

Csk Is Constitutively Associated with a 60-kDa Tyrosine-phosphorylated Protein in Human T Cells*

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The protein-tyrosine kinase Csk is one of the main down-regulators of the Src family of kinases. Csk may be involved in the down-regulation of T cell receptor (TCR) signaling by C-terminal tyrosine phosphorylation of Lck and Fyn; however, it is not known how Csk activity is regulated or how it targets these Src family members. We used Jurkat T cells and normal human T cells to examine proteins that bind to the SH2 domain of Csk. In both Jurkat and normal T cells, the Src homology 2 (SH2) domain of Csk bound constitutively to a tyrosine-phosphorylated protein of 60 kDa (p60). The 60-kDa protein was detected in Csk immunoprecipitates from both unstimulated and CD3-stimulated cells. In addition to p60, a protein of 190 kDa coprecipitated with Csk, and both proteins were phosphorylated on tyrosine residues by the immunocomplex. Small amounts of GTPase-activating protein (GAP) were detected in anti-Csk immunoprecipitates, suggesting that p60 may be a GAP-associated protein. Our data demonstrate that the SH2 domain of Csk specifically associates with at least two tyrosine-phosphorylated proteins in normal human T cells, that this association is independent of TCR/CD3 activation, and that Csk may be a part of a multiprotein complex containing GAP.

In addition to its crucial role in cell growth and development, the Src family of protein-tyrosine kinases plays an essential role in the activation of cells of the immune system (reviewed in Ref. 1). The Src family contains at least 10 known members (reviewed in Refs. 2–4) that show cell type- and receptor-specific distribution; for example, Lck and Fyn are necessary for signal transduction through the T cell receptor (TCR)¹ (reviewed in Ref. 1); Lyn, Blk, and Fyn associate with the B cell receptor (5–7); and Lyn is required for signal transduction through the high affinity IgE receptor (8). Engagement of these receptors with their respective ligands activates the associated Src kinases, leading to tyrosine phosphorylation of multiple cellular substrates, ultimately stimulating the effector functions of the cell, such as proliferation, gene transcription, cyto-

kine production, and mediator release. The basic structure of the Src kinases is highly conserved between family members. In addition to their catalytic domain, Src kinases contain SH2 and SH3 domains, an N-terminal myristylation site that directs attachment to the cell membrane, and two regulatory tyrosine phosphorylation sites (9–15). Autophosphorylation of a tyrosine in the kinase domain serves to increase the activity of the kinases, whereas phosphorylation of a C-terminal tyrosine causes the kinase to fold into an inactive state (reviewed in Ref. 15).

Csk (C-terminal Src kinase) was identified as a protein-tyrosine kinase that phosphorylates the C-terminal tyrosines of the Src kinases, thereby negatively regulating their activity (16–19). The essential role of Csk as a regulator of Src kinases has been extensively demonstrated both *in vivo* and *in vitro* (20–22). The primary structure of Csk is similar to that of the Src family; however, Csk does not have an N-terminal myristylation site, a positive regulatory auto-phosphorylation site, or a negative C-terminal regulatory tyrosine residue (15).

Several studies suggest that Csk may play a role in down-regulating activation signals in T and B cells. Csk is required for normal development of lymphoid cells (23); it is also abundantly expressed in adult thymus and spleen, suggesting that it is actively involved in the normal functioning of mature lymphocytes (1). Furthermore, it has been reported that TCR/CD3 stimulation results in the increased activity of Csk in Jurkat T cells (24). Importantly, overexpression of Csk in T cell hybridomas resulted in inhibition of TCR-induced tyrosine phosphorylation and greatly reduced interleukin-2 production (25). This effect could be reversed by the overexpression of constitutively active Lck. Analysis of B cell receptor signaling in Csk-deficient B cell lines revealed that Lyn was constitutively active and highly phosphorylated on its autophosphorylation site (26). Taken together, these studies suggest that Csk acts to down-regulate Src kinases in the T and B cell receptor systems. It is of interest that only a fraction of Lck and Lyn are found to be phosphorylated on their C termini in unstimulated cells. This indicates that the activity of Csk may be continually balanced by the activity of a protein-tyrosine phosphatase. In this regard it is noteworthy that Csk can phosphorylate CD45 *in vitro*, thereby creating a binding site for Lck (27). The exact details of the interactions between the Src kinases, Csk, and CD45 have yet to be understood.

Little is known about how Csk activity is regulated or with which proteins Csk associates in hematopoietic cells. In this study, we examined proteins that associate with the SH2 domain of Csk in Jurkat cells and normal human T cells. We found that Csk associates directly with two proteins, a heavily phosphorylated protein of 60 kDa and a protein of 190 kDa. Importantly, these associations were constitutive, and phosphorylation of neither protein was affected by TCR/CD3 stim-

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¹ The abbreviations used are: TCR, T cell receptor; SH2, Src homology 2; SH3, Src-homology 3; GST, glutathione S-transferase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; GAP, GTPase-activating protein.

ulation. Small amounts of GAP were detected in anti-Csk immunoprecipitates, suggesting that p60 may be the human analog of the GAP-associated 60-kDa phosphoprotein recently reported to be associated with Csk in Src-transformed murine fibroblasts (28).

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Antibodies—Unless specified, all reagents were purchased from Sigma. Protein A-Sepharose was purchased from Repligen or Pharmacia Biotech Inc. Radioactive isotopes and horseradish peroxidase-labeled second antibodies were purchased from Amersham Corp. The Jurkat cell line was grown in RPMI 1640 medium containing Hepes and supplemented with 5% fetal bovine serum, 50 units/ml penicillin, and streptomycin, and 300 mg/liter L-glutamine. The monoclonal antibody (mAb) OKT3 against human CD3 ϵ chain was purified from hybridoma culture supernatants using protein A-Sepharose column chromatography and used at a concentration of 10 μ g/ml for stimulation of the T cells. Antiserum 3622.3 generated against the 13 C-terminal amino acids of Csk (KEQLEHIKTHELHL) was generously supplied by Jonathan Cooper. Rabbit antiserum against the same peptide was subsequently raised in the laboratory and affinity purified using the same peptide coupled to CNBr-activated Sepharose (Pharmacia). Antisera #387 and #527 against TCR ζ chain were kindly supplied by Lawrence Samelson and Allan Weissman, respectively. Antibodies against ZAP-70 kinase, human Lck, Fyn, Src, phosphotyrosine-specific mAb 4G10, and anti-GAP mAb were purchased from UBI. Antiserum against the GAP-associated protein p62 and Shc were obtained from Santa Cruz Biotechnology, Inc. Antisera against GAP were generously provided by Frank McCormick and Tony Pawson, and anti-c-cbl antiserum was provided by Lawrence Samelson. Anti-FAK antibody and anti-Csk mAb were from Signal Transduction Laboratories.

Culture of Short Term Human T cell Lines—Human T cells from the peripheral blood of healthy volunteer donors were grown in RPMI 1640 medium containing Hepes, 20 units/ml recombinant interleukin-2, 10% fetal bovine serum, penicillin, streptomycin, and glutamine. To guarantee the T cell lineage, cells were stimulated with 3 μ g/ml phytohemagglutinin for 10–14 days before restimulation with phytohemagglutinin in the presence of autologous feeder cells. Following the second stimulation the cells were allowed to rest for at least 10 days before use. After two rounds of stimulation with phytohemagglutinin, over 99% of the viable cells were CD3 $^+$ T cells, as shown by fluorescence-activated cell sorter analysis using the anti-CD3 antibody, OKT3. The cells were harvested, counted, resuspended in RPMI, and stimulated with OKT3 as described for the Jurkat cells.

Expression and Purification of Glutathione S-Transferase-Fusion Proteins—The cDNA clone encoding Csk was generously supplied by Akira Imamoto; the cDNA encoding the SH2 domain of Csk in which the phosphotyrosine-binding FLVRES motif had been mutated to FLVSKI was kindly supplied by Brian Howell. DNA sequences encoding SH2 (amino acids 105–208), SH3 (amino acids 42–96), and SH2/3 (amino acids 42–208) of Csk were amplified by polymerase chain reaction using specific primers containing restriction sites for *Bam*HI and *Eco*RI. The polymerase chain reaction products were subcloned into the pGEX-2T (29) vector using standard procedures (30). The recombinant plasmids were used to transform *Escherichia coli* strain JM109. After confirmation of the DNA sequences by dideoxy sequencing, fusion proteins were produced and purified by affinity chromatography on glutathione-agarose.

Fusion Protein Binding Assays, Immunoprecipitations, and Immunoblots—For fusion protein binding assays, unstimulated cells or cells that had been stimulated with 10 μ g/ml OKT3 for the indicated times were harvested and lysed on ice with Triton lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 mM sodium orthovanadate). Insoluble material was removed by centrifugation at 14,000 \times g for 5 min. The lysed cells were used at the equivalent of 5–10 \times 10 7 cells/gel lane for the Jurkat cells and 1–5 \times 10 7 /gel lane for the normal T cells. Cell lysates were tumbled with 3–10 μ g of glutathione-agarose immobilized GST-fusion proteins at 4 $^{\circ}$ C for 4 h. In the SH2 mutant binding assays, lysate from 2 \times 10 7 cells was incubated with 0.5 μ g of fusion protein. The beads were washed three times in ice-cold lysis buffer and boiled in SDS sample buffer (2% SDS, 50 mM Tris, pH 6.8, 100 mM dithiothreitol, 0.1% bromophenol blue, 10% glycerol). For immunoprecipitation of Csk, the cells (1–5 \times 10 7 /lane) were lysed in a Nonidet P-40 lysis buffer (10 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml aprotinin, 1 mM sodium orthovanadate, 50 mM NaF). After centrifuga-

tion for 5 min at 14,000 \times g, the lysate was incubated with protein A-Sepharose for 30 min to remove the OKT3 mAb used for activation. This was followed by a second preclearing for 30 min with normal rabbit serum, prebound to anti-rabbit Ig-conjugated agarose beads. The lysate was then incubated for 1 h with anti-Csk antibodies, prebound to anti-rabbit Ig conjugated agarose beads, washed three times in the Nonidet P-40 lysis buffer, and boiled in SDS sample buffer. In the coprecipitation experiments in Fig. 2, the antibodies were covalently coupled to protein-A Sepharose beads using dimethylpimelimidate (Pierce).

The eluted material was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The blots were blocked in 10% Carnation milk powder in phosphate-buffered saline containing 0.1% Tween 20. The blots were incubated with the appropriate primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Corp.).

In Vitro Kinase Assays—To analyze proteins that coprecipitate with Csk, Csk was immunoprecipitated from lysates of unstimulated or stimulated Jurkat cells (1 \times 10 7 cells/lane). The immunoprecipitates were washed three times in Nonidet P-40 lysis buffer and once in Csk kinase buffer (10 mM Hepes, pH 7.4, 5 mM MnCl $_2$). The beads were incubated in 20 μ l of Csk kinase buffer with 0.5 μ Ci of [γ - 32 P]ATP for 30 min at 22 $^{\circ}$ C. After three washes with lysis buffer, the immunoprecipitate was suspended in 50 μ l of lysis buffer containing 1% SDS. The samples were boiled for 5 min, the beads were pelleted by centrifugation, and the supernatants were collected. 10% of the supernatant (total kinase reaction) was analyzed directly by SDS-PAGE, whereas the rest was diluted 20 times with Triton lysis buffer. The diluted samples were incubated with 3–5 μ g of immobilized fusion proteins for 1 h at 4 $^{\circ}$ C. The adsorbed proteins were separated by SDS-PAGE, transferred to Immobilon membrane (Bio-Rad), and visualized by autoradiography. To identify proteins that were phosphorylated on tyrosine residues, the filters were treated with 1 N KOH for 2 h at 55 $^{\circ}$ C as described (31).

To assay the specific activity of Csk, variations of two previously published procedures were used (24, 32). In the first procedure, Csk was immunoprecipitated from 1–5 \times 10 6 cells as described above. The precipitate was washed three times in Nonidet P-40 lysis buffer and once in Csk-kinase buffer. The activity of Csk was measured by analyzing the incorporation of [γ - 32 P]ATP into a synthetic copolymer of (Glu, Tyr) 4:1 (Sigma), covalently coupled to CNBr-activated Sepharose (Pharmacia) at 5 mg/ml. The Csk immunoprecipitate was incubated in 40 μ l of kinase buffer containing 0.5 μ Ci of [γ - 32 P]ATP and 20 μ l of the copolymer beads at 30 $^{\circ}$ C for 5 min. The reaction was terminated by the addition of Nonidet P-40 lysis buffer containing 2% SDS. The beads were washed once in lysis buffer followed by several alternate washes in 50 mM sodium bicarbonate, pH 8.5, 0.5 M NaCl, 50 mM sodium acetate, pH 4, 0.5 M NaCl. The beads were washed once in water, and the bound radioactivity was counted by liquid scintillation. In the second procedure, Csk immunoprecipitation was performed using 10% slurry of formaldehyde-fixed *Staphylococcus aureus* (Sigma) instead of anti-rabbit IgG beads. The washed immunoprecipitates were incubated with 100 μ g of soluble copolymer and 0.5 μ Ci of [γ - 32 P]ATP, for 5 min at 30 $^{\circ}$ C. The *S. aureus* precipitate was pelleted by centrifugation, and the supernatant containing the copolymer was subjected to SDS-PAGE followed by scintillation counting of the excised gel lanes.

RESULTS

Csk-SH2 Domain Binds a Distinct Set of Phosphoproteins in Jurkat Cells—To identify proteins with which Csk interacts, recombinant GST fusion proteins encompassing the Csk-SH2, SH3, and SH2/3 domains were used in binding assays with lysates of unstimulated and OKT3-stimulated Jurkat cells (Fig. 1A). Several tyrosine-phosphorylated proteins bound to the Csk-SH2 fusion protein, the two most prominent bands migrating with apparent molecular masses of 60 (p60) and 21 kDa (p21). p60 was constitutively phosphorylated on tyrosine, and its interaction with the Csk-SH2 domain was not activation-dependent. Upon shorter exposures of the film, p60 was shown to consist of two closely spaced bands. p21 also migrated as a doublet and interacted with the Csk-SH2 domain in an activation-dependent manner. Maximal interaction between p21 and Csk-SH2 occurred after 1 min of stimulation and started to decrease after 2 min. Neither the Csk-SH3 domain nor the GST control bound any tyrosine-phosphorylated proteins. Csk-SH2/3 bound the

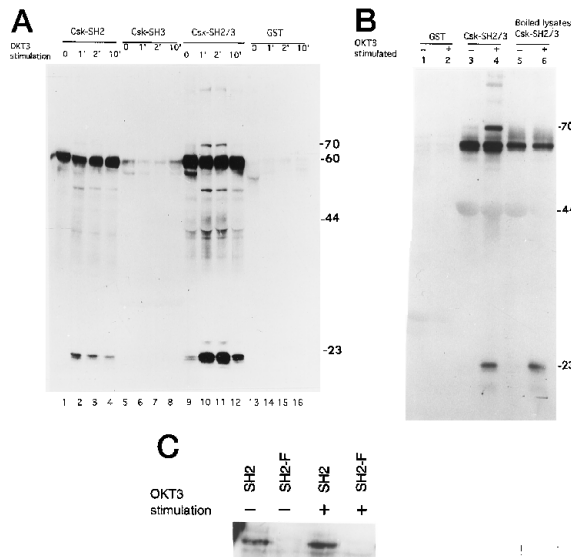


FIG. 1. *A*, binding of tyrosine-phosphorylated proteins to GST fusion proteins. Lysates of Jurkat cells, unstimulated (*lanes 1, 5, 9, and 13*) or stimulated with mAb OKT3 for 1 min (*lanes 2, 6, 10, and 14*), 2 min (*lanes 3, 7, 11, and 15*), or 10 min (*lanes 4, 8, 12, and 16*) were incubated with the GST-SH2 (*lanes 1–4*), GST-SH3 (*lanes 5–8*), GST-SH2/3 (*lanes 9–12*), or GST control (*lanes 13–16*) beads. The adsorbates were separated by SDS-PAGE on 8% acrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine mAb 4G10. *B*, GST (*lanes 1 and 2*) or GST-SH2/3 (*lanes 3–6*) fusion proteins were incubated with unstimulated cell lysate (*lanes 1 and 3*), boiled unstimulated cell lysate (*lane 5*), lysate of cells stimulated for 1 min with OKT3 mAb (*lanes 2 and 4*), and boiled lysate of stimulated cells (*lane 6*). Precipitates were separated by SDS-PAGE on 12% acrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine mAb. *C*, GST-fusion proteins containing the Csk SH2 domain (SH2) or the Csk-SH2 domain in which the FLVRES motif has been mutated to FLVSKI (SH2-F) were incubated with lysates of unstimulated or stimulated Jurkat cells, and the bound proteins were analyzed by phosphotyrosine immunoblotting.

same set of proteins as did Csk-SH2 but apparently with higher affinity, consistent with previous reports concerning the influence of the flanking SH3 domain on the affinity of the SH2 domain toward phosphopeptides (33, 34). Besides p21 and p60, four other tyrosine-phosphorylated proteins with apparent molecular masses of 120, 70, 52, and 25 kDa bound to the Csk-SH2, and more strongly to the SH2/3 domains (after a longer exposure of the blot, all four proteins were clearly seen to bind to Csk-SH2). Their interactions with SH2 and SH2/3 domains were also activation-dependent with a time course similar to the binding of p21. Using TCR immunoprecipitations, followed by reprecipitation with Csk-SH2 fusion proteins, the 21- and 25-kDa proteins were subsequently identified, respectively, as the ζ and ϵ chains of the CD3 complex; the 70-kDa protein was identified by Western blotting analysis as the protein-tyrosine kinase ZAP-70 (35).

To analyze which of the Csk-SH2-associated proteins in Fig. 1A interacts directly with Csk, we utilized the fact that SH2 domains recognize linear phosphopeptide sequences that persist after the three-dimensional structure of the proteins has been disrupted by denaturation. Lysates of unstimulated and stimulated Jurkat cells were boiled in 1% SDS, diluted 10 times with lysis buffer, and incubated with the immobilized Csk-SH2/3 fusion protein (Fig. 1B). Only the two major phosphoproteins, p60, and the ζ (p21) chain, were detected in the Csk-SH2/3 adsorbates; furthermore, when more cells were used, the ϵ chain was also shown to bind directly to Csk-SH2/3 (data not shown). The other three phosphoproteins, ZAP-70, p52, and p120 did not associate with the Csk-SH2/3 protein after denaturation. A Csk-SH2 mutant, in which the phosphotyrosine-binding motif had been changed from FLVRES to

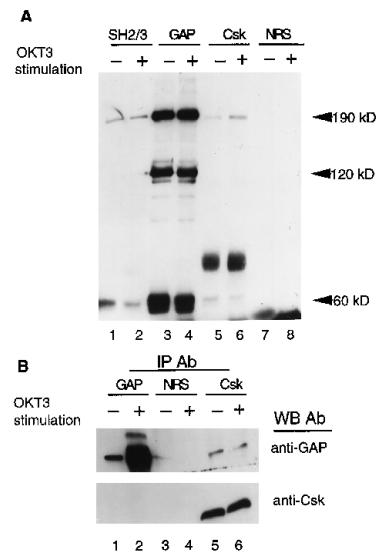


FIG. 2. Coprecipitation studies of Csk. *A*, a 60-kDa tyrosine-phosphorylated protein coprecipitates with Csk in unstimulated and TCR/CD3-stimulated Jurkat cells. Lysates of unstimulated (*lanes 1, 3, 5, and 7*) and OKT3-stimulated (*lanes 2, 4, 6, and 8*) Jurkat cells were immunoprecipitated with GST-SH2/3 fusion protein (*lanes 1 and 2*; 3×10^7 cells/lane), anti-GAP antiserum (1×10^6 cells in *lane 3* and 1×10^7 cells in *lane 4*), rabbit anti-Csk antiserum (*lanes 5 and 6*; 5×10^7 cells/lane), or normal rabbit serum (NRS, *lanes 7 and 8*; 5×10^7 cells/lane). The immunoprecipitates were resolved by SDS-PAGE, blotted onto nitrocellulose, and probed with anti-phosphotyrosine antibody. Molecular masses are shown on the right. *B*, in a separate experiment, anti-Csk and anti-GAP immunoprecipitates were Western blotted and probed with antibodies to GAP and Csk. *IP Ab*, immunoprecipitating antibody; *WB Ab*, blotting antibody.

FLVSKI did not bind p60 (Fig. 1C) nor to any of the other phosphoproteins (data not shown).

The Tyrosine-phosphorylated 60-kDa Protein Coprecipitates with Csk from Jurkat Cells—To examine which of the tyrosine-phosphorylated proteins associate with Csk *in vivo*, Csk was immunoprecipitated from unstimulated and OKT3-stimulated Jurkat cells, and the immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine antibodies (Fig. 2A). A 60-kDa tyrosine-phosphorylated protein was found to be associated with Csk in both unstimulated and stimulated cells; this protein comigrated exactly with the Csk-SH2/3-bound p60, and its phosphorylation state was not altered by OKT3-activation. Two other constitutively phosphorylated proteins of about 190 and 70 kDa were detected in the anti-Csk immunoprecipitate. The 70-kDa protein band was blurry and not consistently detected in all experiments; the 190-kDa band was only detected when a large number of cells was used.

Neet and Hunter (28) recently reported that Csk associates with a GAP-associated protein of 60 kDa in murine fibroblasts overexpressing Src. This 60-kDa protein was initially identified as a substrate of the insulin receptor kinase (36) and subsequently (37) shown to be distinct from the RNA-binding, GAP-associated 62-kDa protein previously described by Wong *et al.* (38). The mAb used to identify the GAP-associated murine p60 does not react with proteins of human origin. Therefore, to test if the human T cell p60 is associated with GAP, we examined whether GAP could be detected in anti-Csk immunoprecipitates and *vice versa*. A small amount of GAP was found to coprecipitate specifically with Csk from both unstimulated and TCR-stimulated T cells (Fig. 2B), suggesting that Csk and GAP may be brought down together by their common association with p60. However, no Csk could be detected in anti-GAP immunoprecipitates (Fig. 2B), further emphasizing the low stoichiometry of this possible indirect association. Previously,

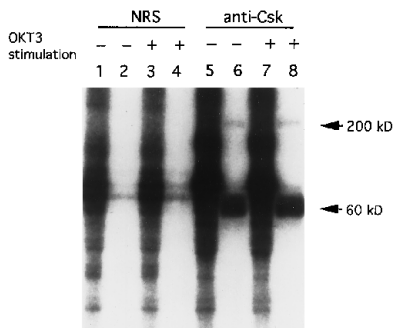


FIG. 3. *In vitro* kinase assay of anti-Csk immunoprecipitates. Lysates of unstimulated (lanes 1, 2, 5, and 6) or OKT3-stimulated (lanes 3, 4, 7, and 8) were incubated with immobilized normal rabbit serum (NRS, lanes 1–4) or anti-Csk antibodies (lanes 5–8). After *in vitro* kinase assay, the beads were boiled in 1% SDS to release bound proteins. 10% of the total kinase sample was removed (lanes 1, 3, 5, and 7), and the rest of the kinase reaction was incubated with Csk-SH2/3-GST fusion protein (lanes 2, 4, 6, and 8). After washing, the bound proteins were analyzed by SDS-PAGE followed by autoradiography. The positions of the 60- and 200-kDa molecular mass markers are indicated by arrows. No proteins other than p60 and p190 were detected in the SH2/3 adsorbates (lanes 6 and 8).

at least three proteins have been described that become phosphorylated and associate with GAP after growth factor stimulation (37–39). These proteins have molecular masses of 60, 62 (Sam 68), and 190 kDa respectively. It is notable that when GAP is immunoprecipitated from unstimulated and TCR-stimulated Jurkat cells, at least three constitutively phosphorylated proteins of the same molecular masses coprecipitate with GAP (Fig. 2A). The constitutive phosphorylation pattern of the GAP-associated proteins in the Jurkat cells supports the hypothesis that the Csk-associated p60 may be a GAP-associated protein.

p60 and p190 Are Phosphorylated by Csk *In Vitro*—To examine which of the Csk-associated proteins are substrates of Csk *in vitro*, Csk was immunoprecipitated from lysates of unstimulated and stimulated Jurkat cells, followed by an *in vitro* kinase assay. A sample of the immunoprecipitate was analyzed by SDS-PAGE to detect radiolabeled proteins. To aid the detection of proteins that bind specifically to Csk-SH2/3, the radiolabeled immunoprecipitates were boiled in the presence of 1% SDS, diluted, and reprecipitated with immobilized Csk-SH2/3-GST fusion protein.

Multiple proteins were phosphorylated in the Csk immunoprecipitates (Fig. 3). There was no detectable difference between the Csk-associated bands in unstimulated and OKT3-stimulated lysates. In both lysates, several bands were specifically associated with Csk; notably, in the total kinase assay, a strong band of 70 kDa and a fainter doublet of about 160 kDa were visible (Lanes 5 and 7). Strikingly, neither of these bands was specifically bound by Csk-SH2/3; instead, Csk-SH2/3 recognized a heavily phosphorylated band of 60 kDa and a fainter band of 190 kDa (Fig. 3, lanes 6 and 8). The 60-kDa band comigrated on gels exactly with p60, suggesting that it may be the same protein. KOH treatment of the phosphorylated protein products demonstrated that both p60 and p190 were primarily phosphorylated on tyrosines, whereas almost all other phosphorylation was due to serine/threonine phosphorylation (data not shown). These two proteins are, therefore, substrates of a tyrosine kinase in the anti-Csk immunoprecipitates, most likely Csk itself.

Phosphorylated p60 Is Associated with Csk in Normal Human T Cells—Because several lines of evidence suggest that Csk may play a role in TCR signaling (24, 40), it was surprising that no change in the phosphorylation state of p60 was observed upon cell activation. Thus, one might speculate that the

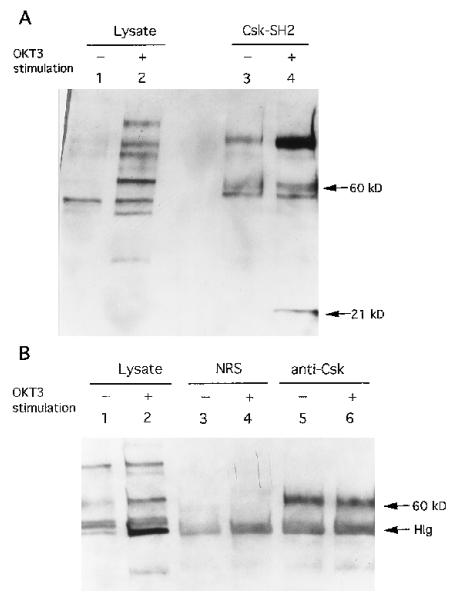


FIG. 4. Csk from normal human T cells is constitutively associated with a 60-kDa phosphorylated protein. A, phosphotyrosine blot of proteins adsorbed to Csk-SH2 fusion protein from lysates of unstimulated (lane 3) and OKT3-stimulated (lane 4) human T cells. Samples of total cell lysate from unstimulated (lane 1) and OKT3-stimulated (lane 2) cells were included on the blot to verify activation. B, lysates of unstimulated (lanes 3 and 5) and OKT3-stimulated (lanes 4 and 6) human T cells were immunoprecipitated with normal rabbit serum (lanes 3 and 4) or anti-Csk antiserum (lanes 5 and 6). The immunoprecipitates were analyzed by SDS-PAGE, blotted onto nitrocellulose, and probed with anti-phosphotyrosine antibody. Lanes 1 and 2 contain samples of total cell lysates of unstimulated and OKT3-stimulated cells. All the experiments were repeated twice using cells from different donors, producing identical results.

leukemic Jurkat cell line has a defect in its signaling machinery leading to constant phosphorylation of p60. To analyze a more physiological situation, we established human T cell lines and examined whether we could detect the same proteins. We were able to reproduce in the normal T cells all of the results we had previously obtained in the Jurkat cells. Thus, the Csk-SH2 fusion protein bound a tyrosine-phosphorylated doublet of 60 kDa in addition to the ζ chain of the TCR (Fig. 4A), and anti-Csk immunoprecipitates contained a 60-kDa phosphorylated protein (Fig. 4B). Identical to the Jurkat cells, the phosphorylation state of p60 did not change following activation. In addition to p60 and ζ , the Csk-SH2 fusion protein consistently precipitated a heavily phosphorylated protein of 120 kDa that was much more prominent than the 120-kDa protein detected in the Jurkat fusion protein assay; however, this protein was not detected in anti-Csk immunoprecipitates. Finally, *in vitro* kinase assays of Csk immunoprecipitates from normal T cells reproduced exactly the results obtained from the Jurkat cells (see Fig. 3), showing that both p60 and p190 were phosphorylated on tyrosines by a kinase activity in the immunoprecipitate and reprecipitated by Csk-SH2 (data not shown).

DISCUSSION

The importance of Csk in T cell development and signal transduction is well documented (23, 25, 40); however, many questions concerning its activity and regulation remain unanswered. In our analysis of Csk-associated proteins, we detected a prominent tyrosine-phosphorylated protein of 60 kDa that binds to the Csk-SH2 domain, both *in vitro* and *in vivo*. Notably, when adsorbed to the recombinant Csk-SH2 fusion protein, p60 presents as a doublet, whereas only a single band coprecipitates with Csk. Several attempts were made to identify the heavily phosphorylated 60-kDa protein doublet. Antibodies to

multiple members of the Src family, including Lck, Fyn, Src, and an antibody raised to a conserved region in all Src kinases failed to specifically identify p60; similarly, antibodies against paxillin (41), the GAP-associated protein p62 (38), or the adaptor protein Shc did not react specifically with p60 (data not shown).

A clue to the identity of p60 was recently provided by Neet and Hunter (28). These investigators showed that Csk associates with a phosphoprotein of 60 kDa in murine fibroblasts that have been transformed by constitutively active Src. This protein was shown to be a recently identified GAP-associated protein that has different characteristics from the previously described RNA-binding GAP-associated protein p62 (37). Because the monoclonal antibody used to identify the GAP-associated protein in the aforementioned report does not react with human homologue(s), we attempted to detect GAP in Csk immunoprecipitates and *vice versa*. Small amounts of GAP were detected in Csk immunoprecipitates, whereas we were unable to detect Csk in anti-GAP precipitates. As this method tests for an indirect association between the two proteins, the sensitivity of the assay may be too low to yield positive results in the latter case. Also, the 190-kDa protein found in anti-Csk immunoprecipitates comigrates exactly with the GAP-associated p190, further suggesting that Csk and GAP may be associated by their common interaction with p60 and p190.

If p60 is associated with GAP, it is possible that the 120-kDa protein observed in the Csk-SH2/3 fusion protein binding assays is GAP itself. When Western blots of the fusion protein precipitated material were probed with anti-GAP serum, the antibody bound reproducibly to a 120-kDa protein in both the Csk-SH2/3 and the GST control. Furthermore, the RNA-binding, GAP-associated p62 described by Wong *et al.* (38) was also found to bind to the control GST beads. This nonspecific binding of p62/GAP to GST precluded the use of the fusion proteins to analyze the potential p60/GAP association. However, as the level of tyrosine phosphorylation of GAP does not change with TCR/CD3 stimulation, it is likely that the 120-kDa protein detected in the fusion protein assays represents a different protein. We have tested the Csk-SH2/3 adsorbates with antibodies to FAK and c-cbl, but neither antibody recognized p120 specifically.

Because Csk has been implicated in TCR signaling, it was of some surprise that the profile of proteins binding to Csk-SH2 was unaffected by TCR/CD3 stimulation. We made several attempts to measure the activity of Csk before and after OKT3 stimulation; however, the reliability of these measurements was questionable because of several confounding factors. First, our data demonstrated that a large amount of Ser/Thr kinase activity coprecipitates with Csk, complicating activity measurements, especially when kinase-deficient Src was used as a substrate. Because this contaminating activity was not reduced when affinity-purified anti-Csk antibodies were used, it is possible that Csk is associated with these kinases *in vivo*. To try to bypass the consequences of the Ser/Thr kinase activity, we used a synthetic Glu-Tyr polymer as a substrate for Csk, either covalently coupled to Sepharose or in solution. However, neither method produced consistent results in our hands. Presently, we believe that if there is a change in the activity of total cellular Csk after TCR/CD3 stimulation, it is too small relative to background to be detected using the tools we have available.

If the cytosolic Csk exerts its effect on TCR signal transduction by phosphorylating the membrane-associated Lck and Fyn, it must be recruited to the membrane. In the light of our results, it is possible that Csk is linked to the membrane through an association with p60 and the GAP complex. However, it is unclear how this constitutive association might affect

TCR signaling. In our fusion-protein assays, Csk-SH2 specifically recognized the phosphorylated ζ and ϵ chains in T cell lysates, suggesting a way by which Csk might be recruited to the TCR. However, we were not able to detect ζ in anti-Csk immunoprecipitates nor Csk in anti-TCR precipitates using Western blotting techniques, possibly because preclearing of the lysates with protein A-Sepharose to remove the OKT3 antibody may have removed a large portion of the CD3 components. In this regard, several lines of evidence support that the interaction between Csk-SH2 and ζ may have physiological significance. First, our own data show that Csk-SH2 recognizes a very limited set of phosphoproteins in T cell lysates, in contrast to the wide variety of proteins recognized by SH2 fusion proteins of other signaling molecules (Ref. 42 and data not shown). Thus, Csk-SH2 behaves in a manner similar to ZAP-70-(SH2)₂, whose highly specific binding to ζ has a crucial role in TCR signaling (43). Second, our data are in accord with the results of other investigators who have analyzed the associations of proteins with the TCR. Takeuchi *et al.* (44) showed that Fyn coprecipitates with Csk from activated T cells, but the nature of this binding was not pursued. These results are consistent with our data if Fyn were co-immunoprecipitated with Csk through the association of both kinases with the TCR. Wange *et al.* (45) reported the co-immunoprecipitation of the TCR with three activated kinases that migrated with apparent molecular masses of 50, 70, and 120 kDa. These kinases were detected as a result of their ability to bind to a nonhydrolyzable photoaffinity analog of ATP. The p50 kinase did not autophosphorylate during the *in vitro* kinase assay, implying that this kinase lacks this ability. Thus, our data are consistent with the p50 kinase being Csk itself.

Our results contrast with a previously published report where Csk-associated proteins in Jurkat cells were analyzed by anti-Csk coprecipitation and *in vitro* binding to Csk-SH2 fusion proteins (24). In that study, only a single 72-kDa tyrosine-phosphorylated protein was found to associate with Csk *in vivo* and *in vitro*. Notably, this protein was shown not to have any kinase activity. We also detected a 70-kDa phosphoprotein that associated indirectly with recombinant Csk-SH2 in Jurkat cell lysates; however, we identified this protein as the protein-tyrosine kinase ZAP-70. Furthermore, a 70-kDa protein coprecipitated with Csk from unstimulated and OKT3-stimulated Jurkat cells, as shown by both phosphotyrosine blotting and *in vitro* kinase assay. However, this protein did not associate directly with the SH2 domain of Csk. Notably, we did not detect a 70-kDa protein in Csk-SH2 adsorbates or anti-Csk immunoprecipitates from normal T cells. We do not have a specific explanation for the observed discrepancies between our results and the previous report, although differences in the Jurkat cell lines and/or reagents used could be responsible.

In summary, we have detected two tyrosine-phosphorylated proteins that associate with Csk-SH2 in T cells. These proteins may be a part of a complex that contains the GTPase-activating protein GAP, linking Csk to proteins that serve as regulators of the Ras pathway. Our current data suggest that TCR/CD3 stimulation does not alter the binding or phosphorylation state of these Csk-associated proteins. Therefore, if Csk plays a role in the early signaling events of the TCR, modulation of its activity must be by a mechanism other than SH2 binding.

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