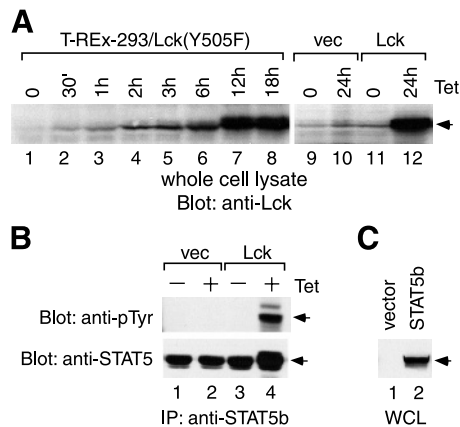


FIGURE 1. Active Lck kinase induces STAT5b tyrosine phosphorylation. **A.** T-REx-293 cells were stably transfected with pcDNA5/TO without (*vec*) or with Lck(Y505F). Cells were either left untreated or treated with tetracycline (*Tet*) for various times. Normalized whole-cell lysates were subjected to SDS-PAGE and subsequent immunoblotting with anti-Lck antibody. Arrowhead, correct position of exogenous Lck protein. **B.** T-REx-293/Lck(Y505F) and the vector control cells were transiently transfected with STAT5b expression construct and then either left untreated or treated with tetracycline for 24 hours. Proteins in normalized whole-cell lysates were immunoprecipitated (*IP*) with anti-STAT5b antibody. The immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (*pTyr*) monoclonal antibody 4G10 (*top*). The membrane was stripped and reblotted with anti-STAT5b antibody (*bottom*). Arrowheads, correct positions of exogenous STAT5b proteins. **C.** T-REx-293/Lck(Y505F) cells were transiently transfected with STAT5b expression construct or the vector control. Normalized whole-cell lysates (*WCL*) were subjected to anti-STAT5b immunoblotting as described for **B**.

examine both short-term and long-term effects of the constitutively active Lck(Y505F) kinase. The transforming properties of Lck(Y505F) are further investigated in BaF3 cells, a well-characterized model for cytokine-dependent growth and cytokine withdrawal-induced apoptosis (30, 31). The role of STAT5b is addressed by exogenous expression of wild-type and dominant-negative STAT5b proteins.

Results and Discussion

To study the biochemical effects of a constitutively active Lck kinase, we established a tetracycline-inducible system to more precisely control the expression of exogenous Lck kinase. A human Lck cDNA with a point mutation of Y505F was cloned into pcDNA5/TO under the control of a tetracycline-regulated promoter and then stably transfected into the T-REx-293 cell line expressing the tetracycline repressor. In the T-REx system, addition of tetracycline to the cells derepresses the promoter of pcDNA5/TO and allows expression of the inserted gene. In T-REx-293/Lck(Y505F), tetracycline induced maximal expression of the active Lck mutant kinase within 12 hours (Fig. 1A, lanes 1-8) and remained stable after 24 hours (Fig. 1A, lane 12). As a negative control, T-REx-293 cells stably transfected with pcDNA5/TO vector alone showed no induction of exogenous Lck (Fig. 1A, lane 10).

Tyrosine phosphorylation is critical for STAT5b activation (32). To determine the effects of the constitutively active Lck kinase on STAT5b phosphorylation, a STAT5b expression construct was transiently transfected into both T-REx-293/Lck(Y505F) and the vector control cells. There is no

detectable expression of endogenous STAT5 proteins in 293 cells (Fig. 1C, lane 1), which enables us to specifically study the exogenously expressed STAT5b (Fig. 1C, lane 2). Tetracycline-regulated expression of Lck(Y505F) strongly induced STAT5b tyrosine phosphorylation (Fig. 1B, compare lanes 3 and 4). As a negative control, tetracycline alone did not induce STAT5b phosphorylation in the vector control cells (Fig. 1B, compare lanes 1 and 2).

To determine whether Lck(Y505F) activates STAT5b DNA-binding activity, we did electrophoretic mobility shift assay using the mammary gland element derived from the β -casein promoter as a probe. Nuclear extracts were prepared from both T-REx-293/Lck(Y505F) and the vector control cells before and after tetracycline treatment. As shown in Fig. 2A, tetracycline-regulated expression of Lck(Y505F) specifically induced a distinct DNA-binding activity. Lck-induced DNA-binding activity could be specifically competed out by unlabeled mammary gland element oligonucleotides but not by unlabeled mammary gland element oligonucleotides with mutations at consensus DNA-binding sites, indicating that the binding was specific (Fig. 2B, lanes 2 and 3). The presence of active STAT5b was further confirmed by supershifting specifically with anti-STAT5b antibody but not with control antibody (Fig. 2B, lanes 4 and 5). This DNA-binding activity also comigrated with interleukin-3 (IL-3)-induced STAT5 activity in BaF3, an IL-3-dependent pro-B-cell line (Fig. 2B, compare lanes 1 and 6). These results clearly show that the constitutively active Lck kinase can activate STAT5b.

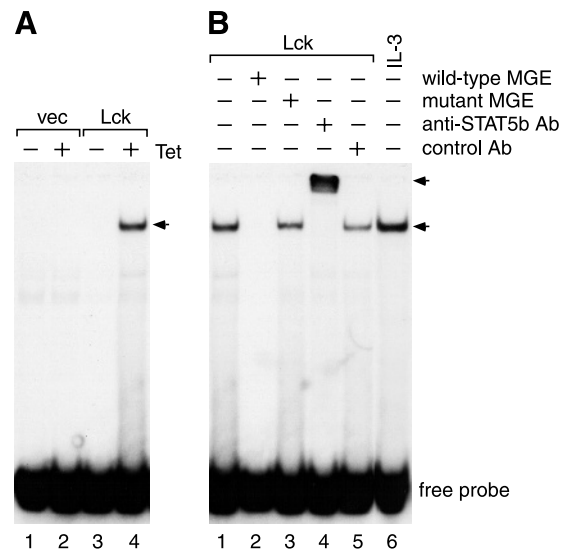


FIGURE 2. Active Lck kinase induces STAT5b DNA-binding activity. **A.** T-REx-293/Lck(Y505F) and the vector control cells were transfected with STAT5b expression construct as described in Fig. 1. Nuclear extracts containing the same amount of total proteins were subjected to electrophoretic mobility shift assay with a 32 P-labeled STAT5 consensus probe. Arrowhead, position of STAT5b-DNA complex. **B.** Nuclear extracts prepared from tetracycline-treated T-REx-293/Lck(Y505F) cells were subjected to electrophoretic mobility shift assay in the absence or presence of 100-fold molar excess of unlabeled wild-type or mutant mammary gland element (*MGE*), anti-STAT5b, or control antibody. Nuclear extract prepared from IL-3-stimulated BaF3 cells was also analyzed by electrophoretic mobility shift assay for comparison. Top arrowhead, supershifted STAT5b-DNA complex.

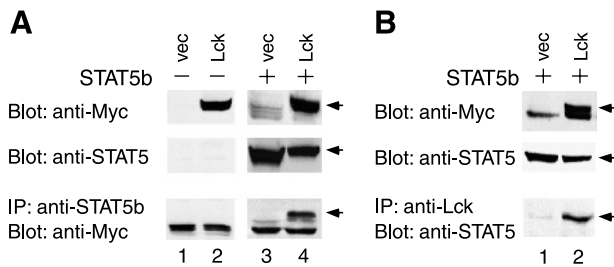


FIGURE 3. Active Lck kinase interacts with STAT5b in cells. T-REx-293/Lck(Y505F) and the vector control cells were transiently transfected with STAT5b expression construct (+) or the vector control (-). A small aliquot of normalized whole-cell lysates was analyzed by immunoblotting with anti-Myc (*top*) and anti-STAT5 (*middle*) antibodies. Proteins in the remaining lysates were immunoprecipitated with anti-STAT5b antibody (**A**) or anti-Lck antibody (**B**). Anti-STAT5b and anti-Lck immunoprecipitates were subjected to SDS-PAGE and subsequent immunoblotting with anti-Myc (**A**) or anti-STAT5 (**B**) antibody, respectively. Arrowheads, correct positions of exogenous STAT5b and Myc-tagged Lck proteins.

Previous studies showed that Lck could interact with STAT3 *in vitro* (33). To determine whether Lck(Y505F) associates with STAT5b in cells, we did reciprocal coimmunoprecipitation experiments in our T-REx-293 system. STAT5b expression construct was transfected into T-REx-293/Lck(Y505F) and the vector control cells as described above. As shown in Fig. 3 (*bottom*), Lck(Y505F) coimmunoprecipitated with STAT5b (Fig. 3A, *lane 4*) and STAT5b coimmunoprecipitated with Lck(Y505F) (Fig. 3B, *lane 2*). Immunoblotting of whole-cell lysates using anti-STAT5 antibody showed comparable expression of exogenous STAT5b proteins (Fig. 3, *middle*). Tetracycline-induced expression of Myc-tagged Lck(Y505F) was also confirmed by anti-Myc immunoblotting of whole-cell lysates (Fig. 3, *top*). Some nonspecific bands of lower molecular weights were detected in anti-Myc immunoblotting of both the vector control and Lck-expressing 293 cells. As another negative control, anti-STAT5b antibody did not bring down Myc-tagged Lck(Y505F) in the absence of STAT5b (Fig. 3A, compare *lanes 1* and *2*). The interaction between Lck(Y505F) and STAT5b suggests that the constitutively active Lck kinase may directly phosphorylate STAT5b.

The constitutively active Lck(Y505F) induced STAT5b DNA-binding activity similar to that of endogenous STAT5 in IL-3-stimulated BaF3 cells (Fig. 2B). BaF3 is an IL-3-dependent mouse pro-B-cell line widely used as a model system to study transforming properties of oncogenes (30, 31). Therefore, we further examined the biological effects of the active Lck kinase in BaF3 cells. To more precisely control the expression of Lck(Y505F), we set up a T-REx system in BaF3 cells similar to T-REx-293 as described above. A T-REx-BaF3 cell line was first generated by stably transfecting pcDNA6/TR, a plasmid expressing the tetracycline repressor, into BaF3 cells. T-REx-BaF3/Lck(Y505F) and the vector control cell lines were established by subsequent stable transfection of pcDNA5/TO/Lck(Y505F) and pcDNA5/TO, respectively.

The kinetics of tetracycline-induced expression of Lck(Y505F) in T-REx-BaF3 cells is slower than that in T-REx-293 cells. There is a gradual increase of exogenous Lck expression within the first 3 days of tetracycline treatment

(Fig. 4A). Tetracycline did not induce any Lck expression in the control T-REx-BaF3 cells with vector alone (Fig. 4B, *lane 1*). To determine the effects of active Lck kinase on cell growth, we calculated the total numbers of cells in the presence of tetracycline for 3 consecutive days. As shown in Fig. 4C, T-REx-BaF3/Lck(Y505F) grew significantly faster than T-REx-BaF3 control cells at each time point. Consistent with the gradual increase of Lck expression, the difference of total cell numbers between Lck-expressing BaF3 and the vector control cells reached the highest level at day 3. These results support the notion that the constitutively active Lck kinase promotes cell proliferation.

Other than stimulating cell proliferation, oncogenic protein tyrosine kinases can protect cells from apoptotic death (34). Prolonged deprivation of IL-3 not only stops cell growth but also induces apoptosis in BaF3 cells (30, 31). Therefore, we further studied the effects of active Lck kinase on IL-3 withdrawal-induced apoptosis in T-REx-BaF3 cells. When IL-3 was removed from the culture medium, we added tetracycline to both T-REx-BaF3/Lck(Y505F) and the control cells. Trypan blue dye exclusion assay indicated that IL-3 deprivation induced cell death of T-REx-BaF3 control and very few viable cells could be detected at day 6 (Fig. 5A). Removal of IL-3 also caused cell death of BaF3 expressing Lck(Y505F) in the first 3 days, but the viability was significantly higher than the vector control (Fig. 5A). One possible explanation for the initial decline of viability could be the slow induction of Lck(Y505F) (Fig. 4A). Nevertheless, T-REx-BaF3/Lck(Y505F) gradually recovered after day 3 and became IL-3 independent (Fig. 5A). The expression of Lck(Y505F) remained high in these cells after continuous passage in the absence of IL-3 (Fig. 5B, *lane 2*). Our findings clearly show that a constitutively active Lck kinase can support IL-3-independent growth of BaF3 cells.

We conducted two additional assays to examine the apoptotic death of T-REx-BaF3 cells at different stages following IL-3 deprivation. First, we used an Annexin V conjugate to detect the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane in apoptotic cells (35). The percentage of cells stained by the fluorescence-conjugated Annexin V was calculated after flow cytometry analysis. Compared with the vector control cells, expression of Lck(Y505F) significantly reduced the percentage of cells recognized by the Annexin V conjugate (Fig. 5C). Second, we examined the characteristic breakdown of the nucleus during apoptosis by chromatin fragmentation (36). In contrast to the vector control cells, expression of Lck(Y505F) greatly reduced the levels of DNA fragmentation shown as a distinct ladder pattern of low molecular weight DNAs (Fig. 5D, compare *lanes 2* and *3*). As a control, exponentially growing T-REx-BaF3 cells showed no sign of DNA fragmentation (Fig. 5D, *lane 4*). These results further illustrate that the active Lck(Y505F) kinase can protect cells from apoptosis induced by cytokine withdrawal.

Activation of STAT5 signaling is important in oncogene-induced cellular transformation and tumorigenesis (24-26). The observation that Lck(Y505F) interacts with and activates STAT5b (Figs. 1-3) prompted us to hypothesize that STAT5b may contribute to the transforming properties of the active

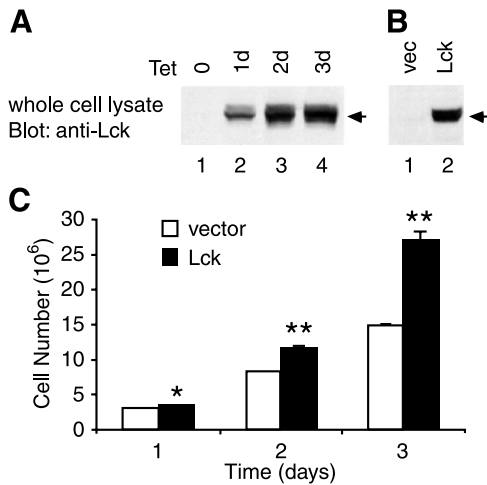


FIGURE 4. Active Lck kinase promotes cell proliferation. T-REx-BaF3 cells were stably transfected with pcDNA5/TO without or with Lck(Y505F). Cells were either left untreated or treated with tetracycline for 1, 2, or 3 days. **A.** Whole-cell lysates prepared from T-REx-BaF3/Lck(Y505F) were analyzed by SDS-PAGE and subsequent anti-Lck immunoblotting. Arrowhead, correct position of exogenous Lck protein. **B.** Whole-cell lysates were prepared from T-REx-BaF3/Lck(Y505F) or the vector control cells after stimulation with tetracycline for 1 day. Normalized lysates were analyzed by anti-Lck immunoblotting as described above. **C.** Tetracycline was added into 1×10^6 cells to a final concentration of $1 \mu\text{g/mL}$. Total numbers of cells were calculated after 1, 2, and 3 days of culture. Statistical difference between Lck-expressing cells and the vector control cells in each group was determined by the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

Lck kinase (Figs. 4 and 5). To test this hypothesis, we transiently transfected T-REx-BaF3/Lck(Y505F) with expression constructs carrying wild-type STAT5b, STAT5b with a point mutation of Tyr⁶⁹⁹ to Phe, or the vector alone. Phosphorylation of the highly conserved Tyr⁶⁹⁹ is critical for

STAT5b functions (20). STAT5b(Y699F) mutant loses its DNA-binding ability and can function as a dominant-negative protein (37, 38). As shown in Fig. 6C, within 2 days after transfection, both wild-type and mutant STAT5b proteins were expressed at comparable levels (*lanes 2, 3, 5, and 6*) and overexpressed compared with endogenous STAT5b (*lanes 1 and 4*). The levels of exogenous STAT5b expression were declined 3 days after transfection (*lanes 8 and 9*).

The effect of exogenous STAT5b on cell proliferation was analyzed as described in Fig. 4C. Compared with the vector control, transfection of wild-type STAT5b significantly accelerated the growth of BaF3 cells expressing Lck(Y505F) (Fig. 6A). Our data suggest that elevated expression of wild-type STAT5b may cooperate with the active Lck kinase in promoting cell proliferation. In contrast, STAT5b(Y699F) functioned as a dominant-negative protein to attenuate Lck-induced cell proliferation (Fig. 6A). We also examined the effects of exogenous STAT5b on cell death of T-REx-BaF3/Lck(Y505F) after IL-3 deprivation. In comparison with the vector control, transfection of wild-type STAT5b significantly reduced the death of Lck(Y505F)-expressing BaF3 cells, whereas transfection of STAT5b(Y699F) augmented the death of Lck(Y505F)-expressing BaF3 cells (Fig. 6B). All together, these results suggest that STAT5b may function as an important effector molecule downstream of the constitutively active Lck kinase.

STAT5 is activated in cells chronically transformed by the oncogenic Lck kinase (39). Using a tetracycline-inducible system to control the expression of a constitutively active Lck kinase, we are able to specifically examine the short-term effects of an oncogenic Lck kinase on STAT5b. The observation that Lck(Y505F) interacts with STAT5b in cells (Fig. 3) suggests that active Lck may directly phosphorylate and activate STAT5b. Consistent with our finding, Lck also interacts with and activates STAT3, another STAT family

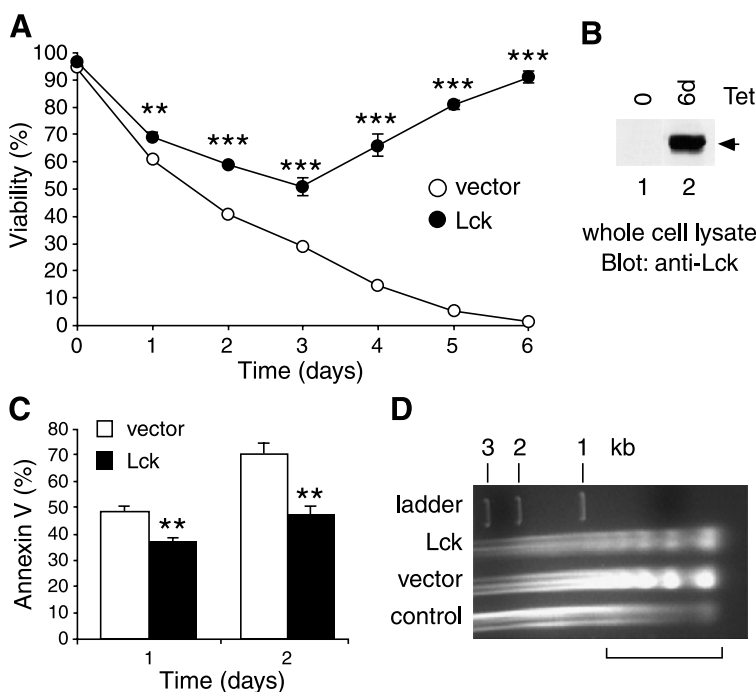


FIGURE 5. Active Lck kinase protects BaF3 cells from IL-3 withdrawal-induced apoptosis. **A.** T-REx-BaF3/Lck(Y505F) and the vector control cells were deprived of IL-3 and treated with tetracycline for 1 to 6 days. At each time point, a small fraction of cells were incubated with 0.1% trypan blue dye. Both viable and dead cells were counted and the percentage of viable cells was calculated. Points, mean of three independent experiments; bars, SE. **, $P < 0.01$; ***, $P < 0.001$. Some error bars overlap with the symbols themselves and are not clearly visible. **B.** Whole-cell lysates were prepared from T-REx-BaF3/Lck(Y505F) cells either untreated or treated with tetracycline for 6 days. Normalized lysates were subjected to anti-Lck immunoblotting as described for Fig. 4. **C.** T-REx-BaF3/Lck(Y505F) and the vector control cells were deprived of IL-3 and treated with tetracycline for 1 and 2 days. Cells were collected, stained with Alexa Fluor 647-conjugated Annexin V, and then analyzed by flow cytometry. Columns, mean of three independent experiments; bars, SE. **, $P < 0.01$. **D.** Genomic DNAs were extracted from T-REx-BaF3/Lck(Y505F) and the vector control cells deprived of IL-3 and treated with tetracycline for 24 hours. Genomic DNAs were also prepared from exponentially growing T-REx-BaF3 in the presence of IL-3 as a negative control (*lane 4*). DNA fragmentation was visualized after electrophoresis and ethidium bromide staining with a marker of 1-kb ladders (*lane 1*). Top, positions of 1, 2, and 3 kb; bottom bracket, lower molecular weight DNA fragments.

member widely implicated in oncogenesis (33). However, we do not exclude the possibility that other cellular proteins may be involved in Lck-induced STAT5b activation. For example, both Jak1 and Jak2 are phosphorylated and activated in cells expressing active Lck kinase (33, 39). Therefore, Lck may also indirectly phosphorylate STAT5b through Jak and other protein tyrosine kinases depending on the intracellular environment.

Although STAT5 is activated in Lck-transformed cells, the role of STAT5 in Lck-mediated oncogenesis has not been fully established. Using the IL-3-dependent BaF3 cells as a model system, we clearly show the oncogenic properties of the constitutively active Lck kinase (Figs. 4 and 5). The observation that expression of exogenous wild-type STAT5b or dominant-negative STAT5b can either potentiate or antagonize Lck-mediated oncogenesis, respectively (Fig. 6), further supports the important role of STAT5b. Other than STAT5b, many intracellular signaling molecules are phosphorylated and modulated by the oncogenic Lck kinase (40). It is likely that STAT5b acts in concert with other signal transduction pathways to exert the full oncogenic potentials of active Lck(Y505F) kinase (16). This may also partly explain why a dominant-negative form of STAT5b cannot completely reverse Lck-induced oncogenesis. Detailed analysis of the signaling networks downstream of oncogenic Lck kinase will further elucidate the role of STAT5b in this cross-talk.

Materials and Methods

Cell Culture

Tetracycline-regulated T-REx-293 cell line (Invitrogen, Inc., Carlsbad, CA) was maintained in DMEM supplemented with 5% FCS, 5% calf serum, and 5 μ g/mL blasticidin. Mouse pro-B-cell line BaF3 was cultured in RPMI supplemented with 5% FCS, 5% calf serum, and 10% conditioned medium containing IL-3. For cytokine stimulation experiments, BaF3 cells were deprived of IL-3 for 16 hours and then stimulated with 10 ng/mL recombinant mouse IL-3 (R&D Systems, Inc., Minneapolis, MN) for 30 minutes. For inducible gene expression in the T-REx system (Invitrogen), tetracycline was added to a final concentration of 1 μ g/mL.

Plasmids

STAT5b Expression Constructs. An *EcoRI-EcoRV* restriction fragment containing the full-length mouse STAT5b cDNA with a FLAG epitope tagged at the carboxyl terminus was cut from pRK5/mSTAT5b and then subcloned into the *EcoRV* site in the pcDNA5/TO (Invitrogen) to make the pcDNA5/TO/mSTAT5b/FLAG expression construct. Subsequently, a point mutation (tAc to tTc) was introduced using QuickChange II Site-Directed Mutagenesis (Stratagene, Inc., La Jolla, CA) and the manufacturer's protocol to construct pcDNA5/TO/mSTAT5b(Y699F)/FLAG.

Lck Expression Constructs. A *XhoI-HindIII* restriction fragment containing the full-length human Lck cDNA was generated by PCR using pEBB/hLck as the template and then inserted into the multiple cloning site in pcDNA3.1/myc-His(-)B (Invitrogen) to make the pcDNA3.1/hLck/myc-His expression construct. A point mutation (tAc to tTc) was introduced as described above to make pcDNA3.1/

hLck(Y505F)/myc-His. Subsequently, a *HindIII-NotI* fragment containing the full-length human Lck(Y505F) with the Myc and histidine tags was inserted into the multiple cloning site in pcDNA5/TO to make the pcDNA5/TO/hLck(Y505F)/myc-His expression construct. All constructs were verified by sequencing for accuracy.

Cell Lines and Transfections

T-REx-293 Cell Lines. T-REx-293 cells were grown to 50% confluence in 60-mm dish and then transfected with 1 μ g pcDNA5/TO or pcDNA5/TO/hLck(Y505F)/myc-His using LipofectAMINE (Invitrogen) and the manufacturer's protocol. Stably transfected cells were selected and maintained in the presence of 200 μ g/mL hygromycin. LipofectAMINE was also used to transiently transfect pCMV/HA/hSTAT5b into T-REx-293 cell lines.

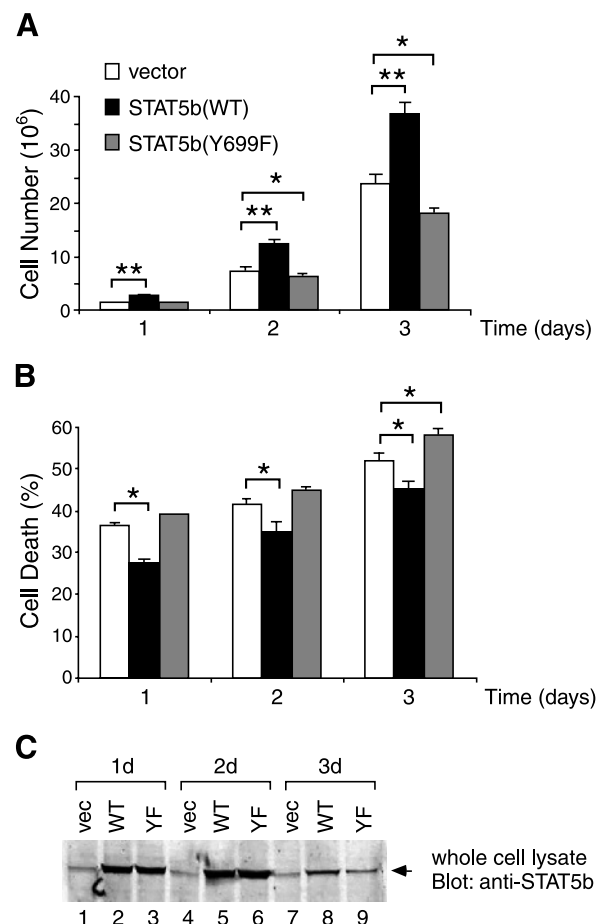


FIGURE 6. STAT5b contributes to Lck-induced cell proliferation and resistance to apoptosis. T-REx-BaF3/Lck(Y505F) cells were transiently transfected with wild-type (WT) STAT5b, STAT5b(Y699F), or the vector control. **A.** Transfected cells (1 million) were cultured in the presence of IL-3 and tetracycline for 1 to 3 days, and the total numbers of cells were counted as described in Fig. 4C. *, $P < 0.05$; **, $P < 0.01$. **B.** The remaining transfected cells were deprived of IL-3 and treated with tetracycline for 1 to 3 days. Cell death was determined by trypan blue staining as described in Fig. 5A. *, $P < 0.05$. **C.** Whole-cell lysates were prepared from an aliquot of transfected cells at each time point, resolved by SDS-PAGE, and then analyzed by anti-STAT5b immunoblotting. Arrowhead, position of STAT5b proteins.

T-REx-BaF3 Cell Lines. To establish our own tetracycline-inducible T-REx-BaF3 cells, 10 μ g pcDNA6/TR (Invitrogen) was first linearized with *SapI* and then electroporated into BaF3 cells. Electroporation was carried out in a single pulse using the Cell-Porator (BRL Life Technologies, Inc., Rockville, MD) set at 300 V, 800 μ F, and low ohms. Stably transfected BaF3 cells were selected and maintained in the presence of 20 μ g/mL blasticidin. T-REx-BaF3 cells were subsequently transfected with 10 μ g *SspI*-linearized pcDNA5/TO or pcDNA5/TO/hLck(Y505F)/myc-His by electroporation. Stably transfected T-REx-BaF3/Lck(Y505F) and vector control cells were selected and maintained in the presence of 1.6 mg/mL hygromycin. Electroporation as described above was also used to transiently transfect pcDNA5/TO/mSTAT5b/FLAG and pcDNA5/TO/mSTAT5b(Y699F)/FLAG into the established T-REx-BaF3/Lck(Y505F) cells.

Immunoprecipitation and Immunoblotting

Preparation of whole-cell lysates and immunoprecipitation were done essentially as described before (41). Anti-Lck, anti-Myc, and anti-STAT5b antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-STAT5 and anti-FLAG monoclonal antibodies used for immunoblotting were from BD Transduction Laboratories (San Diego, CA) and Sigma (St. Louis, MO), respectively. Dilutions of different antibodies for immunoblotting and subsequent detection by the enhanced chemiluminescence system were done as recommended by the manufacturers.

Electrophoretic Mobility Shift Assay

Preparation of nuclear extracts, electrophoretic mobility shift assay, competition, and antibody supershift assays were done essentially as described previously (39). The wild-type and mutant oligonucleotides as well as the anti-STAT5b supershift antibody were from Santa Cruz Biotechnology. The control rabbit antibody was from Southern Biotechnology Associates (Birmingham, AL).

Flow Cytometry

After electroporation, T-REx-BaF3 cells were deprived of IL-3 and cultured in the presence of tetracycline for 24 and 48 hours. Cells were washed in cold PBS [140 mmol/L NaCl, 3 mmol/L KCl, 10 mmol/L Na_2HPO_4 , 2 mmol/L KH_2PO_4 (pH 7.4)] and then resuspended in Annexin V-binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl_2] to 1×10^6 cells/mL. Alexa Fluor 647-conjugated Annexin V (1 μ L; Molecular Probes, Inc., Eugene, OR) was added into 100 μ L cell suspension and then incubated at room temperature for 15 minutes. Cells were diluted 5-fold with Annexin V-binding buffer and kept on ice. Approximately 10,000 cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Inc., San Jose, CA).

DNA Fragmentation Analysis

After electroporation, T-REx-BaF3 cells were deprived of IL-3 and cultured in the presence of tetracycline for 24 hours. Cells were washed in ice-cold PBS and then lysed in DNA digestion buffer [100 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 200 mmol/L NaCl, 0.2% SDS, and 0.5 mg/mL proteinase K]

for 1 hour at 55°C. Genomic DNAs were purified by phenol/chloroform extraction followed by precipitation with 2-propanol. DNA pellet was dissolved in TE buffer [10 mmol/L Tris (pH 7.4), 1 mmol/L EDTA] and treated with 0.5 mg/mL RNase A. Equal amounts of total DNAs were resolved by electrophoresis in a 1.2% agarose gel and then visualized by ethidium bromide staining.

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