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Polyomavirus Middle-T Antigen Associates with the Kinase Domain of Src-Related Tyrosine Kinases

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Middle-T antigen of mouse polyomavirus, an oncogenic DNA virus, associates with and activates the cellular tyrosine kinases c-Src, c-Yes, and Fyn. This interaction is essential for polyomavirus-mediated transformation of cells in culture and tumor formation in animals. To determine the domain of c-Src directing association with middle-T, mutant c-Src proteins lacking either the amino-terminal unique domain and the myristylation signal, the SH2 domain, the SH3 domain, or all three of these domains were coexpressed with middle-T in NIH 3T3 cells. All mutants were found to associate with middle-T, demonstrating that the kinase domain of c-Src, including the carboxy-terminal regulatory tail, is sufficient for association with middle-T. Moreover, we found that Hck, another member of the Src kinase family, does not bind middle-T, while chimeric kinases consisting of the amino-terminal domains of c-Src fused to the kinase domain of Hck or the amino-terminal domains of Hck fused to the kinase domain of c-Src associated with middle-T. Hck mutated at its carboxy-terminal regulatory residue, tyrosine 501, was also found to associate with middle-T. These results suggest that in Hck, the postulated intramolecular interaction between the carboxy-terminal regulatory tyrosine and the SH2 domain prevents association with middle-T. This intramolecular interaction apparently also limits the ability of c-Src to associate with middle-T, since removal of the SH2 or SH3 domain increases the efficiency with which middle-T binds c-Src.

The tumor antigens (T antigens) of polyomaviruses expressed early in the viral life cycle are responsible for the oncogenicity of these viruses (43). One of these proteins, middle-T, has been shown to be sufficient for oncogenic transformation of established cell lines (44), while both middle-T and large-T are required to transform primary cells (17). Middle-T modulates the activities of a variety of cellular proteins acting as regulators of cell proliferation, such as phosphatase 2A (33, 46), tyrosine kinases of the Src family, phosphatidylinositol (PI) 3-kinase, SHC, 14-3-3 proteins, and phospholipase C-γ1. Middle-T encoded by mouse polyomavirus preferentially associates with c-Src (16), c-Yes (25), and, to a lesser extent, Fyn (9, 26), while hamster polyomavirus codes for a middle-T protein which binds only Fyn (15). Upon complex formation with c-Src, c-Yes, or Fyn, middle-T becomes phosphorylated, a prerequisite for binding of PI 3-kinase (47), the adaptor protein SHC (6, 18), and phospholipase C-γ1 (40) to phosphorylated tyrosine residues 315, 250, and 322, respectively.

c-Src is composed of several distinct functional domains (Fig. 1). The first 14 amino acids contain information for myristylation and membrane association (24). They are followed by the unique domain which is not conserved among Src family kinases. The amino-terminal half of c-Src also includes an SH3 and an SH2 domain, two regions of homology shared by members of the Src family as well as a large number of otherwise unrelated proteins involved in cell signaling (35). The carboxy-terminal half of c-Src consists of the kinase (SH1) domain which is highly conserved among Src family members followed by 11 amino acids referred to as the kinase tail encompassing Tyr-527, a site known to negatively regulate c-Src kinase activity upon phosphorylation and conserved in all Src family kinases (12). Mutation of Tyr-527 leads to increased kinase activity unleashing the transforming potential of the kinase (14). It has been proposed that phosphorylated Tyr-527 exerts its repressing function by intramolecular binding to the SH2 domain (28, 37) and that SH2 and SH3 domains cooperate in repressing kinase activity (20, 41). As a consequence of dephosphorylation at Tyr-527 (7, 13), the activity of c-Src associated with middle-T is dramatically increased compared with that of uncomplexed c-Src (5). Middle-T may either increase the susceptibility of Tyr-527 to a cellular phosphatase or prevent phosphorylation of this site by the inhibitory cellular kinase Csk (32).

The finding that amino acids 519 to 525 of c-Src are required for complex formation with middle-T (10) suggests that middle-T may bind to a region close to the carboxy terminus of this kinase. On the other hand, the observation that a chimeric protein consisting of c-Src residues 1 to 516 fused to the tail of Lck was able to bind middle-T indicates that c-Src sequences located amino terminal to position 516 are essential for middle-T binding since Lck itself does not associate with middle-T (30). Therefore, these earlier results did not allow an unambiguous identification of the region of the c-Src molecule responsible for association with middle-T. In particular, it was not clear whether the carboxy terminus of c-Src is sufficient for middle-T binding. Since the amino-terminal unique, SH3, and SH2 domains are known to mediate interactions of Src-related kinases with a variety of proteins, it is conceivable that they are also required for association with middle-T. To address this question, we created a series of c-Src deletion mutants and found that all bound middle-T, demonstrating that the kinase domain including the tail is sufficient for association with T antigen. Investigating why Hck, a kinase highly homologous to c-Src, c-Yes, and Fyn, does not associate with middle-T, we constructed chimeric molecules consisting of domains derived from c-Src and Hck as well as an Hck mutant lacking the carboxy-terminal regulatory tyrosine residue. All mutants bound middle-T, suggesting that the intramolecular interaction between the phosphorylated carboxy-terminal regulatory ty-

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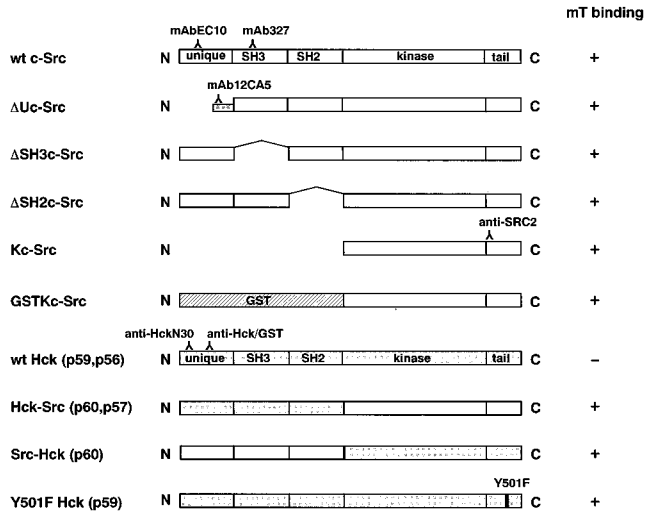

FIG. 1. Structures of c-Src and Hck constructs. The approximate binding regions for the antibodies [MAbs EC10, 327, and 12CA5; polyclonal antibodies anti-SRC2, anti-Hck(N-30), anti-Hck/GST] are indicated. mT, middle-T.

rosine residue and the SH2 domain prevents association of Hck with middle-T. In agreement with this model, we show that deletion of the SH2 or SH3 domain of c-Src dramatically improves the efficiency with which this kinase binds middle-T.

MATERIALS AND METHODS

Plasmid constructs. Plasmids were constructed by using standard molecular cloning techniques. The chicken c-*src* cDNA was subcloned as a *Hin*dIII-*Bgl*II fragment, and the murine *hck* cDNA was subcloned as an *Eco*RI fragment (obtained from A. Dunn) into a pSP72 vector (Promega). Plasmids pSP72c-src and pSP72hck were used as templates for PCR. The deletion mutants ΔSH3c-Src and ΔSH2c-Src and the chimeras derived from the c-*src* and the *hck* cDNAs were constructed by using the method of splicing by overlap extension-PCR (21, 22). The sequences coding for ΔUc-Src (starting at codon 82 of c-*src*) and Kc-Src (starting at codon 258 of c-*src*) were amplified from pSP72c-src by PCR. The primers used introduced a *Hin*dIII site and a novel start codon and fused a nucleotide sequence specifying the nine-amino-acid peptide YPYDVPDYA derived from the influenza virus hemagglutinin protein (HA tag) together with a sequence coding for an 11-amino-acid linker peptide, KLMGLVVMNIT, in frame to the 5′ end of the truncated c-*src* coding sequence. The PCR products were ligated as *Hin*dIII-*Cla*I fragments into a pSV expression vector (31). The sequence coding for Y501FHck (Hck containing a Tyr-501→Phe mutation) was obtained from R. Perlmutter (48). pcDNAmT is an expression vector derived from pcDNA1Neo (Invitrogen) carrying the cDNA sequence of middle-T. The mammalian expression vector pCMV-GX (generously provided by W. G. Kaelin, Jr.) is derived from pCMV-Neo-Bam (1) and carries the coding sequence for glutathione *S*-transferase (GST) under the control of a cytomegalovirus promoter. pCMV-GX-Kc-src contains the coding sequence for the kinase domain of c-Src (residues 258 to 533) fused in frame to the 3′ end of the GST coding sequence. The Kc-Src sequence was amplified by PCR from pSP72c-src, using a 5′ primer that introduced a *Bam*HI site allowing fusion in frame with the GST coding sequence. All mutations were confirmed by restriction enzyme analysis and DNA sequencing.

Antibodies. Using the chicken c-*src* cDNA for constructing the mutants allowed specific detection of wild-type protein, ΔSH3, and ΔSH2c-Src with monoclonal antibody (MAb) EC10, which recognizes only the chicken form of c-Src (34). The epitope-tagged ΔUc-Src protein lacking the epitope recognized by MAb EC10 was recognized by MAb 12CA5 (Boehringer) or by MAb 327 (27). pSVΔUc-src was transfected into src15 cells, a cell line derived from transgenic mice lacking both *src* alleles (42), thus ensuring that MAb 327 recognizes only the gene product of transfected pSVΔUc-Src.

Hck, Hck-Src, and Y501FHck were detected either with rabbit polyclonal antibody anti-Hck(N-30) (Santa Cruz Biotechnology) or with rabbit polyclonal antiserum anti-Hck/GST (29). Anti-Hck(N-30) was raised against a peptide corresponding to amino acids 8 to 37 within the amino-terminal unique domain of human $p59^{hck}$. Anti-Hck/GST was raised against a GST-Hck fusion protein containing residues 8 to 53 of mouse $p59^{hck}$. Anti-Hck(N-30) and anti-Hck/GST recognize both $p59^{hck}$ and $p56^{hck}$ and lack cross-reactivity with other members of the Src family. Anti-SRC2 (Santa Cruz Biotechnology) is a rabbit polyclonal antibody raised against a peptide corresponding to residues 509 to 533 of human c-Src. Middle-T was immunoprecipitated with rat ascites fluid specific for polyomavirus T antigens (BNasc) or with PAb762 (19), a kind gift of S. Dilworth.

Cell lines, DNA transfection, focus assays, and virus infection. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum at 37°C in a 10% CO_2 incubator. NIH 3T3 cell lines expressing wild-type or mutant forms of c-Src and Hck were generated by stable transfection with a pSV expression vector carrying the corresponding cDNA under the control of the simian virus 40 early promoter (31). This expression vector was introduced together with the selectable marker pMOneo^r into 3T3 cells by $Ca_3(PO_4)_2$-mediated transfection as described previously (3).

Transient transfections were performed by the Lipofectamine method. Twenty micrograms of plasmid DNA together with 60 μl of Lipofectamine (GIBCO) in 3 ml of Optimem 1 medium (GIBCO) was added to 10^6 3T3 cells plated on 10-cm-diameter dishes and incubated for 5 h. Three milliliters of DMEM containing 20% calf serum was added to the transfection mixture. The cells were lysed for immunoprecipitation 18 h after transfection.

Focus-forming activities of the various constructs were assayed by using NIH 3T3 mouse fibroblasts as described previously (36).

NIH 3T3 cells were infected with wild-type polyomavirus (NG59RA) for 2 h and lysed after 20 h.

Metabolic labeling with Tran^{35}S-Label. In vivo labeling with Tran^{35}S-Label ([^{35}S]methionine-[^{35}S]cysteine; ICN Pharmaceuticals) was performed as follows. Eighteen hours after transfection or virus infection, cells were washed with DMEM without methionine and cysteine. The cells were incubated for 6 h with 250 μCi of Tran^{35}S-Label in 2.5 ml of DMEM lacking methionine and cysteine and supplemented with 1% calf serum.

Immunoprecipitations and in vitro kinase assays. Cell lysis with buffer containing Nonidet P-40, immunoprecipitations using protein A–Sepharose CL-4B (Pharmacia Biotech), and in vitro kinase assays with [γ-^{32}P]ATP were performed as described previously (23). For reprecipitation, immunoprecipitates were boiled for 10 min in 2% sodium dodecyl sulfate (SDS)–5% 2-mercaptoethanol and centrifuged briefly. The supernatant was lyophilized, dissolved in 20 times the original volume by using Nonidet P-40 lysis buffer, and incubated overnight with BNasc, PAb762, or anti-SRC2. Immune complexes were harvested with protein A–Sepharose CL-4B and washed as described previously (23).

Peptide mapping. *Staphylococcus aureus* V8 protease maps were performed as described previously (23, 36). In brief, the bands corresponding to the respective kinase variants or middle-T were excised, mounted onto an SDS–12.5% polyacrylamide gel, and overlaid with V8 protease solution (10 μg/ml; ICN Pharmaceuticals). Protease digestion occurred as the gel was run at low voltage for 30 min after stacking.

RESULTS

The kinase domain of c-Src including the carboxy-terminal tail is sufficient for complex formation with middle-T. To evaluate the importance of the amino-terminal domains of c-Src for complex formation with middle-T, we introduced deletions into the c-*src* cDNA that resulted in mutant proteins lacking either the unique domain and the myristylation signal (ΔUc-Src; residues 1 to 81 deleted, epitope tagged with the HA tag), the SH3 domain (ΔSH3c-Src; residues 83 to 140 deleted), or the SH2 domain (ΔSH2c-Src; residues 143 to 247 deleted) (Fig. 1). Two methods were applied to assay complex formation between c-Src mutants and middle-T. NIH 3T3 cell lines stably expressing either wild-type or mutant c-Src proteins were infected with polyomavirus. Alternatively, pSVc-src, pSVΔSH3c-src, or pSVΔSH2c-src was transiently cotransfected with pcDNAmT, a mammalian expression vector carrying the cDNA sequence of middle-T, into NIH 3T3 cells. pSVΔUc-src was transfected together with pcDNAmT into src15 cells, a line derived from *src* knockout mice (42). Lysates made from cells expressing either of the c-Src variants together with middle-T were used for immunoprecipitation. The expression levels of the various c-Src mutants were comparable, as determined by Western blotting (immunoblotting) (data not shown). Mock-infected cells or cells transfected with the respective c-Src constructs were included as controls. Lysates were incubated with MAb EC10 (for the wild type, ΔSH3, or ΔSH2c-Src) or with MAb 327 or 12CA5 (for ΔUc-Src). Immunoprecipitates were phosphorylated in vitro with [γ-^{32}P]ATP, resolved by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. As expected from the calculated

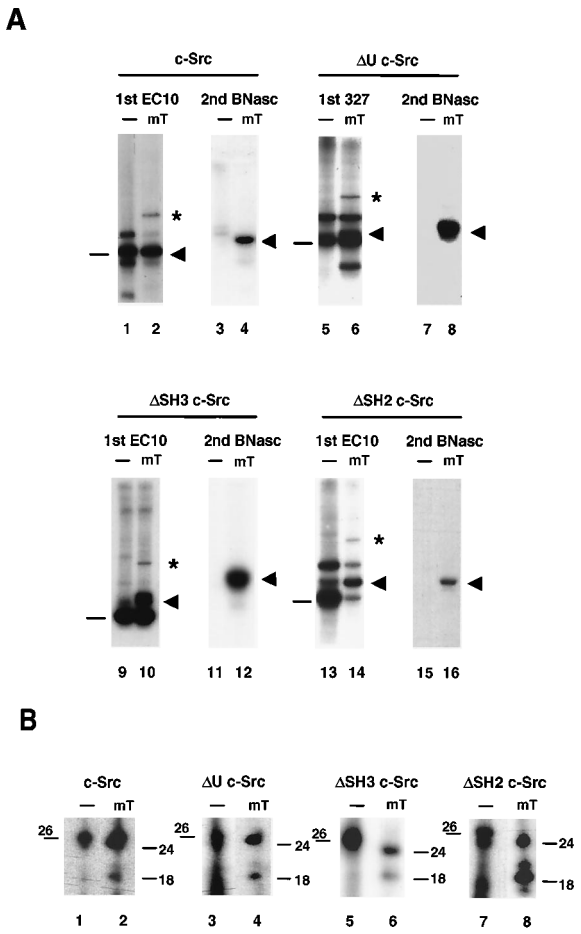

FIG. 2. Coprecipitation of middle-T with c-Src deletion mutants. Immunoprecipitations were carried out with lysates from control (−) or middle-T-expressing (mT) cells transfected with c-Src, ΔUc-Src, ΔSH3c-Src, or ΔSH2c-Src. Association with middle-T was assayed in polyomavirus-infected NIH 3T3 cells expressing the respective kinase variant (c-Src or ΔSH3c-Src). pSVΔUc-Src was transfected together with pcDNAmT into src15 cells. ΔSH2c-Src was transfected together with pcDNAmT into NIH 3T3 cells. (A) Immunoprecipitates obtained with MAb EC10 or 327 were phosphorylated in vitro with [γ-^{32}P]ATP. Immunoprecipitates from cells expressing c-Src (lanes 1 and 2) and ΔSH3c-Src (lanes 9 and 10) were analyzed on SDS–12.5% polyacrylamide gels; ΔUc-Src (lanes 5 and 6) and ΔSH2c-Src (lanes 13 and 14) were analyzed on SDS–10% polyacrylamide gels. Phosphorylated proteins were detected by autoradiography. Bars, bands corresponding to the respective kinase mutants; arrowheads, bands corresponding to middle-T; asterisks, the p85 subunit of PI 3-kinase. Middle-T was identified by reprecipitation with BNasc (lanes 3, 4, 7, 8, 11, 12, 15, and 16). (B) Cleveland mapping of the bands corresponding to c-Src deletion mutants and middle-T, using *S. aureus* V8 protease. All c-Src mutants gave rise to a 26-kDa fragment (lanes 1, 3, 5, and 7); middle-T gave rise to 18- and 24-kDa fragments (lanes 2, 4, 6, and 8).

molecular masses, pSVc-Src, pSVΔUc-src, pSVΔSH3c-src, and pSVΔSH2c-src gave rise to phosphorylated proteins of 60, 51, 53, and 49 kDa, respectively (Fig. 2A, lanes 1, 5, 9, and 13).

Middle-T gave rise to bands corresponding to apparent molecular masses of 56 and 58 kDa (Fig. 2A, arrowheads), as a result of differential phosphorylation (36, 38), which were not separated on all gels. Middle-T was coprecipitated with wild-type c-Src by using MAb EC10 (Fig. 2A, lane 2). MAbs 327 (Fig. 2A, lane 6) and 12CA5 (data not shown) allowed coprecipitation of middle-T with ΔUc-Src. Middle-T was also coprecipitated with ΔSH3c-Src and ΔSH2c-Src by using MAb EC10 (Fig. 2A, lanes 10 and 14). The phosphorylated form of the p85 subunit of PI 3-kinase was detected in all precipitates of c-Src variants capable to bind middle-T (Fig. 2A, lanes 2, 6, 10, and 14, asterisks), indicating that these mutants properly phosphorylated middle-T. The presence of middle-T in immunoprecipitates obtained with antibodies against c-Src was also confirmed by reprecipitation with BNasc. Middle-T was reprecipitated from immunoprecipitates of all kinase mutants coexpressed with middle-T (Fig. 2A, lanes 4, 8, 12, and 16).

The identity of each of the immunoprecipitated proteins was further confirmed by Cleveland mapping (11) with *S. aureus* V8 protease (Fig. 2B). The 60-, 53-, 51-, and 49-kDa bands attributed to the c-Src variants (Fig. 2A, lanes 1, 5, 9, 13) all gave rise to a 26-kDa fragment (Fig. 2B, lanes 1, 3, 5, and 7) encompassing a carboxy-terminal phosphopeptide typical for c-Src (2). V8 digestion of the 56/58-kDa doublet of middle-T (Fig. 2A, lanes 2, 6, 10, and 14) gave rise to fragments of 24 and 18 kDa (Fig. 2B, lanes 2, 4, 6, 8) as described previously (38). The identity of the p85 band (Fig. 2A, lanes 2, 6, 10, and 14) was also confirmed by V8 mapping (data not shown).

Complex formation of middle-T with a c-Src mutant lacking the entire amino-terminal half up to the kinase domain (Kc-Src; residues 1 to 257 deleted; Fig. 1) was investigated in transiently transfected NIH 3T3 cells metabolically labeled with Tran^{35}S-Label expressing Kc-Src or, as a control, c-Src together with middle-T. BNasc (Fig. 3, lanes 1 to 4) and anti-SRC2 (Fig. 3, lanes 5 to 8) immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis followed by fluorography. Reprecipitation from denatured BNasc immunoprecipitates with anti-SRC2 (Fig. 3, lanes 9 to 12) allowed detection of c-Src (Fig. 3, lane 10) or Kc-Src (Fig. 3, lane 12) coprecipitated with middle-T. As expected, Kc-Src migrated with an apparent molecular mass of 32 kDa on SDS-polyacrylamide gels. These data show that the kinase domain including the tail of c-Src is sufficient for association with middle-T.

A fusion protein (GSTKc-Src; Fig. 1) consisting of GST and the c-Src kinase domain (residues 258 to 533) was coexpressed together with middle-T in NIH 3T3 cells transiently transfected with pCMV-GX-Kc-src and pcDNAmT and precipitated by using glutathione-Sepharose. As shown in Fig. 3B, middle-T specifically associated with GSTKc-Src and was labeled together with p85 upon phosphorylation in vitro. These findings indicate that the kinase domain of c-Src is sufficient for both association with middle-T and subsequent phosphorylation of the bound protein at Tyr-315, the site required for p85 binding.

Hck does not associate with middle-T. Hck is a Src family kinase primarily expressed in hematopoietic cells (29). The high homology with c-Src, c-Yes, and Fyn makes it a good candidate for association with middle-T. We investigated the ability of Hck to bind middle-T in NIH 3T3 mouse fibroblasts (Fig. 4) and in the hematopoietic cell lines FDC-P1 and HL-60 (data not shown). pSVhck-transfected cell clones were infected with polyomavirus. Alternatively, association of Hck with middle-T was assayed by cotransfection of pSVhck with pcDNAmT. Hck was immunoprecipitated with polyclonal antibody anti-Hck(N-30) or anti-Hck/GST (Fig. 1) and phosphorylated in vitro with [γ-^{32}P]ATP. The murine *hck* cDNA gave rise to 56- and 59-kDa protein isoforms as a consequence of alternative translation initiation (29) (Fig. 4A, lanes 3 to 6). V8 digestion of both bands generated a 26-kDa fragment similar to the carboxy-terminal fragment of c-Src (Fig. 4B, lanes 1 and 2). Neither anti-Hck(N-30) nor anti-Hck/GST immunoprecipitates from polyomavirus-infected Hck-expressing NIH 3T3 cells contained middle-T (Fig. 4A, lanes 4 and 6). No middle-T protein was reprecipitated with BNasc following anti-Hck immunoprecipitation (Fig. 4A, lanes 17 to 20), and the absence of the indicative middle-T V8 fragments (Fig. 4B, lane 2) con-

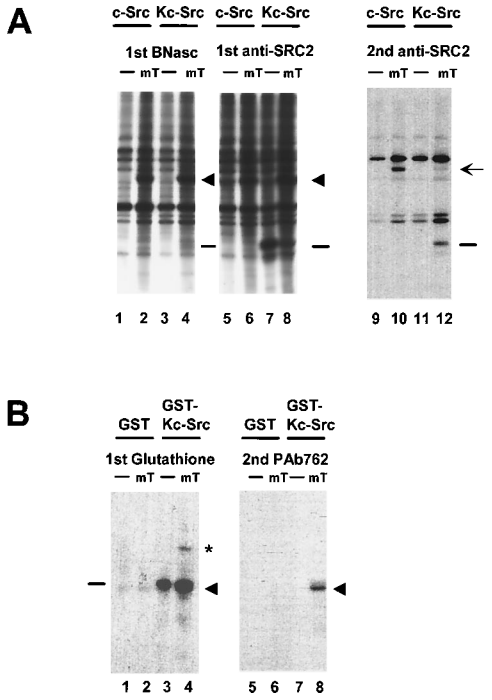

FIG. 3. The c-Src kinase domain is sufficient for association with middle-T. (A) Coprecipitation of the c-Src kinase domain (residues 258 to 533) with middle-T. NIH 3T3 cells were transfected with pSV plasmids coding for wild-type c-Src or Kc-Src (c-Src kinase domain) alone (−) or together with pcDNAmT (mT) and labeled with Tran^{35}S-Label. Middle-T was immunoprecipitated with BNasc (lanes 1 to 4), c-Src (lanes 5 and 6), and Kc-Src (lanes 7 and 8) with anti-SRC2. Immunoprecipitates were analyzed on SDS–10% polyacrylamide gels and fluorographed. c-Src (lane 10) and Kc-Src (lane 12) were reprecipitated from BNasc immunoprecipitates with anti-SRC2. Arrowheads, middle-T; arrow, c-Src; bars, Kc-Src. (B) Phosphorylation of middle-T by GSTKc-Src, a fusion protein consisting of GST fused to the c-Src kinase domain. Lysates of NIH 3T3 cells expressing GST or GSTKc-Src fusion protein alone (−) or together with middle-T (mT) were used for precipitation with glutathione-Sepharose. Precipitated GST (lanes 1 and 2) and GSTKc-Src (lanes 3 and 4) were incubated in vitro with [γ-^{32}P]ATP. Autophosphorylated GSTKc-Src (lane 3, indicated by the bar) and phosphorylated coprecipitated middle-T (arrowhead) and p85 (asterisk) (lane 4) are indicated. The presence of middle-T in the precipitate of lane 4 was confirmed by reprecipitation with PAb762 (lane 8).

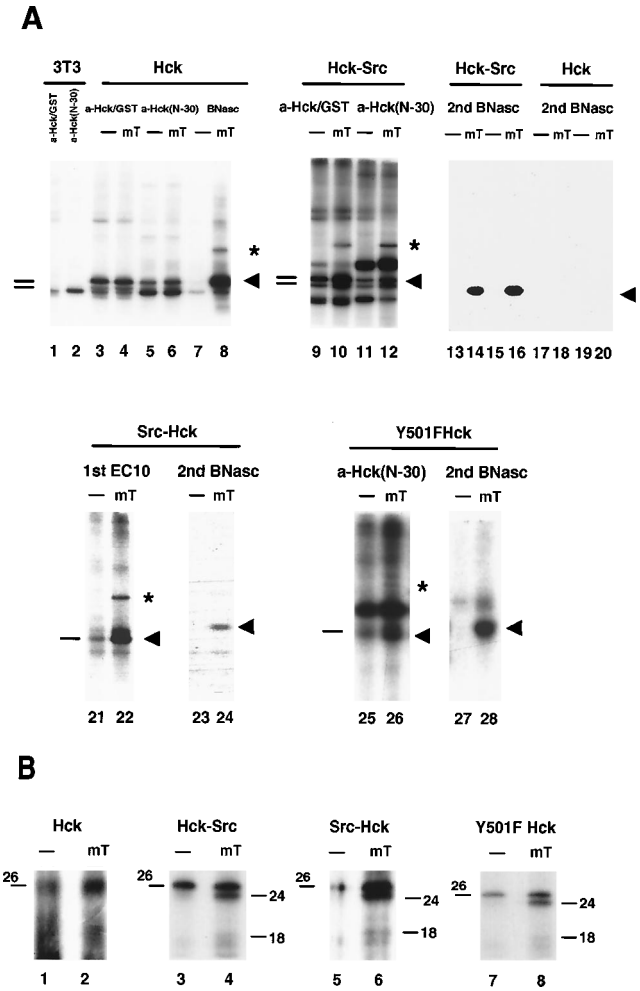

FIG. 4. Coprecipitation of middle-T with chimeric kinases and Y501FHck. Immunoprecipitations were carried out by using lysates from cells expressing Hck, Hck-Src, and Src-Hck without (−) or with middle-T (mT). Association with middle-T was assayed in virus-infected NIH 3T3 cells stably expressing Hck, Hck-Src, or Src-Hck. pSVY501Fhck was transiently cotransfected with pcDNAmT into NIH 3T3 cells. (A) Immunoprecipitates obtained with anti-Hck/GST, anti-Hck(N-30), or MAb EC10 were phosphorylated in vitro with [γ-^{32}P]ATP. Immunoprecipitates from cells expressing Hck (lanes 3 to 8), Hck-Src (lanes 9 to 12), and Src-Hck (lanes 21 and 22) were analyzed on SDS–12.5% polyacrylamide gels; Y501FHck was analyzed on SDS–10% polyacrylamide gels. Bars, bands corresponding to the respective kinase variants; arrowheads, middle-T; asterisks, the p85 subunit of PI 3-kinase. Anti-Hck/GST and anti-Hck(N-30) immunoprecipitates from NIH 3T3 control cells are shown in lanes 1 and 2. BNasc precipitates from mock-infected (lane 7) or virus-infected (lane 8) cells expressing Hck were loaded as controls. Middle-T was identified by reprecipitation with BNasc (lanes 13 to 20, 23, 24, 27, and 28). (B) Cleveland mapping of the bands corresponding to the kinase variants and middle-T, using *S. aureus* V8 protease. All kinase variants gave rise to a 26-kDa fragment (lanes 1, 3, 5, and 7). Middle-T gave rise to 18- and 24-kDa fragments (lanes 2, 4, 6, and 8).

firmed the conclusion that middle-T was not coprecipitated with Hck.

To exclude the possibility that Hck associates with but fails to phosphorylate middle-T, polyomavirus-infected cells expressing Hck were labeled with Tran^{35}S-Label and immunoprecipitated with BNasc or anti-Hck(N-30). Middle-T and Hck were present in BNasc and anti-Hck(N-30) immunoprecipitates, respectively, while middle-T was absent from anti-Hck(N-30) immunoprecipitates (data not shown). Middle-T was also absent from anti-Hck immunoprecipitates prepared from middle-T-transformed FDC-P1 and HL-60 cells, demonstrating that Hck also fails to associate with middle-T in these hematopoietic cells (data not shown).

Chimeric kinases derived from c-Src and Hck associate with middle-T. To address the question why Hck was not able to associate with middle-T, we constructed chimeric kinases consisting of sequences derived from both c-Src and Hck. One of the chimeras, Hck-Src, carried the unique, SH3, and SH2 domains of Hck (residues 1 to 229 of p56^{hck} and 1 to 250 of p59^{hck}) fused to the carboxy-terminal half of c-Src (residues 258 to 533) (Fig. 1). The reciprocal chimera, Src-Hck, had the unique, SH3, and SH2 domains of c-Src (residues 1 to 257) fused to the carboxy-terminal half of Hck (residues 230 to 503 of p56^{hck}) (Fig. 1). NIH 3T3 cell lines stably expressing these variants were generated and infected with polyomavirus. The expression levels of the two chimeras were comparable, as determined by Western blotting (not shown). Hck-Src was immunoprecipitated with anti-Hck(N-30) or anti-Hck/GST, and Src-Hck was immunoprecipitated with MAb EC10 (Fig. 1). The *hck-src* construct gave rise to two isoforms of 57 and 60 kDa, since the two alternative initiation codons of *hck* were maintained in this construct (Fig. 4A, lanes 9 and 11). The *src-hck* construct gave rise to a 60-kDa protein (Fig. 4A, lane

TABLE 1. c-Src activity in control or middle-T-expressing cells[a]

Expt	Src mutant	Relative kinase activity of Src	% Src bound to middle-T	Relative kinase activity of middle-T-bound Src
1	c-Src	1.0		
	c-Src with middle-T	3.3	15	16.3
	ΔSH3c-Src	15.3		
	ΔSH3c-Src with middle-T	18.6	69	20.0
	ΔSH2c-Src	16.8		
	ΔSH2c-Src with middle-T	17.1	90	17.1
	Src-Hck	1.3		
	Src-Hck with middle-T	2.5	10	13
2	c-Src	1.0		
	c-Src with middle-T	1.9	10	10.0
	ΔUc-Src	0.6		
	ΔUc-Src with middle-T	1.1	10	5.6
3	Hck	1.0		
	Hck-Src	1.5		

[a] Relative kinase activities were measured by phosphorylation of enolase in immunoprecipitations performed with MAb EC10 (experiment 1), MAb 327 (experiment 2), or anti-Hck(N-30) (experiment 3).

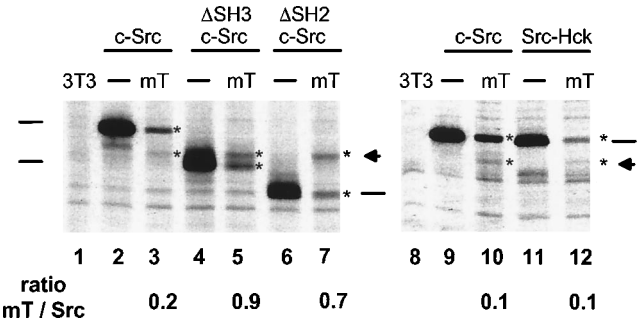

FIG. 5. Quantification of the interactions between various c-Src mutants and middle-T. Immunoprecipitations were performed with MAb EC10, using lysates from cells labeled with Tran^{35}S-Label expressing c-Src (lanes 2 and 3), ΔSH3c-Src (lanes 4 and 5), ΔSH2c-Src (lanes 6 and 7), or Src-Hck (lanes 11 and 12) alone (−) or together with middle-T (mT). MAb EC10 immunoprecipitates from mock-transfected NIH 3T3 cells were included as controls (lanes 1 and 8). Quantification was performed with a Molecular Dynamics PhosphorImager. For precise determination of the relative amounts of the proteins, the band intensities were corrected for the specific activities of the respective proteins. The ratio of the amount of middle-T to the amount of c-Src determined for this representative experiment is indicated below the corresponding lane. Arrows show the positions of middle-T; bars indicate the positions of the kinase variants; asterisks indicate the relevant bands.

21). Middle-T was coprecipitated with both Hck-Src (Fig. 4A, lanes 10 and 12) and Src-Hck (Fig. 4A, lane 22). Complex formation between these chimeric kinases and middle-T was confirmed by reprecipitation of middle-T with BNasc (Fig. 4A, lanes 14, 16, and 24). V8 digestion of the 57- and 60-kDa forms of Hck-Src (Fig. 4A, lanes 9 and 11) and of Src-Hck (Fig. 4A, lane 21) gave rise to 26-kDa fragments (Fig. 4B, lane 3 and 5). Middle-T bands were also mapped with V8 protease, resulting in the characteristic 24- and 18-kDa fragments (Fig. 4B, lanes 4 and 6).

The transforming potential of the chimeric kinases was determined in focus formation assays. Both chimeras were nontransforming (data not shown). Their relative kinase activities were not significantly different from those of wild-type c-Src and Hck, as determined in vitro by using enolase as substrate (Table 1).

The fact that Hck-Src bound middle-T was in agreement with results obtained with deletion mutants of c-Src demonstrating that the carboxy-terminal half of c-Src is responsible for middle-T binding. It was, however, surprising that Src-Hck also bound middle-T. This result suggests that the kinase domain of Hck possesses the ability to bind middle-T but that either the unique, the SH3, or the SH2 domain of Hck interferes with middle-T binding to wild-type Hck.

Y501FHck associates with middle-T. On the basis of the hypothesis that intramolecular interaction between the phosphorylated carboxy-terminal regulatory residue (tyrosine 501) and the SH2 domain of Hck may prevent middle-T from binding, we investigated the ability of Y501FHck to associate with middle-T. In this mutant, Tyr-501 was converted to phenylalanine, a mutation that is presumed to disrupt the intramolecular association. Y501FHck has previously been shown to be oncogenic although it does not have increased in vitro kinase activity, in contrast to the analogous mutant Y527Fc-Src (48).

pSVY501FHck was transiently transfected alone or together with pcDNAmT into NIH 3T3 fibroblasts. Cells were lysed, and Y501FHck was precipitated with anti-Hck(N-30). Middle-T was coprecipitated with Y501FHck (Fig. 4A, lane 26) and detected by reprecipitation with BNasc (Fig. 4A, lane 28). V8 digestion of Y501FHck yielded the typical 26-kDa fragment (Fig. 4B, lane 7), while middle-T gave rise to the characteristic 24- and 18-kDa fragments (Fig. 4B, lane 8). These data suggest that in order to be able to bind middle-T, Hck must be in an open conformation.

c-Src mutants lacking the SH2 or SH3 domain associate better than wild-type c-Src with middle-T. The observation that wild-type Hck did not associate with middle-T whereas Y501FHck and Src-Hck were able to do so can be explained by the fact that the proposed intramolecular interaction between the phosphorylated kinase tail and the SH2 domain interferes with middle-T binding. In Y501FHck and Src-Hck, the intramolecular interaction might be weakened, thus favoring an open conformation allowing middle-T binding.

It has been shown previously that only a small fraction of the c-Src molecules present in a cell bind middle-T, even if the latter is overexpressed (4). If our model is valid, one might predict that c-Src mutants lacking the SH2 and SH3 domains will be better able than wild-type c-Src to bind middle-T, since deletion of the SH2 and SH3 domains is likely to disrupt the closed conformation of the kinase. To test this hypothesis, NIH 3T3 cells were transiently transfected with pSVc-src, pSVΔSH3c-src, or pSVΔSH2c-src alone or in combination with pcDNAmT and metabolically labeled with Tran^{35}S-Label. Half of each lysate was used for immunoprecipitation of the c-Src variants with MAb EC10, and the other half was used for immunoprecipitation of middle-T with PAb762. The expression levels of the c-Src variants coexpressed with middle-T were similar (Fig. 5, lanes 3, 5, and 7), and the amounts of middle-T protein coexpressed with the various c-Src variants were approximately the same, as estimated by immunoprecipitation with PAb762 (data not shown). Middle-T expression was always higher than expression of the various c-Src mutants. Thus, the amount of middle-T available for complex formation with any of the c-Src mutants used in this experimental setup was not limiting. Three independent experiments were performed to quantify the interactions of c-Src, ΔSH3c-Src, and ΔSH2c-Src with middle-T. Figure 5 shows a fluorogram of an SDS-polyacrylamide gel from a representative experiment. Quantification of the bands showed that in all three experiments, the amount of middle-T protein coprecipitated with

c-Src was 10 to 20% of the total immunoprecipitable c-Src (Fig. 5, lane 3). Since it is known that one kinase molecule is present in a middle-T–c-Src complex (8), we conclude that 10 to 20% of the immunoprecipitated wild-type c-Src molecules were bound to middle-T, even though middle-T expression was higher than expression of c-Src. This result is in agreement with previously published estimates indicating that 5 to 10% of immunoprecipitable c-Src is complexed with middle-T (4).

The finding that ΔSH3c-Src (Fig. 5, lane 5) and ΔSH2c-Src (Fig. 5, lane 7) immunoprecipitates contained significantly higher amounts of middle-T protein than wild-type c-Src immunoprecipitates further supports our model that middle-T preferentially associates with kinase molecules assuming the open conformation. In all experiments, 60 to 90% of ΔSH3c-Src and 60 to 90% of ΔSH2c-Src molecules were found to be complexed with middle-T.

The relative amount of middle-T protein coprecipitated with Src-Hck was also determined by using the same experimental approach. We observed that 10 to 20% of the Src-Hck molecules formed a complex with middle-T when Src-Hck and middle-T were expressed together (Fig. 5, lane 12). This ratio is the same as that determined for the interaction between c-Src and middle-T (Fig. 5, lane 10), indicating that the capacity of Src-Hck to bind middle-T is similar to that of c-Src. We were not able to quantitate the amount of middle-T coprecipitated with Hck-Src and Y501FHck, since immunoprecipitation from metabolically labeled cells with the polyclonal anti-Hck antisera available yielded a high unspecific background, making detection of coprecipitated middle-T difficult. Middle-T could be detected by reprecipitation in such experiments, but an accurate quantification is not possible with this protocol.

We also determined the relative amount of middle-T coprecipitated with ΔUc-Src by metabolic labeling with Tran^{35}S-Label in src15 cells lacking endogenous c-Src. Wild-type c-Src and ΔUc-Src expressed alone or together with middle-T were immunoprecipitated with MAb 327 (data not shown). Five to 10% of wild-type c-Src or ΔUc-Src was associated with middle-T. This result indicates that deletion of the c-Src unique domain does not influence the ability of the kinase to bind middle-T, and deletion of this domain therefore seems not to alter the conformation of c-Src required for middle-T binding.

The kinase activities of c-Src, ΔUc-Src, ΔSH3c-Src, ΔSH2c-Src, and Src-Hck expressed alone or together with middle-T were measured in two independent experiments. Immunoprecipitates obtained with MAb EC10 (for c-Src, ΔSH3c-Src, ΔSH2c-Src, and Src-Hck) and MAb 327 (for c-Src and ΔUc-Src) were used for in vitro kinase assays with enolase as the substrate. Half of each immunoprecipitate was used for the kinase assay; the other half was used for quantification of complex formation between the various c-Src variants and middle-T. Table 1 shows the results of one representative experiment. ΔSH3c-Src and ΔSH2c-Src were about 15-fold activated compared with wild-type c-Src. These results are in agreement with published data indicating that c-Src mutants lacking the SH3 domain or parts of the SH2 domain have a 14- to 30-fold increase in kinase activity (39). Src-Hck and ΔUc-Src have approximately the same level of activity as wild-type c-Src. When wild-type c-Src was coexpressed with middle-T, its activity was two- to fourfold increased. Since only 5 to 20% of the c-Src molecules were complexed with middle-T, the actual kinase activity of middle-T-bound c-Src was approximately 20-fold greater than that of uncomplexed c-Src. In contrast, ΔSH3c-Src and ΔSH2c-Src were activated no more than twofold upon complex formation with middle-T (Table 1). ΔUc-Src and Src-Hck behaved like wild-type c-Src.

DISCUSSION

In this work, we identified the domain of the c-Src tyrosine kinase responsible for association with polyomavirus middle-T. Previously published data (10) have shown that c-Src truncated at residue 525 is able to bind middle-T, indicating that the eight carboxy-terminal amino acids are dispensable for middle-T binding. c-Src truncated at residue 518 as well as v-Src, which carries a completely different sequence derived from cellular DNA fused to the *src* coding sequence after codon 514, does not associate with middle-T (10). Others showed that a chimeric kinase consisting of amino acids 1 to 516 of c-Src fused to the last 15 amino acids from Lck was able to form a complex with middle-T (30). Since no association between wild-type Lck and middle-T was observed, this result led to the conclusion that sequences between residues 1 and 516 of c-Src in addition to residues 519 to 525 are necessary for association with middle-T.

Since the unique domain is the region which diverges most among the different kinases of the Src family, it is believed to account for functional specialization of these enzymes. This domain is a candidate binding site for cellular proteins, and it has been shown in Lck to mediate association with the CD4 and CD8 receptors in T cells (45). It is also known that the SH3 and SH2 domains are required for protein-protein interactions regulating both localization and activity of these kinases (35). We thus investigated whether the unique, SH3, and SH2 domains of c-Src are necessary for complex formation with middle-T. Deletion analysis led us to conclude that they are not required for binding middle-T and that the kinase domain of c-Src is sufficient both for binding and for phosphorylation of middle-T. Our results, together with those published earlier, allow delineation of the middle-T binding region of c-Src as residues 248 to 525, i.e., the kinase domain including the first three residues of the tail. The middle-T binding site in the carboxy-terminal half of Src-related tyrosine kinases may also be required for association with cellular regulatory proteins. It will therefore be interesting to search for cellular proteins interacting with the carboxy-terminal half of c-Src.

The Src-related tyrosine kinase Hck, which has 58.5% sequence identity with c-Src, does not bind middle-T. Investigating the ability of chimeric proteins derived from c-Src and Hck to bind middle-T, we found that both Hck-Src carrying the unique, SH3, and SH2 domains of Hck fused to the kinase domain of c-Src and the reciprocal chimera, Src-Hck, bound and phosphorylated middle-T. The fact that Hck-Src associates with middle-T is in agreement with the finding that the amino-terminal domains of c-Src are dispensable for middle-T binding. Apparently, the amino-terminal half of c-Src can be replaced by the corresponding Hck sequence with no negative effect on middle-T binding. It was, however, unexpected that Src-Hck bound middle-T. The kinase domain of c-Src including residues 519 to 525 is not fully conserved in Hck, but the fact that Src-Hck associates with middle-T shows that these sequence differences do not affect middle-T binding. Apparently, the carboxy-terminal half of Hck has the ability to bind middle-T, but sequences located in the amino-terminal half of Hck hinder binding of the wild-type Hck protein to middle-T. The amount of middle-T coprecipitated with Src-Hck is similar to that found in wild-type c-Src immunoprecipitates, indicating that the affinity of the Hck kinase domain for middle-T is comparable to that of c-Src.

Various lines of evidence (28, 37) suggest that the carboxy-terminal tail of Src-related tyrosine kinases associates intramolecularly with the SH2 domain through conserved phosphorylated tyrosine residues, Tyr-527 in c-Src and Tyr-501 in Hck.

The SH3 domain may further stabilize this interaction in the inactive, closed conformation, perhaps upon dimerization of two kinase molecules as suggested for Lck on the basis of an X-ray structure (20). We propose that the intramolecular interaction between the tail of Hck and its SH2 domain may interfere with middle-T binding. Our concept is supported by the fact Y501FHck, which carries a mutation believed to disrupt the intramolecular interaction, bound middle-T. Association of wild-type Hck with middle-T might not be possible because the intramolecular interaction in Hck is stronger than the affinity of the kinase domain of Hck for middle-T. One should bear in mind that Hck may not be susceptible to activation in NIH 3T3, FDC-P1, or HL-60 cells and may therefore be permanently locked in a closed conformation, thus preventing middle-T binding. The interaction between the carboxy-terminal regulatory tail and the SH2 domain is apparently not disrupted in Hck-Src and Src-Hck, since these chimeric kinases are not transforming and not significantly activated compared with c-Src and Hck. Our data suggest that the intramolecular interaction in these chimeras is slightly weaker than that in Hck, favoring an intermolecular interaction with middle-T. It will be interesting to look for association of Hck with middle-T in cells in which Hck can be activated.

It has been shown previously that only a small fraction (about 10%) of the c-Src molecules present in a cell associate with middle-T (4). This might be due to the fact that only a small percentage of the c-Src molecules are in the activated state and thus have an open conformation allowing binding to middle-T. In agreement with our concept that the intramolecular interaction between the SH2 and SH3 domains and the kinase tail hinders middle-T binding, we provide evidence that c-Src mutants lacking the SH2 or SH3 domain bind middle-T significantly better than wild-type c-Src. Sixty to 90% of the immunoprecipitated ΔSH2c-Src and ΔSH3c-Src molecules were complexed with middle-T. These c-Src deletion mutants have kinase activity approximately 15-fold greater than that of wild-type c-Src. Wild-type c-Src complexed with middle-T was activated about 20-fold compared with uncomplexed c-Src, while ΔSH2c-Src and ΔSH3c-Src were only slightly activated upon middle-T binding.

In conclusion, our results suggest that middle-T preferentially binds to and thereby stabilizes the open conformation of activated c-Src molecules, and we propose that it might mimic the function of cellular proteins involved in regulating the activity of Src family kinases.

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REFERENCES

1. **Baker, S. J., S. Markowitz, E. R. Pearon, J. K. V. Willson, and B. Vogelstein.** 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science **249:**912–915.
2. **Ballmer-Hofer, K., and T. L. Benjamin.** 1985. Phosphorylation of polyoma middle T antigen and cellular proteins in purified plasma membranes of polyoma virus-infected cells. EMBO J. **4:**2321–2327.
3. **Ballmer-Hofer, K., G. Mandel, D. V. Faller, T. M. Roberts, and T. L. Benjamin.** 1987. Expression of influenza hemagglutinin-polyoma T-antigen fusion proteins in a rat embryo fibroblast cell line. Virus Res. **6:**345–361.
4. **Bolen, J. B., V. Deseau, J. O Shaughnessy, and S. Amini.** 1987. Analysis of middle tumor antigen and pp60$^{c\text{-}src}$ interactions in polyomavirus-transformed rat cells. J. Virol. **61:**3299–3305.
5. **Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge.** 1984. Enhancement of cellular src gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. Cell **38:**767–777.
6. **Campbell, K. S., E. Ogris, B. Burke, W. Su, K. R. Auger, B. J. Druker, B. S. Schaffhausen, T. M. Roberts, and D. C. Pallas.** 1994. Polyoma middle tumor antigen interacts with SHC protein via the NPTY (Asn-Pro-Thr-Tyr) motif in middle tumor antigen. Proc. Natl. Acad. Sci. USA **91:**6344–6348.
7. **Cartwright, C. A., P. L. Kaplan, J. A. Cooper, T. Hunter, and W. Eckhart.** 1986. Altered sites of tyrosine phosphorylation in pp60$^{c\text{-}src}$ associated with polyomavirus middle tumor antigen. Mol. Cell. Biol. **6:**1562–1570.
8. **Cheng, S. H., P. C. Espino, J. Marshall, R. Harvey, and A. E. Smith.** 1990. Stoichiometry of cellular and viral components in the polyomavirus middle-T antigen-tyrosine kinase complex. Mol. Cell. Biol. **10:**5569–5574.
9. **Cheng, S. H., R. Harvey, P. C. Espino, K. Semba, T. Yamamoto, K. Toyoshima, and A. E. Smith.** 1988. Peptide antibodies to the human c-fyn gene product demonstrate pp59$^{c\text{-}fyn}$ is capable of complex formation with the middle-T antigen of polyomavirus. EMBO J. **7:**3845–3855.
10. **Cheng, S. H., H. Piwnica-Worms, R. W. Harvey, T. M. Roberts, and A. E. Smith.** 1988. The carboxy terminus of pp60$^{c\text{-}src}$ is a regulatory domain and is involved in complex formation with the middle-T antigen of polyomavirus. Mol. Cell. Biol. **8:**1736–1747.
11. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. W. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252:**1002–1106.
12. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr527 is phosphorylated in pp60$^{c\text{-}src}$: implications for regulation. Science **231:**1431–1434.
13. **Courtneidge, S. A.** 1985. Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. **4:**1471–1477.
14. **Courtneidge, S. A., S. Fumagalli, M. Koegl, G. Superti Furga, and S. G. M. Twamley.** 1993. The Src family of protein tyrosine kinases: regulation and functions. Dev. Suppl. **1993:**57–64.
15. **Courtneidge, S. A., L. Goutebroze, A. Cartwright, A. Heber, S. Scherneck, and J. Feunteun.** 1991. Identification and characterization of the hamster polyomavirus middle T antigen. J. Virol. **65:**3301–3308.
16. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-src cellular gene. Nature (London) **303:**435–439.
17. **Cuzin, F.** 1984. The polyoma virus oncogenes. Biochim. Biophys. Acta **781:**193–204.
18. **Dilworth, S. M., C. E. Brewster, M. D. Jones, L. Lanfrancone, G. Pelicci, and P. G. Pelicci.** 1994. Transformation by polyoma virus middle T-antigen involves the binding and tyrosine phosphorylation of Shc. Nature (London) **367:**87–90.
19. **Dilworth, S. M., and V. P. Horner.** 1993. Novel monoclonal antibodies that differentiate between the binding of pp60$^{c\text{-}src}$ or protein phosphatase 2A by polyomavirus middle T antigen. J. Virol. **67:**2235–2244.
20. **Eck, M. J., S. K. Atwell, S. E. Shoelson, and S. C. Harrison.** 1994. Structure of the regulatory domains of the Src-family tyrosine kinase Lck. Nature (London) **368:**764–769.
21. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
22. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:**61–68.
23. **Kaech, S., L. Covic, A. Wyss, and K. Ballmer-Hofer.** 1991. Association of p60$^{c\text{-}src}$ with polyoma virus middle-T antigen abrogating mitosis-specific activation. Nature (London) **350:**431–433.
24. **Kaplan, J. M., H. E. Varmus, and J. M. Bishop.** 1990. The Src protein contains multiple domains for specific attachment to membranes. Mol. Cell. Biol. **10:**1000–1009.
25. **Kornbluth, S., M. Sudol, and H. Hanafusa.** 1987. Association of the polyomavirus middle-T antigen with c-yes protein. Nature (London) **325:**171–173.
26. **Kypta, R. M., A. Hemming, and S. A. Courtneidge.** 1988. Identification and characterization of p59^{fyn} (a src-like protein tyrosine kinase) in normal and polyoma virus transformed cells. EMBO J. **7:**3837–3844.
27. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48:**352–360.
28. **Liu, X., S. R. Brodeur, G. Gish, S. Zhou, L. C. Cantley, A. P. Laudano, and T. Pawson.** 1993. Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. Oncogene **8:**1119–1126.
29. **Lock, P., S. Ralph, E. Stanley, I. Boulet, R. Ramsay, and A. R. Dunn.** 1991. Two isoforms of murine *hck*, generated by utilization of alternative translational initiation codons, exhibit different patterns of subcellular localization. Mol. Cell. Biol. **11:**4363–4370.

30. **Louie, R. R., C. S. King, A. Macauley, J. D. Marth, R. M. Perlmutter, W. Eckhart, and J. A. Cooper.** 1988. p56^{lck} protein-tyrosine kinase is cytoskeletal and does not bind to polyomavirus middle T antigen. J. Virol. **62:**4673–4679.
31. **Muser, J., S. Kaech, C. Moroni, and K. Ballmer-Hofer.** 1989. Stimulation of pp60$^{c\text{-}src}$ kinase activity in FDC-P1 cells by polyoma middle-T antigen and hematopoietic growth factors. Oncogene **4:**1433–1439.
32. **Okada, M., S. Nada, Y. Yamanashi, T. Yamamoto, and H. Nakagawa.** 1991. CSK: a protein-tyrosine kinase involved in regulation of src family kinases. J. Biol. Chem. **266:**24249–24252.
33. **Pallas, D. C., L. K. Shahrik, B. L. Martin, S. Jaspers, T. B. Miller, D. L. Brautigan, and T. M. Roberts.** 1990. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell **60:**167–176.
34. **Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons.** 1984. Monoclonal antibodies to Rous sarcoma virus pp60^{src} react with enzymatically active cellular pp60^{src} of avian and mammalian origin. J. Virol. **51:**272–282.
35. **Pawson, T.** 1994. SH2 and SH3 domains in signal transduction. Adv. Cancer Res. **64:**87–110.
36. **Pérez, L., A. Paasinen, B. Schnierle, S. Kaech, M. Senften, and K. Ballmer-Hofer.** 1993. Mitosis-specific phosphorylation of polyomavirus middle-sized tumor antigen and its role during cell transformation. Proc. Natl. Acad. Sci. USA **90:**8113–8117.
37. **Roussel, R. R., S. R. Brodeur, D. Shalloway, and A. P. Laudano.** 1991. Selective binding of activated pp60$^{c\text{-}src}$ by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60$^{c\text{-}src}$. Proc. Natl. Acad. Sci. USA **88:**10696–10700.
38. **Schaffhausen, B., and T. L. Benjamin.** 1981. Comparison of phosphorylation of two polyoma virus middle T antigens in vivo and in vitro. J. Virol. **40:**184–196.
39. **Seidel-Dugan, C., B. E. Meyer, S. M. Thomas, and J. S. Brugge.** 1992. Effects of SH2 and SH3 deletions on the functional activities of wild-type and transforming variants of c-Src. Mol. Cell. Biol. **12:**1835–1845.
40. **Su, W., W. Liu, B. S. Schaffhausen, and T. M. Roberts.** 1995. Association of polyomavirus middle tumor antigen with phospholipase C-gamma 1. J. Biol. Chem. **270:**12331–12334.
41. **Superti Furga, G., S. Fumagalli, M. Koegl, S. A. Courtneidge, and G. Draetta.** 1993. Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src. EMBO J. **12:**2625–2634.
42. **Thomas, J. E., A. Aguzzi, P. Soriano, E. F. Wagner, and J. S. Brugge.** 1993. Induction of tumor formation and cell transformation by polyoma middle T antigen in the absence of Src. Oncogene **8:**2521–2529.
43. **Tooze, J.** 1980. DNA tumor viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Treisman, R., U. Novak, J. Favaloro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. Nature (London) **292:**595–600.
45. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrosine kinase p56^{lck} with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell **60:**755–765.
46. **Walter, G., R. Ruediger, C. Slaughter, and M. Mumby.** 1990. Association of protein phosphatase 2A with polyoma virus medium tumor antigen. Proc. Natl. Acad. Sci. USA **87:**2521–2525.
47. **Whitman, M., D. R. Kaplan, B. S. Schaffhausen, L. Cantley, and T. M. Roberts.** 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature (London) **315:**239–242.
48. **Ziegler, S. F., S. D. Levin, and R. M. Perlmutter.** 1989. Transformation of NIH 3T3 fibroblasts by an activated form of p59^{hck}. Mol. Cell. Biol. **9:**2724–2727.