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Requirement of the SH3 and SH2 Domains for the Inhibitory Function of Tyrosine Protein Kinase p50^{csk} in T Lymphocytes

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Previous studies from our laboratory have shown that the cytosolic tyrosine protein kinase p50^{csk} is involved in the negative regulation of T-cell activation (L. M. L. Chow, M. Fournel, D. Davidson, and A. Veillette, Nature [London] 365:156–160, 1993). This function most probably reflects the ability of Csk to phosphorylate the inhibitory carboxy-terminal tyrosine of p56^{lck} and p59^{fynT}, two Src-related enzymes abundantly expressed in T lymphocytes. Herein, we have attempted to better understand the mechanisms by which Csk participates in the inhibitory phase of T-cell receptor signalling. Our results demonstrated that the Src homology 3 (SH3) and SH2 domains of p50^{csk} are crucial for its negative impact on T-cell receptor-mediated signals. As these two sequences were not essential for phosphorylation of the carboxy-terminal tyrosine of a Src-like product in yeast cells, we postulated that they mediate protein-protein interactions allowing the recruitment of p50^{csk} in the vicinity of activated Lck and/or FynT in T cells. In complementary studies, it was observed that linkage of a constitutive membrane targeting signal to the amino terminus of Csk rescued the deleterious impact of a point mutation in the SH2 domain of p50^{csk}. This observation suggested that the SH2 sequence is in part necessary to translocate p50^{csk} from the cytoplasm to the plasma membrane, where Src-related enzymes are located. Nevertheless, constitutive membrane localization was unable to correct the effect of complete deletion of the SH3 or SH2 sequence, implying that these domains provide additional functions necessary for the biological activity of p50^{csk}.

Activation of T lymphocytes by antigen or anti-T-cell receptor (TCR) antibodies requires the induction of a rapid intracellular tyrosine protein phosphorylation signal (reviewed in references 24, 27, 32, and 34). Because the antigen receptor, as well as the associated CD3 and ζ chains, are devoid of intrinsic tyrosine protein kinase activity, this biochemical response necessitates the participation of cytoplasmic tyrosine protein kinases. Evidence indicates that p56^{lck} and p59^{fynT}, two Src-related tyrosine protein kinases expressed in T lymphocytes, are responsible for the initiation of TCR-mediated signals. Furthermore, Zap-70, which belongs to a separate class of tyrosine protein kinases, is involved in the amplification of this response.

The catalytic function of Src-related enzymes is tightly regulated by phosphorylation of a conserved carboxy-terminal tyrosine (tyrosine 505 for p56^{lck}; tyrosine 528 for p59^{fynT}) (reviewed in references 8 and 9). Phosphorylation at this site is postulated to trigger an intramolecular association between the phosphorylated carboxy terminus and the Src homology 2 (SH2) domain of Src-related products, thereby disallowing interactions between their kinase domain and intracellular substrates. This reaction is not caused by autophosphorylation but rather is mediated by a distinct cytoplasmic tyrosine protein kinase termed p50^{csk} (3, 11, 18). In turn, dephosphorylation of the inhibitory tyrosine of Lck and, to a lesser extent, FynT, is catalyzed by CD45, a transmembrane tyrosine protein phosphatase expressed on all nucleated hemopoietic cells (4, 15, 21, 28).

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While p50^{csk} accumulates in all cell types, it is contained in higher amounts in cells of hemopoietic lineages, including T lymphocytes (11, 18). The basic structure of Csk is similar to that of Src family tyrosine protein kinases (18). It possesses amino-terminal SH3 and SH2 regions, as well as a carboxy-terminal kinase domain. In contrast to Src-related products, however, Csk is devoid of myristylation signal, site of autophosphorylation, and carboxy-terminal site of tyrosine phosphorylation. The creation of Csk-deficient mice by homologous recombination in embryonic stem cells revealed that p50^{csk}-mediated functions are crucial for normal embryonic development, most noticeably for the maturation of the central nervous system (16, 19). p50^{csk} also plays an important role in normal T-cell physiology. In support of this idea, we previously reported that overexpression of p50^{csk} in an antigen-specific T-cell line (BI-141) inhibited TCR-induced tyrosine protein phosphorylation and lymphokine secretion (6). Importantly, this uncoupling of TCR signalling from the tyrosine protein kinase pathway could be overcome by expression of Lck or FynT polypeptides carrying a mutation of the negative regulatory tyrosine, thereby being refractory to regulation by Csk (6, 7). In addition to its function as a negative regulator of T-cell activation, recent studies have demonstrated that Csk is crucial for normal development of the lymphoid lineage in vivo (13). Indeed, mouse ES cells lacking p50^{csk} failed to differentiate into T or B cells when introduced into blastocysts.

The regulation of Csk in T lymphocytes is not well understood. Csk is not detectably phosphorylated in vivo, implying that it is unlikely to be regulated through phosphorylation. Because p50^{csk} is mostly cytosolic, it has been hypothesized that its function may be activated by recruitment to the plasma membrane, where Src-related enzymes are localized. This concept is in agreement with our earlier finding that constitutive membrane targeting of Csk through the addition of a myristy-

lation signal from Src, or farnesylation and palmitylation signals from Ras, increased the ability of Csk to inhibit TCR signalling (6). Under physiological conditions, recruitment of $p50^{csk}$ to the plasma membrane could be mediated by its SH3 and/or SH2 domains, which are capable of binding proline-rich proteins and phosphotyrosine-containing polypeptides, respectively (reviewed in reference 22). This model is in part supported by studies carried out with fibroblasts, in which the capacity of $p50^{csk}$ to colocalize with activated $p60^{c\text{-}src}$ in podosomes was abrogated by deletion of the SH3 or SH2 sequence (14). Furthermore, in another study (26), the ability of Csk to reduce cell transformation caused by the combined actions of c-Src and Crk was significantly reduced by removal or mutation of these motifs. However, the importance of the SH3 and SH2 domains of $p50^{csk}$ in the control of physiological functions mediated by Src-like kinases was not addressed in any of these reports.

The present study was aimed at better understanding the regulation of Csk in T lymphocytes. We found that complete deletion of the SH3 or SH2 domain abrogated the inhibitory effect of Csk on antigen receptor-mediated T-cell activation. Moreover, mutation of a conserved arginine residue in the SH2 motif (arginine 107-to-lysine 107 [K107] mutation) significantly reduced the negative influence of Csk on TCR-triggered signals. These effects did not seem to relate to a decrease in the catalytic activity of $p50^{csk}$, as all mutants efficiently phosphorylated the inhibitory carboxy-terminal tyrosine of Lck in yeast cells. Interestingly, addition of a membrane targeting signal from c-Src rectified the inability of the K107 mutant to inhibit TCR signalling, suggesting that part of the function of the SH2 domain is to mediate translocation of Csk to cellular membranes.

MATERIALS AND METHODS

Cells. BI-141, a CD4-negative, CD8-negative, class II major histocompatibility complex (MHC)-restricted, beef insulin-specific mouse T-cell hybridoma (25), was propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum. Derivatives expressing neomycin phosphotransferase (Neo) alone or in combination with either wild-type $p50^{csk}$, a membrane-targeted version of Csk (Src-Csk), or the myristylation-defective glycine 2-to-alanine 2 (A2) Lck were described elsewhere (1, 5, 6). All BI-141 T-cell derivatives were maintained in growth medium supplemented with the aminoglycoside G418 (0.6 mg ml^{-1}). ψ-2 retroviral packaging cells were grown in α minimal essential medium and 10% fetal bovine serum.

Generation of *csk* mutants. Mutant *csk* cDNAs were generated by PCR, using a rat *csk* cDNA (18) provided by Masato Okada (Osaka, Japan) as the template. ΔSH3 *csk* was created by overlap extension based on the following oligonucleotide sequence (sense): 5′ CCATCCGGTACAGAACGTGAGGGTGTGAAG 3′. This allowed the removal of amino acids 14 to 67 of $p50^{csk}$. ΔSH2 *csk* was produced by creating *Sma*I and *Sna*BI sites at the beginning and end, respectively, of the sequences coding for the SH2 domain. The following oligonucleotides were used: 5′ CGTGGAACCCGGGCATAAG 3′ (antisense) and 5′ ATC AAACCATACGTAATGGAGGGC 3′ (sense). The *Sma*I-*Sna*BI fragment was then removed by DNA digestion, and the resulting large fragment was religated. This modification deleted amino acids 82 to 171 of $p50^{csk}$. The K107 *csk* cDNA was created by overlap extension based on the oligonucleotide sequence (sense) 5′ TCCTGGTGAAAGAAAGCAC 3′. The various *src-csk* chimeras were produced through standard recombinant DNA technology, using these mutant *csk* cDNAs and the previously reported wild-type *src-csk* cDNA.

Retrovirus-mediated gene transfer. The *csk* cDNAs were cloned into the multiple cloning site of the retroviral vector pLXSN (17). Retroviral constructs were subsequently transfected by calcium phosphate precipitation (1) in ψ-2 packaging cells, and polyclonal virus-producing cell lines were established by growth in medium containing G418 (0.4 mg ml^{-1}). BI-141 T-cells were infected with the appropriate retroviral stocks as described elsewhere (2). Infected cells were selected in medium containing G418 (0.75 mg ml^{-1}), and monoclonal cell lines were derived by limiting dilution. Cells were screened by immunoblotting of total cell lysates with the appropriate antisera (see below). All cell lines used in our experiments expressed unaltered levels of TCR, CD3ε, Thy1, and CD45 and remained CD4 negative (data not shown).

Immunoblots. Immunoblots were performed as previously described (30, 31, 33). Anti-Csk immunoblotting was done with polyclonal rabbit antisera directed against either the kinase domain (7) or the carboxy-terminal 13 residues (amino acids 438 to 450 [14]) of Csk. Anti-Src immunoblotting was done with mouse monoclonal antibody (MAb) LA-074 (Quality Biotech, Camden, N.J.), which was generated against a synthetic peptide corresponding to amino acids 2 to 17 of the c-Src sequence. Antiphosphotyrosine immunoblotting was done with either affinity-purified rabbit antiphosphotyrosine antibodies (our unpublished data) or mouse MAb 4G10 (Upstate Biotechnology, Inc., Lake Placid, N.Y.). For anti-Lck immunoblotting, we used an antiserum generated against a fusion protein encompassing amino acids 2 to 148 of $p56^{lck}$ (1). After incubation with ^{125}I-protein A (Amersham) or ^{125}I-labeled goat anti-mouse immunoglobulin G (IgG) (ICN), immunoreactive products were detected by autoradiography and quantitated with a phosphorimager (BAS 2000; Fuji).

Antibody-mediated T-cell activation. T cells were activated by stimulation with anti-TCR Vβ8 mouse MAb F23.1 (29) and sheep anti-mouse (SAM) IgG as outlined elsewhere (1, 10). Following activation, cells were lysed in boiling sample buffer, and lysates were processed for antiphosphotyrosine immunoblotting. Alternatively, for in vitro binding assays, cells were lysed by adding an equal volume of 2× TNE buffer (1× TNE is 50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, and 20 mM EDTA [pH 8.0]) containing 20 μg each of leupeptin, aprotinin, *N*-tosyl-L-phenylalanine chloromethyl ketone, *N*-*p*-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride per ml, as well as phosphatase inhibitors sodium fluoride (100 mM) and sodium orthovanadate (2 mM).

Lymphokine production assays. Cells were stimulated in 96-well plates with various concentrations of anti-TCR MAb F23.1 coated on plastic as previously outlined (1, 10). After 24 h, supernatants were harvested and frozen at −70°C to destroy carryover cells. The presence of secreted lymphokines was then measured in the standard bioassay, evaluating tritiated thymidine incorporation in the interleukin-2 (IL-2)-dependent HT-2 cell line. All assays were done in triplicate and were repeated at least four times.

Cell fractionation studies. For cell fractionation, cells were incubated for 15 min in hypotonic buffer (10 mM Tris [pH 7.4], 2 mM EDTA [pH 8.0]) supplemented with the protease and phosphatase inhibitors specified above. Then, membranes were mechanically broken in a Dounce homogenizer. In all cases, staining with trypan blue confirmed that over 95% of cells had been lysed (data not shown). After adjustment of the homogenates to 0.15 M NaCl, intact cells, nuclei, and large membrane sheets were removed by two successive centrifugations at 480 × *g* for 5 min. Supernatants were then separated into soluble (S100) and particulate (P100) fractions by ultracentrifugation at 100,000 × *g* for 30 min. The various fractions were extracted in boiling sample buffer. Particulate fractions were washed once with hypotonic buffer prior to detergent extraction. Lysates corresponding to defined cell numbers were subjected to immunoblotting with anti-Csk or anti-Src antibodies (see above). To avoid overloading the protein gels, lysates from five-times-lower cell numbers were used for the S100 fraction. This factor was taken into consideration in the subsequent calculation of the relative distribution of Csk. The validity of the cell fractionation procedure was confirmed by studying the distribution of a myristylation-defective (A2) Lck mutant, as well as of the ζ chain of TCR, in BI-141 cells. These two polypeptides are expected to primarily localize to the S100 and P100 fractions, respectively.

Yeast coexpression studies. The various *csk* mutants were cloned in the yeast expression vector pGAL-A4 and introduced in *Saccharomyces cerevisiae* S150-2B expressing either R273 (lysine 273-to-arginine 273) $p56^{lck}$ or R273F505 $p56^{lck}$ (R273 Lck variant bearing an additional tyrosine 505-to-phenylalanine 505 mutation) as described previously (11). Expression of both Csk and Lck was induced by growth in medium containing galactose (2%). After being washed in water, yeast cells were resuspended in a buffer containing 20 mM morpholine propanesulfonic acid (MOPS; pH 7.0), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), supplemented with protease and phosphatase inhibitors, and lysed by vigorous vortexing in the presence of glass beads. Lck was immunoprecipitated and probed by immunoblotting as described above. Csk was immunoprecipitated and subjected to immunoblotting with rabbit anti-Csk antibodies.

Generation of bacterial fusion proteins. DNA constructs allowing the synthesis of glutathione *S*-transferase (GST) fusion proteins encompassing the SH3 (amino acids 13 to 82), SH2 (amino acids 80 to 174), or SH3 plus SH2 (amino acids 13 to 174) domains of $p50^{csk}$ were created by PCR and standard recombinant DNA technology. All PCR-generated DNA fragments were cloned in the pGEX-2T vector (Pharmacia). They were fully sequenced to ensure that no unwanted mutation was introduced in the process (data not shown). Constructs encoding the SH2 sequence of Vav, phospholipase C-γ1, or $p56^{lck}$ were previously described (23). Production and purification of GST fusion proteins were done as outlined elsewhere (23). The concentration of the fusion proteins was estimated by resolving aliquots in SDS-polyacrylamide gels and subsequent staining with Coomassie blue.

In vitro binding assays. BI-141 derivatives expressing an activated version of $p56^{lck}$ (F505 $p56^{lck}$ [1]) were pharmacologically activated by treatment for 10 min with 20 μM bPV (PIC), a synthetic analog of the tyrosine protein phosphatase inhibitor pervanadate. Lysates were then incubated with immobilized GST fusion proteins in the presence of dithiothreitol at a final concentration of 5 mM. In some cases, phosphotyrosine or phosphoserine was also added to the reaction mixtures at the indicated concentration. The agarose beads were washed three times with 1 ml of 1× TNE buffer containing 1 mM sodium orthovanadate. Bound proteins were subsequently eluted with sample buffer, boiled, and resolved by SDS-polyacrylamide gel electrophoresis. Tyrosine-phosphorylated

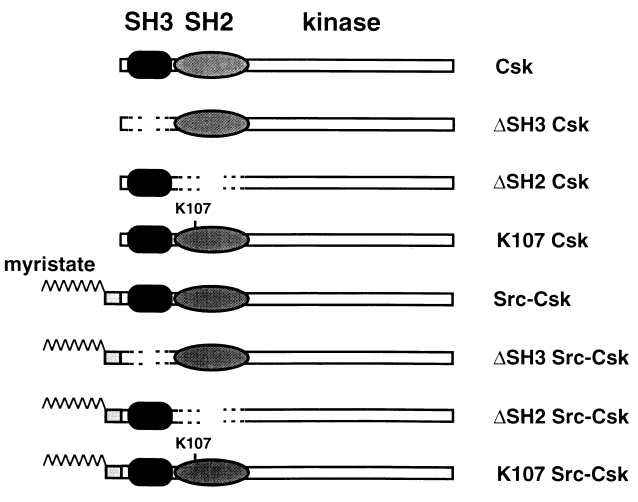

FIG. 1. Structures of Csk mutants. See text for details.

polypeptides were detected by immunoblotting with antiphosphotyrosine antibodies.

RESULTS

Creation and expression of Csk variants bearing mutations in the SH3 or SH2 domain. To better understand the regulation of p50^{csk} in T cells, we wished to examine the roles of its SH3 and SH2 domains in the ability to negatively regulate TCR signalling. To this end, the sequences coding for the Csk SH3 or SH2 motif were deleted through a PCR-based approach. These deletions removed amino acids 14 to 67 and 82 to 171, respectively, of p50^{csk} (Fig. 1). In addition, a point mutation was created at a highly conserved arginine in the Csk SH2 domain (K107 mutation). On the basis of the structure of other SH2 domains (reviewed in reference 22), this arginine is presumed to directly participate in phosphotyrosine binding and thus to be essential for the function of the Csk SH2 motif.

The mutant *csk* cDNAs were inserted into the retroviral vector pLXSN. After transfection in ψ-2 packaging cells, retroviral supernatants were used to infect the antigen-specific mouse T-cell line BI-141. As pLXSN also contains the *neo* gene (17), infected cells were selected for growth in medium supplemented with the aminoglycoside G418. Monoclonal cell lines were subsequently established by limiting dilution and screened for Csk overexpression by immunoblotting of total cell lysates with an antiserum directed against the Csk kinase domain (data not shown). An immunoblot of representative cell lines is depicted in Fig. 2. As a result of their deletions, ΔSH3 Csk (lane 3) and ΔSH2 Csk (lane 4) migrated at approximately 42 and 39 kDa, respectively, in SDS-polyacrylamide gels. In contrast, K107 Csk (lane 5) occasionally exhibited a slightly retarded electrophoretic mobility compared with wild-type p50^{csk} (lanes 1 and 2 and data not shown). The basis for this anomaly is not known. Quantitation of this immunoblot demonstrated that the Csk mutants (lanes 3 to 5) accumulated at levels comparable to those found in BI-141 cells overexpressing wild-type p50^{csk} (lane 2), thus causing a three- to fourfold increase in the abundance of Csk in this cell line. This moderate degree of overexpression most likely reflected the abundant quantities of p50^{csk} present in parental BI-141 cells. Alternatively, it may indicate that expression of greater amounts of p50^{csk} was incompatible with cellular proliferation. All cell lines used for further experiments expressed unaltered levels of TCR, CD3, CD45 and Thy1 and remained CD4 negative (data not shown).

Mutation of the SH3 or SH2 domain diminishes the ability of p50^{csk} to inhibit antigen receptor signalling. The ability of ΔSH3 Csk, ΔSH2 Csk, and K107 Csk to inhibit TCR signalling was next evaluated. Clones were stimulated with anti-TCR mouse MAb F23.1 and SAM IgG for 2 min, and the changes in intracellular tyrosine protein phosphorylation were monitored by immunoblotting of total cell lysates with antiphosphotyrosine antibodies (Fig. 3A). Compared with cells expressing Neo alone (lanes 1 to 4), cells containing ΔSH3 Csk (lanes 7 to 10), ΔSH2 Csk (lanes 11 to 14), or K107 Csk (lanes 15 to 18) did not exhibit a significant reduction in TCR-induced tyrosine protein phosphorylation. In contrast, cells overexpressing wild-type p50^{csk} (lanes 5 and 6) demonstrated a strikingly diminished TCR-triggered response, in accordance with our previous report (6).

In addition, we ascertained the influence of the Csk mutants on antigen receptor-induced lymphokine secretion, a more distal event of T-cell activation. Cells were stimulated for 24 h with increasing amounts of MAb F23.1 coated on plastic, and the release of IL-2 was determined in a standard IL-2 bioassay (Fig. 3B). As previously reported (6), overexpression of wild-type Csk caused a pronounced inhibition of TCR-mediated lymphokine production. However, introduction of equivalent amounts of K107 Csk had a mitigated, albeit reproducible, impact on this response, whereas expression of ΔSH3 Csk or ΔSH2 Csk had no effect. Similar results were obtained with other BI-141 clones (data not shown). When lower concentrations of MAb F23.1 were used for stimulation, significant clonal variation in the degree of IL-2 production was noted (data not shown). Hence, these amounts of antibody were not included in our assays.

Mutation of the SH3 or SH2 sequence does not measurably affect the catalytic activity of p50^{csk} in yeast cells. To ensure that the diminished function of ΔSH3 Csk, ΔSH2 Csk, and K107 Csk in BI-141 cells was not consequent to a decrease in catalytic activity toward Src-like enzymes, the behavior of each of these mutants was also evaluated in yeast cells. Previous reports have demonstrated that p50^{csk} can efficiently phosphorylate the carboxy-terminal tyrosine of Src family members

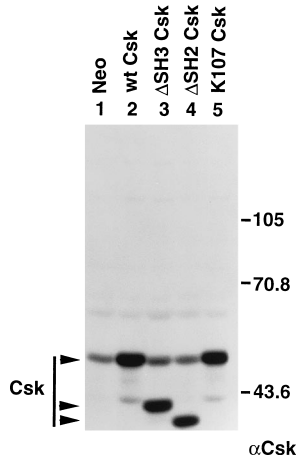

FIG. 2. Expression of Csk mutants in BI-141 T-cells. Lysates from representative cell lines were immunoblotted with a rabbit antiserum directed against the kinase domain of Csk (αCsk). Lanes: 1, Neo.5; 2, wt.28; 3, ΔSH3.19; 4, ΔSH2.57; 5, K107.28. The migrations of the Csk mutants are indicated on the left, while those of prestained molecular mass markers are shown on the right in kilodaltons. Exposure time, 15 h. wt, wild type.

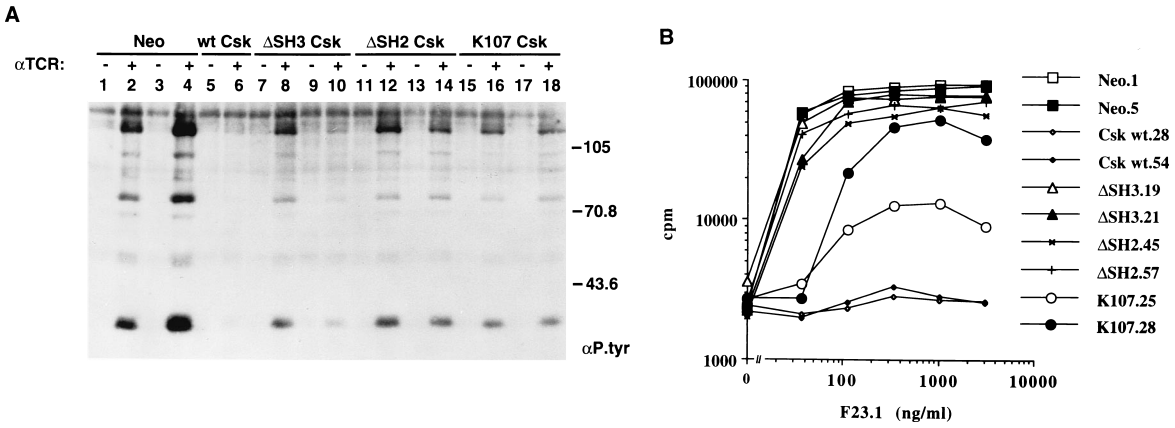

FIG. 3. Effects of Csk mutants on TCR signalling. (A) TCR-mediated tyrosine protein phosphorylation. Representative clones were stimulated for 2 min with anti-TCR MAb F23.1 (αTCR) and SAM IgG and lysed in boiling sample buffer. The abundance of phosphotyrosine-containing proteins was monitored by immunoblotting of total cell lysates with antiphosphotyrosine antibodies (αP.Tyr). Lanes: 1 and 2, Neo.1; 3 and 4, Neo.5; 5 and 6, wt.28; 7 and 8, ΔSH3.19; 9 and 10, ΔSH3.21; 11 and 12, ΔSH2.45; 13 and 14, ΔSH2.57; 15 and 16, K107.25; 17 and 18, K107.28. The positions of prestained molecular weight markers are indicated on the right in kilodaltons. Exposure time, 48 h. wt, wild type. (B) Lymphokine production assay. Cells were stimulated in triplicate for 24 h with increasing amounts of anti-TCR MAb F23.1 (coated on plastic). The production of lymphokines was then measured in a standard IL-2 bioassay.

in this system (11). Hence, the Csk variants were individually introduced in yeast cells containing a kinase-defective variant of $p56^{lck}$ (R273 Lck). After induction in galactose-containing medium, cells were lysed in detergent-containing buffer, and $p56^{lck}$ molecules were recovered by immunoprecipitation with anti-Lck antibodies. The extent of tyrosine phosphorylation of $p56^{lck}$ was determined by immunoblotting with antiphosphotyrosine MAb 4G10 (Fig. 4A, top panel), while its abundance was verified by immunoblotting with an anti-Lck serum (middle panel). In addition, the levels of Csk were evaluated by immunoblotting of anti-Csk immunoprecipitates with an antiserum directed against the carboxy terminus of Csk (bottom panel).

As reported elsewhere (11), in the absence of Csk expression (lane 1), R273 Lck was not detectably tyrosine phosphorylated in yeast cells. However, upon introduction of wild-type $p50^{csk}$ (lane 2), tyrosine phosphorylation of $p56^{lck}$ was readily observed. In a similar way, expression of ΔSH2 Csk (lanes 3 and 4), ΔSH3 Csk (lanes 5 and 6), or K107 Csk (data not shown) also caused tyrosine phosphorylation of the kinase-defective Lck molecules. To verify that R273 Lck was exclusively phosphorylated at the inhibitory carboxy-terminal tyrosine (tyrosine 505), comparable experiments were performed with yeast cells expressing an R273 $p56^{lck}$ variant bearing an additional tyrosine 505-to-phenylalanine 505 substitution (R273F505 $p56^{lck}$) (Fig. 4B). Contrary to R273 $p56^{lck}$ (Fig. 4A; Fig. 4B, lane 1), R273F505 $p56^{lck}$ was not tyrosine phosphorylated by either wild-type $p50^{csk}$ (Fig. 4B, lane 2), ΔSH2 Csk (lanes 3 and 4), ΔSH3 Csk (lanes 5 and 6), or K107 Csk (data not shown). These findings suggested that deletions or mutations in the SH3 or SH2 motif did not affect the catalytic activity of $p50^{csk}$ toward Src-related enzymes.

Expression of membrane-targeted versions of Csk mutants in BI-141 T cells. It has been hypothesized that the SH3 and SH2 domains of $p50^{csk}$ mediate its recruitment to the plasma membrane, where it is more prone to act on Src-like enzymes (6, 9, 14, 20). To test this model, we examined the ability of constitutive membrane targeting to rescue the function of ΔSH3 Csk, ΔSH2 Csk, and K107 Csk. The myristylation sequence from $p60^{c\text{-}src}$ (amino acids 1 to 15) was linked to the amino terminus of the Csk mutants described above through standard recombinant DNA technology (Fig. 1). The resulting Src-Csk polypeptides were introduced in BI-141 cells by retrovirus-mediated gene transfer, and monoclonal cell lines were established as described earlier.

Immunoblot analysis of representative cell lines by using an antibody directed against amino acids 2 to 17 of Src (MAb LA-074) demonstrated that all mutant Src-Csk chimeras (Fig. 5A, top panel, lanes 3 to 5) were expressed in amounts similar to those of wild-type Src-Csk (lane 2). Analogous results were obtained when an antiserum directed against the catalytic domain of $p50^{csk}$ was used (Fig. 5A, bottom panel). This last experiment also revealed that the Src-Csk chimeras accumulated at levels comparable to those of the endogenous $p50^{csk}$. To verify the adequacy of the Src-derived membrane targeting

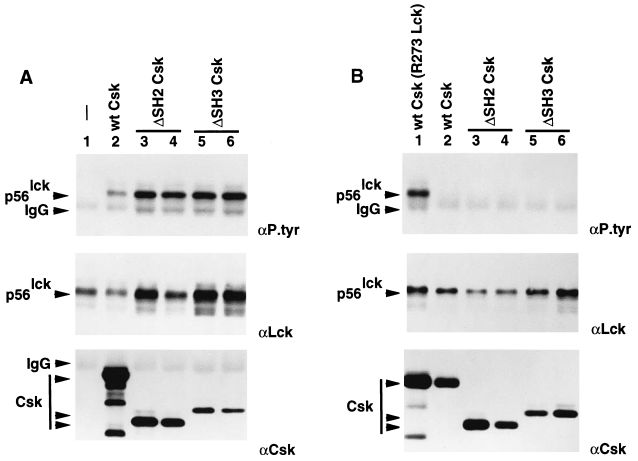

FIG. 4. Yeast coexpression studies. (A) Coexpression of Csk mutants with R273 $p56^{lck}$. The enzymatic activity of wild-type (wt) Csk and each of the Csk mutants was determined by measuring the ability to phosphorylate the carboxy-terminal tyrosine (tyrosine 505) of a kinase-defective (R273) Lck mutant expressed in yeast cells. The migrations of $p56^{lck}$, heavy chain of IgG, and Csk are indicated on the left. Exposure times: top panel, 48 h; middle panel, 6 h; bottom panel, 16 h. (B) Coexpression of Csk mutants with R273F505 $p56^{lck}$. Conditions are as for panel A except that in lanes 2 to 6, yeast cells contained R273F505 $p56^{lck}$. Exposure times: top panel, 4 days; middle panel, 12 h; bottom panel, 12 h. αP.tyr, αLck, and αCsk, antibodies against phosphotyrosine, Lck, and Csk.

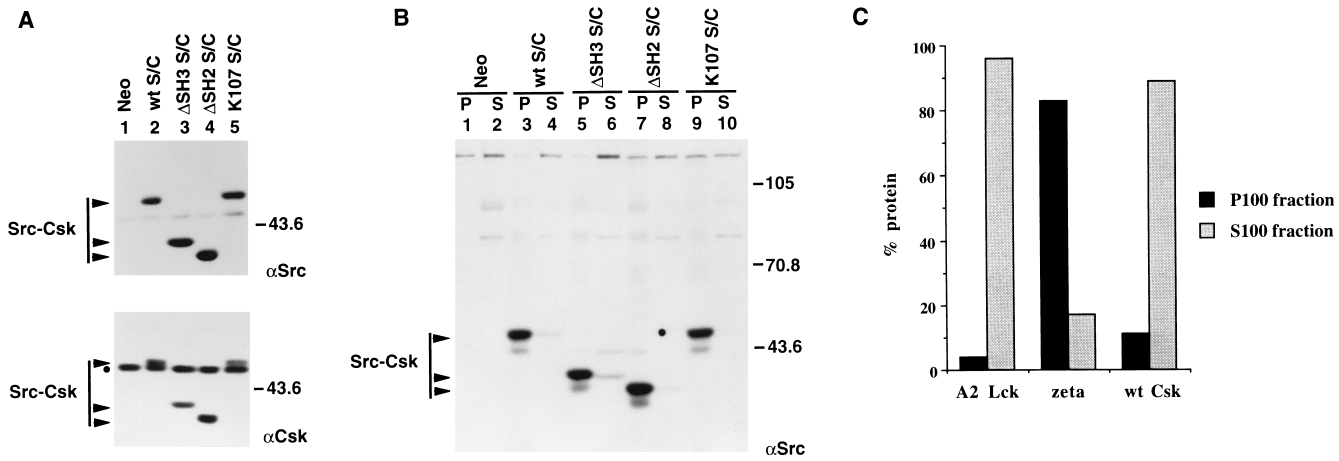

FIG. 5. Expression of membrane-targeted Csk variants in BI-141 cells. (A) The abundance of each of the Src-Csk (S/C) mutants and the wild type (wt) was measured by immunoblotting of total cell lysates with an antibody directed against the Src amino terminus (MAb LA-074; αSrc; top panel) or the catalytic domain of $p50^{csk}$ (αCsk; bottom panel). Lanes: 1, Neo.5; 2, S/C wt.50; 3, S/C ΔSH3.20; 4, S/C ΔSH2.42; 5, S/C K107.1. The migrations of the Src-Csk polypeptides are indicated on the left, while that of endogenous $p50^{csk}$ is shown by a dot (bottom panel). Positions of size markers are shown on the right in kilodaltons. Exposure time, 18 h. (B) Cell fractionation studies. The abundance of each of the Src-Csk proteins in the particulate (P100) and cytosolic (S100) fractions was determined by cell fractionation and subsequent immunoblotting with anti-Src MAb LA-074. Note that lysates corresponding to five-times-greater cell numbers were used in the P100 lanes. Lanes: 1 and 2, Neo.5; 3 and 4, S/C wt.50; 5 and 6, S/C ΔSH3.20; 7 and 8, S/C ΔSH2.42; 9 and 10, S/C K107.1. The positions of the Src-Csk polypeptides are indicated on the left, while those of prestained molecular mass markers are indicated on the right in kilodaltons. Exposure time, 3 days. (C) Distribution of control polypeptides in BI-141 cells. The distributions of A2 Lck, ζ, and wild-type Csk are shown as a control.

signal, the cellular distribution of the Src-Csk chimeras was determined by cell fractionation (Fig. 5B). Cells were mechanically lysed in hypotonic buffer, and postnuclear supernatants were separated into particulate (P100) and cytosolic (S100) fractions. The abundance of Src-Csk in each fraction was determined by immunoblotting of lysates with anti-Src MAb LA-074. After correction for the fact that lysates from five-times-greater cell numbers were used for the P100 fraction, it was estimated that 50 to 70% of the various Src-Csk chimeras was present in the particulate fraction. In contrast, only 10% of wild-type $p50^{csk}$ accumulated in the P100 fraction (Fig. 5C and data not shown). This observation implied that all chimeras were stably associated with cellular membranes. To verify the adequacy of our cell fractionation procedure, the distribution of a myristylation-defective $p56^{lck}$ mutant (A2 Lck) and of the ζ chain of TCR was evaluated in BI-141 cells (Fig. 5C and data not shown). Quantitative analyses showed that, as expected, over 90% of A2 Lck was present in the S100 fraction, while approximately 85% of ζ was detected in the P100 fraction.

Constitutive membrane targeting can rescue the defective function of K107 Csk. The impact of the membrane-targeted Csk variants on TCR-induced tyrosine protein phosphorylation was compared with that of the non-membrane-targeted Csk molecules (Fig. 6). Importantly, an anti-Csk immunoblot of total cell lysates demonstrated that the membrane-targeted versions (lanes 6 to 9) were expressed in amounts lower than or

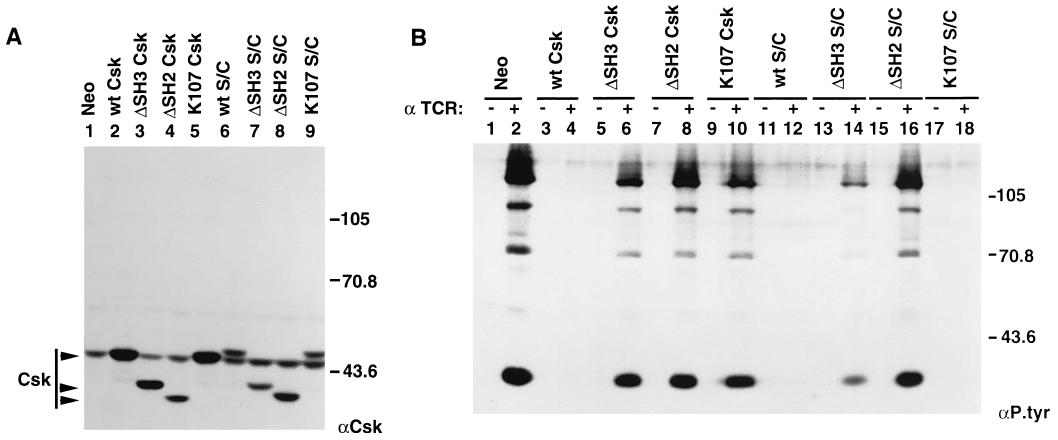

FIG. 6. Impact of membrane-targeted Csk variants on TCR-mediated tyrosine protein phosphorylation. (A) Comparison of the expression levels of wild-type (wt) Csk and non-membrane-targeted and membrane-targeted Csk variants. Lysates from representative clones were immunoblotted with anti-Csk antibodies (αCsk). Lanes: 1, Neo.5; 2, wt.28; 3, ΔSH3.19; 4, ΔSH2.57; 5, K107.28; 6, Src-Csk (S/C) wt.50; 7, S/C ΔSH3.20; 8, S/C ΔSH2.42; 9, S/C K107.1. The migrations of the Csk mutants are indicated on the left, while those of prestained molecular mass markers are shown on the right in kilodaltons. Exposure time, 4 h. (B) Antiphosphotyrosine immunoblot. Cells were stimulated for 2 min with anti-TCR MAb F23.1 (αTCR) and SAM IgG and lysed in boiling sample buffer. The abundance of phosphotyrosine-containing proteins was monitored by immunoblotting of total cell lysates with antiphosphotyrosine antibodies (αP.tyr). Lanes: 1 and 2, Neo.1; 3 and 4, wt.28; 5 and 6, ΔSH3.19; 7 and 8, ΔSH2.45; 9 and 10, K107.25; 11 and 12, S/C wt.39; 13 and 14, S/C ΔSH3.26; 15 and 16, S/C ΔSH2.55; 17 and 18, S/C K107.12. The positions of prestained molecular mass markers are indicated on the right in kilodaltons. Exposure time, 15 h.

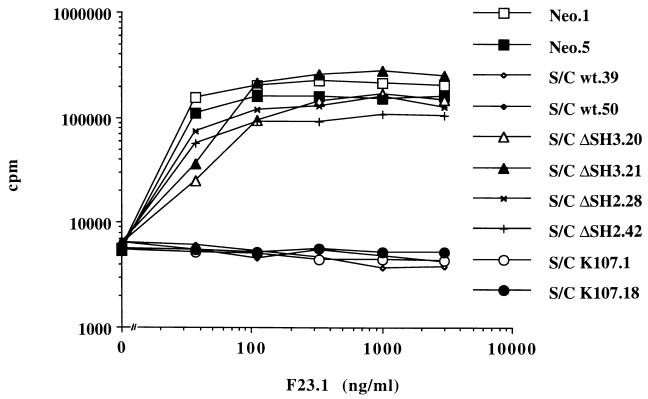

FIG. 7. Impact of membrane-targeted Csk variants on TCR-mediated lymphokine secretion. For details, see the legend to Fig. 3B.

comparable to those of the non-membrane-targeted variants (lanes 2 to 5). Cells were stimulated for 2 min with anti-TCR MAb F23.1 and SAM IgG, and the abundance of phosphotyrosine-containing polypeptides was measured by immunoblotting with antiphosphotyrosine antibodies (Fig. 6B). In agreement with our earlier report (6), wild-type Src-Csk completely abrogated TCR-triggered tyrosine protein phosphorylation in BI-141 cells (lanes 11 and 12). Expression of ΔSH3 Src-Csk led to a partial reduction of TCR-mediated tyrosine protein phosphorylation (lanes 13 and 14), while introduction of ΔSH2 Src-Csk did not repress the TCR-induced response (lanes 15 and 16). In contrast, however, K107 Src-Csk fully inhibited antigen receptor-induced tyrosine protein phosphorylation (lanes 17 and 18). Hence, addition of the Src myristylation signal partially rescued the function of ΔSH3 Csk and completely restored the effect of K107 Csk. Similar results were obtained with other clones (data not shown).

The consequences of expression of the Src-Csk mutants on antigen receptor-mediated lymphokine secretion were next examined (Fig. 7). In contrast to wild-type Src-Csk, which completely blocked TCR-induced IL-2 secretion, ΔSH3 Src-Csk and ΔSH2 Src-Csk had no measurable effect on this response, even when lower concentrations of anti-TCR antibodies were used for stimulation (data not shown). However, as noted for tyrosine protein phosphorylation, K107 Src-Csk completely prevented lymphokine secretion induced by antigen receptor stimulation.

The Csk SH2 domain can interact with several phosphotyrosine-containing proteins from activated T cells. Because SH2 domains have the ability to associate with phosphotyrosine-containing proteins (22), we evaluated the capacity of the Csk SH2 domain to bind tyrosine-phosphorylated polypeptides from activated BI-141 cells. Pervanadate-treated BI-141 T cells expressing a constitutively activated form of $p56^{lck}$ (F505 Lck) were used as a source of abundant tyrosine-phosphorylated substrates in these assays. After immobilization on agarose-glutathione beads, recombinant GST fusion proteins were incubated with BI-141 cell lysates. Following several washes, bound proteins were detected by antiphosphotyrosine immunoblotting (Fig. 8). Contrary to GST alone (Fig. 8A, lane 1) and GST-Csk SH3 (lane 2), which failed to detectably associate with tyrosine-phosphorylated polypeptides, GST-Csk SH2 (lane 3) bound several phosphotyrosine-containing proteins from activated BI-141 cells. The most prominent products migrated at approximately 120, 68, and 62 kDa. A comparable set of polypeptides was recovered by a fusion protein containing the SH3 and SH2 domains of $p50^{csk}$ (lane 4). In contrast, the SH2 region of the putative guanine nucleotide exchange factor Vav bound additional tyrosine-phosphorylated polypeptides of 160, 76, 74, and 70 kDa (lane 5), while the amino-terminal SH2 sequence of phospholipase C-γ1 reacted with a supplementary protein of 76 kDa (lane 6). Moreover, the SH2 domain of $p56^{lck}$ (lane 7) associated with essentially

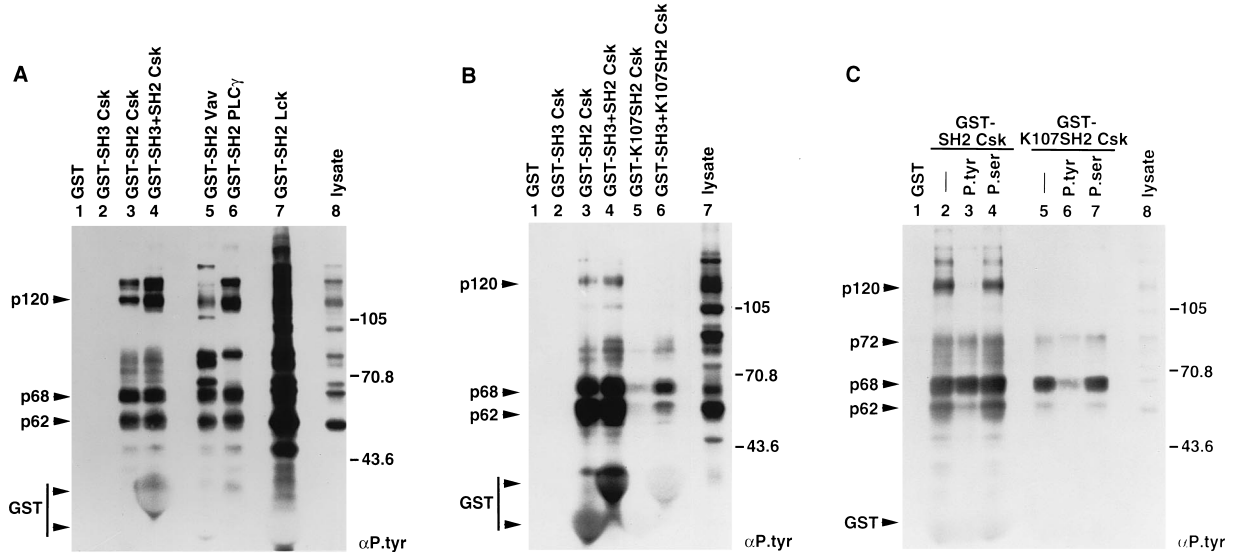

FIG. 8. In vitro association of the Csk SH2 domain with phosphotyrosine-containing proteins. (A) The abilities of bacterial fusion proteins to associate with phosphotyrosine-containing proteins from pervanadate-treated F505 Lck-expressing BI-141 cells were examined by antiphosphotyrosine (αP.tyr) immunoblotting. The positions of the major tyrosine-phosphorylated polypeptides and of the GST fusion proteins are indicated on the left, whereas those of prestained molecular mass markers are shown on the right in kilodaltons. Exposure time, 4 h. PLCγ, phospholipase C-γ1. (B) Same as for panel A except that K107 Csk SH2 domains were included in the analysis. Exposure time, 9 h. (C) Same as for panel B except that 50 mM of phosphotyrosine (P.tyr) or phosphoserine (P.ser) was added during the binding reactions. Exposure time, 3 h.

all tyrosine-phosphorylated proteins that could be detected in BI-141 cell lysates (lane 8). Hence, the SH2 domain of Csk reacted with a rather selected subset of phosphotyrosine-containing substrates in activated BI-141 T cells.

The ability of the Src membrane association signal to rescue the function of K107 Csk but not that of ΔSH2 Csk suggested that the K107 mutation did not completely abrogate the properties of the Csk SH2 domain. To test this possibility, we evaluated the capacity of the mutant Csk SH2 domain to associate with phosphotyrosine-containing proteins in vitro (Fig. 8B). These assays showed that the K107 Csk SH2 domain recovered significantly lower amounts of tyrosine-phosphorylated proteins (lanes 5 and 6). However, residual association with products of 72, 68, and 62 kDa could be observed.

The equivalent of arginine 107 in other SH2 domains has been shown to directly bind the phosphate group of phosphotyrosine, thus explaining its requirement for the association with tyrosine-phosphorylated proteins (reviewed in reference 22). To evaluate whether the residual function of the K107 SH2 domain was still dependent on phosphotyrosine-mediated interactions, we tested the ability of free phosphotyrosine to compete with these associations. In the case of wild-type Csk SH2 domains (Fig. 8C, lanes 2 to 4), addition of phosphotyrosine (50 mM) diminished the recovery of p120 and p62 without significantly affecting that of p72 and p68 (lane 3). In contrast, addition of phosphoserine did not affect binding of any substrate (lane 4). When similar experiments were performed with K107 Csk SH2 domains (lanes 5 to 7), phosphotyrosine competed for the binding of p68 and p62, while it did not markedly influence the recovery of p72 (lane 6). Together, these results indicated that the K107 Csk SH2 domain had residual phosphotyrosine-binding activity, which may explain the distinct behaviors of ΔSH2 Csk and K107 Csk in vivo. Nevertheless, as phosphotyrosine failed to compete for the binding of wild-type and K107 Csk SH2 domains to some cellular proteins, it cannot be excluded that a subset of complexes was formed through a phosphotyrosine-independent mechanism.

DISCUSSION

In this report, we have attempted to further elucidate the processes by which $p50^{csk}$ inhibits antigen receptor signalling in T lymphocytes. Previous studies from our laboratory had shown that this function was dependent on the intrinsic kinase activity of Csk and that it was enhanced by constitutive membrane targeting of $p50^{csk}$ (6). In the present study, we determined that the noncatalytic SH3 and SH2 domains are also essential for the negative influence of Csk on TCR signalling. Indeed, complete deletion of either the SH3 or SH2 sequence completely abolished the capacity of Csk to inhibit TCR-induced tyrosine protein phosphorylation and lymphokine secretion. Moreover, mutation of a conserved arginine residue in the SH2 sequence (K107 Csk mutation) significantly reduced the negative impact of $p50^{csk}$ on T cells.

In contrast to the striking effects of deletion of the SH3 or SH2 sequence in T cells, these alterations had no appreciable effect on the ability of Csk to phosphorylate the inhibitory carboxy-terminal tyrosine of a Src-like kinase ($p56^{lck}$) in yeast cells. Similarly, Sabe et al. (26) previously reported that deletion or mutation of the SH3 or SH2 domain did not affect the ability of $p50^{csk}$ to phosphorylate poly(Glu,Tyr) in vitro. However, it is worth mentioning that these results differed somewhat from those published by Howell and Cooper (14). Whereas these authors also noted that deletion of the SH3 region did not interfere with the ability of $p50^{csk}$ to phosphorylate substrates in vitro, they nevertheless observed that complete or partial removal of the SH2 domain abrogated the function of Csk in these assays. Whereas the precise basis for this discrepancy is not known, it is possible that the enzymatic activity of ΔSH2 Csk was lost in the study of Howell and Cooper (14) during the process of cell lysis and immunoprecipitation.

In any case, given our data and those reported by Sabe et al. (26), it is not likely that the defective function of ΔSH3 Csk and ΔSH2 Csk in BI-141 T-cells was consequent to a decrease in Csk catalytic activity. Rather, as SH3 and SH2 domains mediate protein-protein interactions (reviewed in reference 22), we speculated that it was caused by defects in critical associations between Csk and other cellular proteins. In keeping with this model, we showed that recombinant Csk SH2 domains bound tyrosine-phosphorylated proteins of 120, 68, and 62 kDa from activated BI-141 T cells. Moreover, Oetken et al. (20) reported that GST-Csk SH2 domains could associate with a 72-kDa tyrosine-phosphorylated polypeptide from activated Jurkat T cells. Whereas the exact identity of these molecules is not known, they are candidate docking proteins mediating the recruitment of $p50^{csk}$ in the TCR signalling cascade.

In fibroblasts, the SH3 and SH2 sequences of Csk have been found to be required for its colocalization with activated c-Src in podosomes (14). While no change in the extent of Csk membrane association has been observed during T-cell activation (7), our laboratory found that addition of a Src-derived membrane targeting signal significantly increased the ability of wild-type $p50^{csk}$ to inhibit TCR signalling (6). Moreover, in the current report, we showed that constitutive membrane association corrected the altered ability of K107 Csk to repress TCR signalling. Therefore, it seems that at least part of the function of the SH2 domain in T cells is to reversibly translocate Csk to the plasma membrane. Intriguingly though, the Src myristylation sequence failed to restore the function of ΔSH3 Csk and ΔSH2 Csk. Hence, it would seem that the SH3 and SH2 domains may not exclusively serve for membrane recruitment of Csk. They may also permit lateral positioning of Csk at the membrane, thereby placing it in the appropriate context to interact with Lck and FynT. Alternatively, they may allow the recruitment of other molecules important for the negative regulatory effect of Csk on TCR signalling.

Intriguingly, some discrepancies between the abilities of variants of Csk to inhibit TCR-induced tyrosine protein phosphorylation and block antigen receptor-mediated lymphokine secretion were observed in our study. First, K107 Csk did not convincingly affect TCR-mediated phosphorylation, while it reproducibly reduced lymphokine secretion. Conversely, even though ΔSH3 Src-Csk repressed TCR-regulated tyrosine protein phosphorylation, it did not influence subsequent IL-2 release. These disparities may imply that Csk regulates TCR-triggered cellular activation at multiple levels, one of these being the initiation of TCR-induced tyrosine protein phosphorylation. In addition, $p50^{csk}$ may have the ability to inhibit later steps of TCR signalling, and the structural domains required for this effect may be distinct from those necessary for down-regulation of TCR-mediated tyrosine protein phosphorylation. Obviously, a better understanding of the downstream events of T-cell activation may help explain these observations.

In this study, we evaluated the mechanisms by which the tyrosine protein kinase $p50^{csk}$ negatively regulates antigen receptor signalling in T lymphocytes. We found that the inhibitory function of $p50^{csk}$ was greatly reduced by deletion of its SH3 or SH2 sequence or by mutation of a conserved arginine residue in its SH2 motif (K107 mutation). These deleterious effects did not appear to be caused by a reduction in $p50^{csk}$-

associated catalytic activity, as indicated by the fact that ΔSH3 Csk, ΔSH2 Csk, and K107 Csk efficiently phosphorylated the inhibitory carboxy-terminal tyrosine of a Src-related enzyme in yeast cells. Instead, they potentially reflected a lack of association with docking proteins involved in recruiting Csk in the TCR signalling cascade or a defect in binding other polypeptides involved in Csk-mediated functions. It was also noted that addition of a constitutive membrane targeting signal rescued the diminished ability of K107 Csk to inhibit TCR signalling. This result suggested that part of the purpose of the Csk SH2 domain is to recruit $p50^{csk}$ to the plasma membrane, where Src-related enzymes and the antigen receptor complex are located. However, the membrane localization signal was incapable of restoring the efficacy of ΔSH3 Csk and ΔSH2 Csk, indicating that additional functions are also provided by the two noncatalytic domains.

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