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p56^{lck} Protein-Tyrosine Kinase Is Cytoskeletal and Does Not Bind to Polyomavirus Middle T Antigen

RONALD R. LOUIE,[1] CONNIE S. KING,[1] ALASDAIR MACAULEY,[1] JAMEY D. MARTH,[2]†
ROGER M. PERLMUTTER,[2] WALTER ECKHART,[3] AND JONATHAN A. COOPER[1]*

Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104[1]; Howard Hughes Medical Institute and Departments of Biochemistry and Medicine, University of Washington, Seattle, Washington 98195[2]; and Molecular Biology and Virology Laboratory, the Salk Institute, San Diego, California 92138[3]

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p56^{lck} and p60$^{c\text{-}src}$ are closely related protein-tyrosine kinases that are activated by similar oncogenic mutations. We have used fibroblast cell lines that express p56^{lck} from introduced DNA molecules to compare the subcellular localizations of p60$^{c\text{-}src}$ and p56^{lck} and their abilities to bind polyomavirus middle T antigen (mT). p56^{lck} is associated with the detergent-insoluble matrix, as defined by extraction with solutions containing nonionic detergents, whereas p60$^{c\text{-}src}$ is soluble under these conditions. p56^{lck} is also associated with detergent-insoluble structures in a lymphoid cell line, LSTRA. p60$^{c\text{-}src}$ binds to mT, but p56^{lck} does not bind detectably. In terms of both solubility and mT interactions, the nononcogenic p56^{lck} more closely resembles oncogenically activated p60$^{c\text{-}src}$ mutants than it resembles p60$^{c\text{-}src}$. Because published results show that an intact carboxy terminus is required for p60$^{c\text{-}src}$ to bind mT and be soluble, we tested whether the different localization and mT binding properties of p56^{lck} and p60$^{c\text{-}src}$ were dictated by their different carboxy termini. A protein consisting largely of p60$^{c\text{-}src}$ sequences but carrying a p56^{lck} carboxy terminus was soluble and bound to mT. We suggest that both the solubility and mT-binding properties of p60$^{c\text{-}src}$ not only require sequences common to the carboxy termini of p60$^{c\text{-}src}$ and p56^{lck}, but also require sequences unique to the body of p60$^{c\text{-}src}$.

p56^{lck} and p60$^{c\text{-}src}$ are homologous membrane-associated protein-tyrosine kinases (J. A. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). Both proteins have tyrosines near their carboxy termini that are normally phosphorylated (12, 37). These phosphorylations correlate with suppression of the kinase activities and transforming abilities of p60$^{c\text{-}src}$ and p56^{lck} (13, 15, 25, 37). Oncogenic mutations in *lck* and c-*src* include missense mutations that substitute the carboxy-terminal tyrosines with other residues and nonsense mutations that cause truncation prior to these tyrosines (2, 8, 29, 37, 42, 43).

Despite these apparent similarities in regulation, p56^{lck} and p60$^{c\text{-}src}$ may participate in different cellular control circuits. p56^{lck} is expressed most abundantly in T lymphocytes, and p60$^{c\text{-}src}$ is present in many cell types, including fibroblasts, but is most plentiful in neurons and blood platelets (14, 20, 39). It seems probable that in diverse cell lineages, protein-tyrosine kinases interact with different effector molecules and substrates. Furthermore, even though p56^{lck} and p60$^{c\text{-}src}$ have 60% amino acid identity in their carboxy-terminal 450 residues, they diverge almost completely in their amino-terminal regions (13% amino acid identity) (39, 47, 50). This sequence divergence could allow functional specialization.

The middle T antigen (mT) of polyomavirus, a DNA tumor virus, is able to transform rodent and chicken fibroblasts (27, 30). mT associates with p60$^{c\text{-}src}$ and stimulates its protein-tyrosine kinase activity (5, 17). mT mutants that do not bind to p60$^{c\text{-}src}$ are transformation defective, and cells expressing mT become less transformed when their content of p60$^{c\text{-}src}$ is artificially decreased 4- to 10-fold (1, 36, 48). Association of p60$^{c\text{-}src}$ and mT requires sequences near the amino terminus of mT and near the carboxy terminus of p60$^{c\text{-}src}$ (11, 36, 48). Populations of p60$^{c\text{-}src}$ and mT sediment together on velocity gradient centrifugation as an approximately 250-kilodalton (kDa) complex and can be immunoprecipitated with antibodies directed against either protein (17, 23). Other proteins which can complex with mT include p62$^{c\text{-}yes}$ (another protein-tyrosine kinase related to p60$^{c\text{-}src}$ and p56^{lck}) and cellular proteins of 88, 61, and 37 kDa of unknown functions (16, 22, 26, 31). Large populations of both p60$^{c\text{-}src}$ and mT also exist separately in the cell, suggesting that other factors are limiting for formation of the complex (4, 17).

p60$^{c\text{-}src}$ and its derivative, p60$^{v\text{-}src}$ (the Rous sarcoma virus transforming protein), are myristoylated, membrane-associated proteins (7). Upon extraction of cellular lipids with nonionic detergents, most p60$^{c\text{-}src}$ is dissolved (24, 34). In contrast, p60$^{v\text{-}src}$ and other transforming versions of p60$^{c\text{-}src}$ are retained in the detergent-insoluble material, which includes the submembranous, cytoplasmic, and nuclear cytoskeletons (6, 24, 34). Enhanced kinase activity is required but not sufficient for cytoskeletal association. Membrane association is also necessary (24). The enzymatically activated, membrane-associated p60$^{c\text{-}src}$ that is complexed with mT is also cytoskeletal (44).

The recent creation of fibroblast cell lines expressing p56^{lck} (37) has enabled us to test whether p56^{lck} resembles p60$^{c\text{-}src}$ in its localization and its affinity for mT. We report here that p56^{lck} differs from p60$^{c\text{-}src}$ in both respects. A chimeric protein, consisting of p60$^{c\text{-}src}$ with the carboxy terminus of p56^{lck} (p60$^{src/lck}$), behaves like p60$^{c\text{-}src}$. We suggest that sequence divergence in the amino-terminal or kinase domain, or both, accounts for the different behaviors.

MATERIALS AND METHODS

Cells and virus. All cells were maintained routinely in Dulbecco modified Eagle medium containing 10% calf serum. BALB/c 3T3 and NIH 3T3 cells infected with the LSHL*lck* retrovirus and NIH 3T3 cells transfected with

* Corresponding author.
† Present address: Oncogen, Seattle, WA 98121.

pNUT*lck* (clone Y2) have been described before (37). These cells were selected in media containing hygromycin. NIH 3T3 cells infected with derivatives of pLJ (42) that carry the chicken c-*src* gene or a c-*src*/*lck* recombinant gene have been described before (35). These cells were selected in media containing G418. BALB/c 3T3 and LSHL*lck*-infected BALB/c 3T3 cells (lck3T3) were infected with PyMLV (a retrovirus carrying the mT sequence [18]), with Moloney murine leukemia virus as helper. The cultures were passaged three times following infection, until all the cells in the cultures appeared morphologically transformed. These uncloned cultures are designated mT3T3 and mTlck3T3. When polyomavirus infection was required, 9-cm dishes were seeded with 10^6 cells and incubated for 24 h, and then the cells were infected with 1 ml of wild-type or *dl*8 (21) polyomavirus for 1 h, fed with 10 ml of Dulbecco modified Eagle medium for 28 h, and harvested for immunoprecipitation.

Antisera and immunoprecipitations. Hybridoma 327 (33) was kindly provided by J. Brugge (State University of New York, Stony Brook, N.Y.). The cells were grown as an ascites in nude mice. Ascites fluid was used for immunoprecipitation. T1.1 is an ascites fluid from rats bearing a tumor caused by polyomavirus (9). Antiserum 195 was from a rabbit immunized with a *trpE-lck* fusion protein containing residues 5 to 148 of $p56^{lck}$. The immunogen was prepared by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of insoluble proteins from bacteria containing a pATH10-derived plasmid (41; J. Marth, unpublished plasmid). Antiserum 7080 was obtained from a rabbit immunized with a synthetic peptide containing residues 492 to 509 of $p56^{lck}$, conjugated with glutaraldehyde to bovine serum albumin. All antibodies were used in excess over antigen. Cell lysates were prepared in modified RIPA buffer (13) and centrifuged at 20,000 × *g* for 30 min before samples were immunoprecipitated with antibody and *Staphylococcus aureus* cells as described before (45). Immunoprecipitations with 327 and T1.1 were supplemented with rabbit antiserum to mouse immunoglobulin G (IgG) and rabbit or goat antiserum to rat IgG, respectively. For in vitro phosphorylation, immunoprecipitates were washed once in 100 mM NaCl–10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0)–20 μg of aprotinin per ml and then incubated for 5 or 15 min at 30°C in 10 μl of 20 mM PIPES (pH 7.0)–10 mM $MnCl_2$ containing 5 μCi of [γ-^{32}P]ATP. Reactions were stopped with 0.5 ml of RIPA buffer, the radioactive immunoprecipitates were recovered by centrifugation, and proteins were analyzed by SDS-PAGE (12.5% acrylamide–0.1% bisacrylamide [3] or 6.7% acrylamide–0.35% bisacrylamide [32] gels).

Detergent extraction. Confluent 35-mm dish cultures of cells were washed three times with cold Tris-buffered saline and then extracted with 0.5 ml of CSK buffer, consisting of 100 mM KCl, 10 mM PIPES (pH 7.0), 0.5% Nonident P-40, 300 mM sucrose, 3 mM $MgCl_2$, and 20 μg of aprotinin per ml, at 0°C for 5 min, with gentle rocking every 30 s. The soluble fraction was collected, the insoluble fraction was extracted once more with 0.5 ml of the same solution, and the combined soluble fractions were adjusted to contain 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS. The insoluble material was scraped from the dish in RIPA buffer (13). LSTRA cells were extracted similarly, except that the soluble and insoluble fractions were separated by centrifugation for 30 s at 8,000 × *g*. Both fractions were centrifuged at 20,000 × *g* for 30 min prior to immunoprecipitation as described previously (45).

Density gradient sedimentation. Lysates were prepared from 50-mm dish cultures by using 0.5 ml of RIPA buffer supplemented with 1 mM leupeptin, 3 mM $MgCl_2$, and 1 mM dithiothreitol and lacking SDS. Following centrifugation at 20,000 × *g* for 30 min, supernatants were supplemented with 5 μl of rabbit serum and 50 μg of thyroglobulin (to serve as internal markers) and layered over linear 4.5-ml gradients of 5 to 20% glycerol in the same buffer. Gradients were centrifuged at 40,000 rpm in the SW55 rotor (Beckman Instruments, Inc.) for 17 h at 4°C. Fractions (20 drops each) were collected from the bottoms of the tubes. Samples (20 μl) were analyzed by SDS-PAGE, and the gels were stained to locate the albumin (68 kDa), immunoglobulin (140 kDa), and thyroglobulin (600 kDa) markers. The markers sedimented similarly on all the gradients. One-tenth of the unfractionated lysate and one-half of each fraction were analyzed by immunoprecipitation with appropriate antisera. Immunoprecipitates were labeled in vitro and analyzed on 12.5% acrylamide–0.1% bisacrylamide gels as described above.

Immunoblotting. Samples of cell lysates and of immunoprecipitates were analyzed on a 10% acrylamide–0.13% bisacrylamide gel, and proteins were transferred to nitrocellulose by published procedures (28). The blot was soaked in 1% nonfat dry milk–0.05% Tween 20 in Tris-buffered saline and then probed sequentially with antiserum 195, biotinylated horse anti-rabbit IgG, a conjugate of avidin and biotinylated alkaline phosphatase, and, finally, a chromogenic substrate (Vector Laboratories).

RESULTS

Detergent solubility of $p56^{lck}$. Gacon et al. (19) have shown that extraction of LSTRA cells (lymphoma cells that express high levels of $p56^{lck}$ [10]), with nonionic detergents leaves protein-tyrosine kinase activity in the insoluble fraction. We used the procedure of Burr et al. (6) to examine the localization of $p56^{lck}$ expressed artificially in fibroblasts.

Confluent cultures of BALB/c 3T3 cells and lck3T3 cells (BALB/c 3T3 cells infected with LSHL*lck* virus [37]) were extracted with CSK buffer, and the soluble and insoluble fractions were dissolved in modified RIPA buffer (see Materials and Methods). Samples were immunoprecipitated with monoclonal antibody 327, which recognizes $p60^{c\text{-}src}$ (33), or with rabbit serum 195, which recognizes $p56^{lck}$ (see Materials and Methods). $p60^{c\text{-}src}$ and $p56^{lck}$ were detected in the immunoprecipitates by their incorporation of $^{32}P_i$ when incubated with [γ-^{32}P]ATP. Reaction products were separated by SDS-PAGE and detected by autoradiography (Fig. 1). $p60^{c\text{-}src}$ was detected as a soluble protein in both cell types. $p56^{lck}$ was absent from 3T3 cells and was detected as a doublet (49) in lck3T3 cells. Less than 5% of $p56^{lck}$ kinase activity was soluble. $p56^{lck}$ of Y2 cells (NIH 3T3 cells transfected with pNUT*lck* [37]) was also insoluble (data not shown). The interactions between $p56^{lck}$ and detergent-insoluble structures of fibroblasts resisted CSK buffers with the following modifications: KCl concentrations of 0 or 0.4 M, replacement of $MgCl_2$ with 2 mM EDTA, and supplementation with 0.5% sodium deoxycholate (data not shown). LSTRA cell $p56^{lck}$ (some of which is in an active form [37]) was also insoluble in CSK buffer (Fig. 1). We have not investigated the solubility of $p56^{lck}$ in other lymphoid cell lines.

We and others (49) have noticed that $p56^{lck}$ frequently contaminates immunoprecipitates formed with preimmune sera or with antisera to other proteins (e.g., $p56^{lck}$ was detected in antibody 327 immunoprecipitates of lck3T3 cells

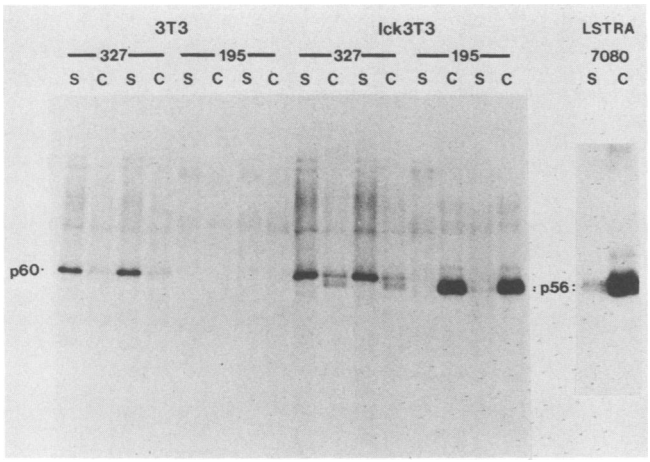

FIG. 1. Detergent extraction of 3T3, lck3T3, and LSTRA cells. Duplicate 35-mm dish cultures of 3T3 and lck3T3 cells were extracted with CSK buffer (see Materials and Methods). A total of 10^7 LSTRA cells were extracted similarly. Soluble (lanes S) and insoluble (cytoskeletal lanes C) fractions were immunoprecipitated with excess antibody 327 (to precipitate p60$^{c\text{-}src}$), antiserum 195, or antiserum 7080 (to precipitate p56^{lck}). Immunoprecipitates were incubated with [γ-^{32}P]ATP. Radioactive proteins were separated by SDS-PAGE (12.5% acrylamide–0.1% bisacrylamide gels) and detected by autoradiography.

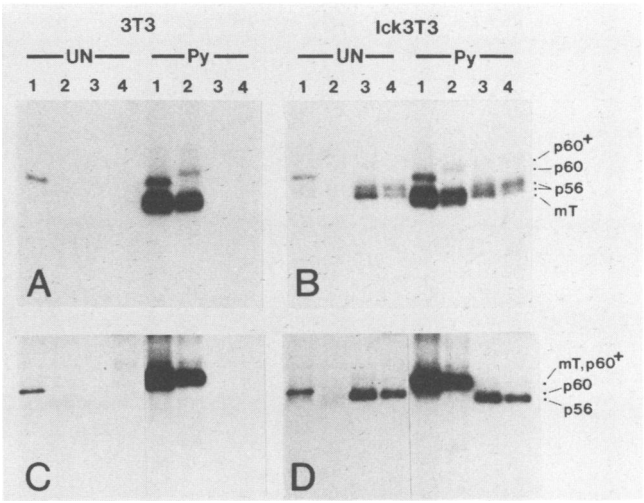

FIG. 2. Immunoprecipitation analysis of 3T3 cells expressing mT and p56^{lck}. Cultures of 3T3 cells (A, C) and lck3T3 cells (B, D) were infected (lanes Py) or mock infected (lanes UN) with wild-type polyomavirus. At 28 h later, lysates were prepared and analyzed by immunoprecipitation with four different antisera: antibody 327 (to p60$^{c\text{-}src}$, lanes 1), T1.1 ascites (to mT, lanes 2), antiserum 195 (to p56^{lck}, lanes 3), and antiserum 7080 (to p56^{lck}, lanes 4). Immunoprecipitates were incubated with [γ-^{32}P]ATP. Radioactive proteins were separated by SDS-PAGE with 6.7% acrylamide–0.35% bisacrylamide (panels A and B) or 12.5% acrylamide–0.1% bisacrylamide (panels C and D) gels.

[Fig. 1; also see Fig. 5]). Immunoprecipitates of p56^{lck} are frequently contaminated with other cellular proteins. In view of the association of p56^{lck} with insoluble structures and the persistence of this association in RIPA buffer (see below and Fig. 3), we suggest that aggregated p56^{lck} may contaminate immune complexes. Aggregation could also hide epitopes on p56^{lck} and decrease the efficiency of immunoprecipitation.

Interactions between p56^{lck} and mT. BALB/c 3T3 and lck3T3 cells were infected with polyomavirus and 28 h later proteins were precipitated with antibodies to p60$^{c\text{-}src}$ (antibody 327), mT (T1.1 ascites fluid), and p56^{lck} (antisera 195 and 7080, from rabbits immunized with a p56^{lck} amino-terminal fusion protein and a p56^{lck} carboxy-terminal peptide, respectively). Immunoprecipitates were incubated with [γ-^{32}P]ATP and then analyzed by SDS-PAGE (6.7% acrylamide–0.35% bisacrylamide gels) (Fig. 2A and B). As expected, polyomavirus infection of 3T3 cells stimulated p60$^{c\text{-}src}$ phosphorylation in an antibody 327 immunoprecipitate (Fig. 2A, Py, lane 1) and resulted in labeling of mT in immunoprecipitates formed with either antibody 327 or T1.1 ascites (Fig. 2A, Py, lanes 1 and 2). The p60$^{c\text{-}src}$ that was labeled in immunoprecipitates formed with T1.1 ascites migrated slowly (Fig. 2B, p60$^+$), possibly owing to amino-terminal phosphorylation in the p60$^{c\text{-}src}$-mT complex (51). Identical results for p60$^{c\text{-}src}$ were obtained with lck3T3 cells infected with polyomavirus (Fig. 2B, Py, lanes 1 and 2). In contrast, polyomavirus infection appeared not to change the quantity or kinase activity of p56^{lck} precipitated from lck3T3 cells with either antiserum 195 or 7080 (Fig. 2B, Py, lanes 3 and 4). Importantly, mT was not labeled in either immunoprecipitate of p56^{lck}.

Since mT (421 residues) and p56^{lck} (509 residues) have similar electrophoretic mobilities in gels containing 6.7% acrylamide and 0.35% bisacrylamide, we also analyzed the products of in vitro phosphorylation reactions in a gel system having different sieving properties (12.5% acrylamide, 0.1% bisacrylamide [Fig. 2C and D]). Under these conditions, mT has a lower mobility than p60$^{c\text{-}src}$ and comigrates with p60$^+$ (Fig. 2C). It is clearly separated from the doublet of p56^{lck} (Fig. 2D). The identities of various bands were confirmed by peptide mapping with protease V8 and elastase and by experiments with *dl*8 mutant polyomavirus, which encodes a smaller mT (data not shown). When the second gel system was used, it was clear that mT was not labeled in immunoprecipitates of p56^{lck} (Fig. 2D, lanes 3 and 4) and that p56^{lck} was not labeled in immunoprecipitates of mT (lane 2). Similar results were found with mTlck3T3 cells, lck3T3 cells transformed with an mT-encoding retrovirus (18).

The results in Fig. 2 suggested that if p56^{lck} associates with mT, the bound p56^{lck} phosphorylates neither itself nor mT. We reasoned that if p56^{lck} and mT were associated, some fraction of p56^{lck} and mT should migrate together when sedimented on velocity gradients.

BALB/c 3T3 and lck3T3 cells were transformed with an mT retrovirus (18). The resulting mT3T3 and mTlck3T3 cells, and their nontransformed counterparts, were lysed in SDS-free RIPA buffer (see Materials and Methods) and analyzed by sedimentation on glycerol gradients. Gradient fractions were immunoprecipitated and phosphorylated in vitro. The p60$^{c\text{-}src}$ of 3T3 cells sedimented at about 60 to 70 kDa (Fig. 3A), as expected (17). In mT3T3 cells, a complex of mT and p60$^{c\text{-}src}$ sedimented at about 280 kDa and was detectable with either antibody 327 (Fig. 3B) or T1.1 ascites (Fig. 3C). Unexpectedly, the p56^{lck} kinase activity of lck3T3 cells sedimented near the bottom of the gradient, at S values expected for proteins of 600 kDa and larger (Fig. 3D). Indeed, most of the p56^{lck} kinase activity seemed to be lost from the gradient, presumably being pelleted (compare the total lysate lanes [T] with the individual gradient fractions [Fig. 3]). For this reason, phosphoproteins that were minor in the unfractionated lysate were conspicuous in the

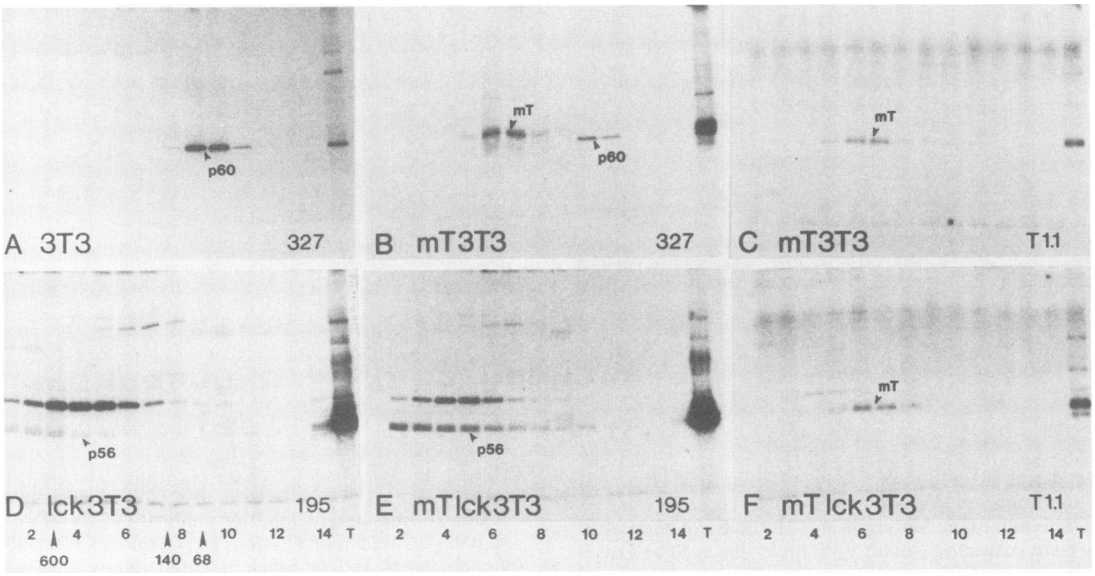

FIG. 3. Density gradient analysis of cells expressing mT and $p56^{lck}$. Lysates of (A) 3T3, (B and C) mT3T3, (D) lck3T3, and (E and F) mTlck3T3 cells were prepared and analyzed by glycerol gradient sedimentation (see Materials and Methods). Samples of fractions 1 to 14 and of the total lysate (T) were immunoprecipitated with antibody 327 (to $p60^{c\text{-}src}$) (panels A and B), T1.1 ascites (to mT) (panels C and F), or antiserum 195 (to $p56^{lck}$) (panels D and E) and labeled with [γ-^{32}P]ATP in vitro. Reaction products were analyzed by SDS-PAGE (12.5% acrylamide–0.1% bisacrylamide gels) and detected by autoradiography. Positions of 600-, 140-, and 68-kDa markers are shown.

gradient fractions (e.g., the band of about 65 kDa and S value corresponding to 500 kDa in Fig. 3D and E). Rapid sedimentation of $p56^{lck}$ was also evident in mTlck3T3 cells (Fig. 3E). More importantly, the kinase-associated population of mT sedimented at 280 kDa in mTlck3T3 cells (Fig. 3F). This suggested that kinase-associated mT was not bound to the 600-kDa form of $p56^{lck}$.

Since the experiments shown in Fig. 2 and 3 would not detect a population of $p56^{lck}$ bound to mT if that population lacked kinase activity, we attempted to analyze immunoprecipitates of [^{35}S]methionine-labeled mTlck3T3 cells. Severe nonspecific precipitation occurred with antibodies to $p56^{lck}$, making it difficult to detect any associated mT; therefore, we tested whether $p56^{lck}$ could be detected by immunoblotting of immunoprecipitates of mT (Fig. 4). Lysates of mTlck3T3 cells and control cells were immunoprecipitated with or without T1.1 ascites, goat anti-rat IgG, and *S. aureus* and analyzed by SDS-PAGE. The gel was transferred to nitrocellulose and probed with antiserum 195, biotinylated horse anti-rabbit IgG, and avidin-biotin-alkaline phosphatase conjugate (see Materials and Methods). Although various bands, presumably IgG, were detected in all lanes (Fig. 4, lanes 9 to 16), no band corresponding to $p56^{lck}$ was detected specifically in the T1.1 immunoprecipitate of mTlck3T3 cells (Fig. 4, lane 16). Immunoblotting of total cell lysates, without immunoprecipitation, showed that 0.5% of the cellular content of $p56^{lck}$ could have been detected by this procedure (Fig. 4, lanes 1 to 8). We conclude that less than 0.5% of the $p56^{lck}$ is complexed with mT in a form that can be precipitated by T1.1 antibodies, even though these antibodies precipitate a population of $p60^{c\text{-}src}$ that is bound to mT (Fig. 2 and 3).

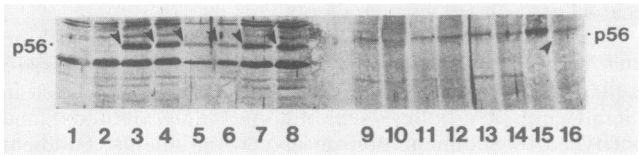

FIG. 4. Immunoblot analysis of immunoprecipitates. Samples of 3T3 (lanes 9 and 10), mT3T3 (lanes 11 and 12), lck3T3 (lanes 13 and 14), and mTlck3T3 (lanes 15 and 16) cells, each containing 500 μg of total protein, were incubated with *S. aureus*, goat antiserum to rat IgG, and 0 (lanes 9, 11, 13, and 15) or 10 μl (lanes 10, 12, 14, and 16) of T1.1 ascites. The immunoprecipitates were analyzed by SDS-PAGE (10% acrylamide–0.13% bisacrylamide gels), together with samples of total cell lysates: 25 μg of BALB/c 3T3 protein (lane 1), 25 μg of mT3T3 protein (lane 2), 25 μg of lck3T3 protein (lane 3), 12.5 μg of lck3T3 protein (lane 4), 2.5 μg of lck3T3 protein (lane 5), 2.5 μg of mTlck3T3 protein (lane 6), 12.5 μg of mTlck3T3 protein (lane 7), and 25 μg of mTlck3T3 protein (lane 8). The gel was immunoblotted and probed with antiserum 195 (to $p56^{lck}$) as detailed in Materials and Methods. Arrowheads point to $p56^{lck}$.

Properties of a chimeric protein, $p60^{src/lck}$. We describe elsewhere the construction of NIH 3T3 cell lines that express high levels of either wild-type avian $p60^{c\text{-}src}$ or a chimeric protein containing residues 1 to 516 of avian $p60^{c\text{-}src}$ and 495 to 509 of murine $p56^{lck}$ (35). The latter protein ($p60^{src/lck}$) has the amino-terminal region and kinase domain of $p60^{c\text{-}src}$ but the regulatory tail (including Tyr-505) of $p56^{lck}$. In regard to its phosphorylation state and kinase activity, $p60^{src/lck}$ resembles both its parents (35). Since the localization and mT binding properties of $p60^{src}$ and $p56^{lck}$ are different, we assayed these properties of $p60^{src/lck}$.

Figure 5 shows the results of extracting NIH 3T3 cells expressing $p60^{c\text{-}src}$, $p60^{src/lck}$, and $p56^{lck}$ with CSK buffer. More 60-kDa protein phosphorylation was detected with antibody 327 in the first two cell lines than in the third, showing that the introduced c-*src* and *src/lck* genes were expressed at about 10 times above the endogenous level. The overexpressed avian $p60^{c\text{-}src}$, $p60^{src/lck}$, and endogenous $p60^{c\text{-}src}$ were all soluble (Fig. 5, lanes S). Antiserum 7080 (to the carboxy terminus of $p56^{lck}$) weakly immunoprecipitated $p60^{src/lck}$ from the soluble fraction of cells expressing the

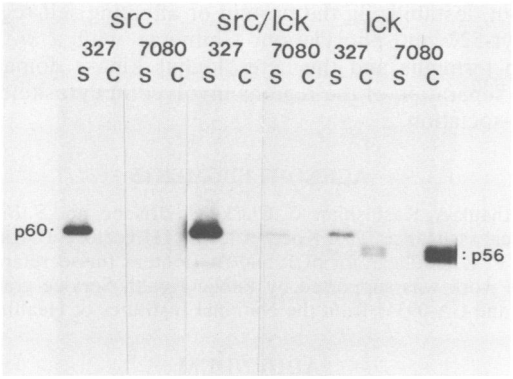

FIG. 5. Detergent solubility of p60$^{src/lck}$. NIH 3T3 cells (expressing avian p60$^{c\text{-}src}$ or p60$^{src/lck}$) and lck3T3 cells were extracted with a solution containing nonionic detergent and separated into soluble (lanes S) and insoluble (cytoskeletal, lanes C) fractions. Samples were immunoprecipitated with antibody 327 (to p60$^{c\text{-}src}$) or antiserum 7080 (to p56^{lck}). Immunoprecipitates were incubated with [γ-^{32}P]ATP. Radioactive proteins were separated by SDS-PAGE (12.5% acrylamide–0.1% bisacrylamide gels) and detected by autoradiography (immunoprecipitates with antiserum 7080 were exposed eight times as long as antibody 327 immunoprecipitates).

hybrid gene and also precipitated p56^{lck} from the detergent-insoluble fraction of lck3T3 cells.

To test whether p60$^{src/lck}$ was associated with mT, cells expressing the hybrid protein were infected with PyMLV. Immunoprecipitates were prepared with various antibodies, incubated with [γ-^{32}P]ATP, and analyzed by SDS-PAGE (Fig. 6). Overexpression of avian p60$^{c\text{-}src}$ did not increase the phosphorylation of mT in immunoprecipitates made with either antibody 327 or T1.1 ascites. This was expected, because the amount of p60$^{c\text{-}src}$ mT complex is not limited by the supply of p60$^{c\text{-}src}$ (4). To distinguish mT bound to

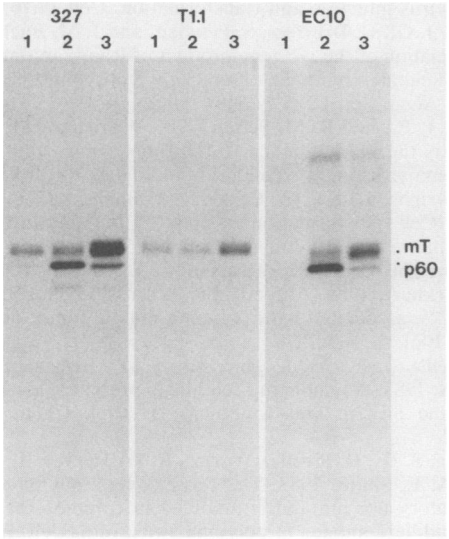

FIG. 6. Binding of p60$^{src/lck}$ to mT. Cultures of cells infected with PyMLV were lysed and immunoprecipitated with antibody 327 (to p60$^{c\text{-}src}$), T1.1 ascites (to mT), and antibody EC10 (to avian p60$^{c\text{-}src}$). Immunoprecipitates were incubated with [γ-^{32}P]ATP. Radioactive proteins were separated by SDS-PAGE (12.5% acrylamide–0.1% bisacrylamide gels) and detected by autoradiography. Lanes: 1, NIH 3T3 cells, 2, NIH 3T3 cells expressing avian p60$^{c\text{-}src}$; 3, NIH 3T3 cells expressing p60$^{src/lck}$.

endogenous (murine) p60$^{c\text{-}src}$ from that associated with avian p60$^{c\text{-}src}$ and p60$^{src/lck}$, we immunoprecipitated lysates with antibody EC10, which is specific for amino-proximal epitopes in avian p60$^{c\text{-}src}$ (41). As expected, EC10 did not immunoprecipitate any kinase activity from control cells, but did immunoprecipitate activity from cells expressing avian p60$^{c\text{-}src}$ and p60$^{src/lck}$ (Fig. 6). mT was also labeled in both these immunoprecipitates, showing that mT is complexed with both avian p60$^{c\text{-}src}$ and p60$^{src/lck}$. The relative labeling of p60 and mT in immunoprecipitates varied from experiment to experiment and did not differ reproducibly between p60$^{c\text{-}src}$ and p60$^{src/lck}$ immunoprecipitates.

DISCUSSION

We have found that murine p56^{lck}, artificially expressed in mouse fibroblasts, binds tightly to detergent-insoluble structures but not to polyomavirus mT. In both respects, p56^{lck} differs from the products of the endogenous c-*src* gene, an introduced avian c-*src* gene, and an introduced chimeric gene that codes for a p60$^{c\text{-}src}$ protein in which the last 17 residues have been replaced with 15 residues from p56^{lck}. Instead, p56^{lck} resembles several transforming variants of p60$^{c\text{-}src}$, including p60$^{v\text{-}src}$.

p56^{lck} may be bound to cytoskeletal structures in living cells or may bind only during cell lysis. Distinguishing these possibilities requires immunofluorescence and cross-linking studies on intact cells, with antisera of greater specificity than are presently available. Either way, some feature of the primary sequence of p56^{lck} must dictate a conformation that exposes a binding site for one or more cytoskeletal proteins. Such a binding site is apparently not exposed in p60$^{c\text{-}src}$. The cytoskeletal proteins to which p56^{lck} binds may be common to lymphocytes and fibroblasts, since the p56^{lck} of LSTRA cells is also insoluble. Because most p56^{lck} is membrane associated, the cytoskeletal binding sites for p56^{lck} may underlie the membrane. Interactions with the cortical cytoskeleton could be important for p56^{lck} function in lymphocytes: p56^{lck} expression changes rapidly during lymphocyte activation, leading to the hypothesis that p56^{lck} may regulate one of the early morphological responses to antigen, perhaps receptor capping or secretion of lymphokines (38).

It is possible that p56^{lck} and p60$^{v\text{-}src}$ associate with the cytoskeleton in the same manner. Both proteins are insoluble in CSK buffers containing a range of concentrations of KCl and $MgCl_2$ (24). One superficial difference is the solubility of p60$^{v\text{-}src}$ but not p56^{lck} in CSK buffer supplemented with 0.5% sodium deoxycholate (24). However, the extraction of p60$^{v\text{-}src}$ by deoxycholate may result from breakdown of the cytoskeleton of the v-*src*-transformed cells (24), which may not occur with nontransformed cells expressing p56^{lck}. Given the similarities in extraction conditions, might equivalent domains of p56^{lck} and p60$^{v\text{-}src}$ be responsible for cytoskeletal association? Hamaguchi and Hanafusa studied various mutant c-*src* and v-*src* proteins (24). They found excellent correlation between cytoskeletal association and malignant transformation. Transformation by v-*src* proteins requires both high protein kinase activity and membrane localization. Kinase-negative p60$^{v\text{-}src}$ mutants are soluble, as are mutants that are not membrane associated. Long amino-terminal deletions in p60$^{v\text{-}src}$ reduce, but do not abolish, cytoskeletal association without reducing kinase activity or membrane association. Since oncogenic mutations in c-*src* simultaneously stimulate its kinase activity, increase phosphorylation at Tyr-416, and induce cytoskeletal association, it is possible that Tyr-416 phosphorylation induces a confor-

mation change that stabilizes interactions between an amino-terminal region and the cytoskeleton. One region of interest in the amino-terminal half of $p60^{c\text{-}src}$ is similar to part of phospholipase C (46), a membrane-associated protein. In contrast to $p60^{v\text{-}src}$, $p56^{lck}$ has repressed kinase activity (relative to the Phe-505 mutant of $p56^{lck}$ [2, 37]), lacks phosphate at the residue homologous to Tyr-416 (Tyr-394), and is nononcogenic (2, 37). Even so, it binds to the cytoskeleton, and it is possible that a homologous cytoskeletal binding site is exposed in $p56^{lck}$ and $p60^{v\text{-}src}$. Cytoskeletal association of $p56^{lck}$ is not due to the altered sequence of the carboxy-terminal tail (Fig. 5). Construction of further chimeras between *src* and *lck* may help to map the binding region of $p56^{lck}$.

Polyomavirus mT binds to both $p60^{c\text{-}src}$ and $p62^{c\text{-}yes}$ but apparently not to $p56^{lck}$. We estimate that less than 0.5% of $p56^{lck}$ is bound to mT. Bolen et al. (4) have shown that 5 to 10% of rat cell $p60^{c\text{-}src}$ is complexed with mT. Since overexpression of $p60^{c\text{-}src}$ does not increase the amount of complex proportionally, it is likely that the quantity (as opposed to the percentage) of complexed $p60^{c\text{-}src}$ is biologically significant. Although it is difficult to estimate the amount of $p56^{lck}$ in our cells relative to the amount of endogenous $p60^{c\text{-}src}$, 0.5% of the $p56^{lck}$ could be a significant quantity. Importantly, however, neither of the antisera to $p56^{lck}$ that we tested revealed any stimulation of $p56^{lck}$ kinase activity due to mT expression, and so no effect on cell phenotype would be expected.

mT does not bind to $p60^{v\text{-}src}$ (J. Bolen, personal communication) or to $p60^{c\text{-}src}$ truncated at residue 518 (8, 11), but can bind to $p60^{c\text{-}src}$ truncated at residue 525 or 527 (11). These data show that the carboxy terminus of $p60^{c\text{-}src}$ is necessary for mT binding. $p56^{lck}$ differs from $p60^{c\text{-}src}$ at three of the seven residues from 519 to 525 in $p60^{c\text{-}src}$ (39, 47, 50). These carboxy-terminal changes do not, however, account for the nonassociation of mT with $p56^{lck}$, since $p60^{src/lck}$ binds to mT (Fig. 6). The role of the body of $p60^{c\text{-}src}$ in mT binding can be interpreted in two ways. First, sequences peculiar to $p56^{lck}$ may block its binding to mT, for example, by driving its association with the cytoskeleton. This explanation seems unlikely, because some activated mutants of $p60^{c\text{-}src}$ demonstrate that cytoskeletal binding does not preclude mT binding (8, 11, 24). Second, $p56^{lck}$ may lack one or more sequences that are present in $p60^{c\text{-}src}$ and $p62^{c\text{-}yes}$ and that are necessary, together with the carboxy terminus, for mT binding. The ability of antibodies to the carboxy terminus of $p60^{c\text{-}src}$ to immunoprecipitate the $p60^{c\text{-}src}$-mT complex (15) and the inability of synthetic carboxy-terminal peptides to disrupt the $p60^{c\text{-}src}$-mT complex (our unpublished results) also suggest that mT interacts with other regions of $p60^{c\text{-}src}$. One possible role for an intact carboxy terminus is to ensure a correct conformation of the body of the protein for interaction with mT.

Activation of $p60^{src}$ by mT appears to involve reduced phosphorylation, or increased dephosphorylation, of Tyr-527 of $p60^{c\text{-}src}$ (9). Because $p60^{src/lck}$ appears to be stimulated by mT, the enzymes involved presumably recognize the $p60^{c\text{-}src}$ and $p56^{lck}$ carboxy termini similarly.

The different behaviors of $p60^{c\text{-}src}$ and $p56^{lck}$ show that the two proteins interact with different molecules in the cell and suggest that they may also recognize different substrates. Mapping of sequences necessary for binding to mT and the cytoskeleton may be aided by the construction of additional chimerae of c-*src* and *lck*. The apparent structural autonomy of the kinase region of $p60^{c\text{-}src}$ means that it may be possible to interchange the kinase regions of $p60^{c\text{-}src}$ and $p56^{lck}$ without destabilizing the protein or affecting self-regulation by Tyr-527 phosphorylation. Chimerae with a *src* or *lck* amino terminus and the heterologous kinase domain may allow separation of the regions involved in cytoskeletal and mT association.

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ADDENDUM

S. H. Cheng, R. Harvey, P. C. Espino, K. Semba, T. Yamamoto, K. Toyoshima, and A. E. Smith (EMBO J., in press) and S. A. Courtneidge (personal communication) have found that the *fyn* gene product associates with mT. In this respect, it differs from $p56^{lck}$ and resembles $p60^{c\text{-}src}$ and $p62^{c\text{-}yes}$.

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