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Genetic Variation of Herpesvirus Saimiri Subgroup A Transforming Protein and Its Association with Cellular src

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Herpesvirus saimiri strain 11 of subgroup A contains a gene called the saimiri transformation-associated protein, STP, which is not required for viral replication but is required for in vitro immortalization and for the lymphoma-inducing capacity of the virus. To assess the effects of sequence variation on STP function, *STP* genes from six subgroup A isolates were cloned and sequenced. Sequence comparisons revealed extensive amino acid substitutions within the central region, but the acidic amino terminus and the hydrophobic carboxyl terminus were well conserved. Amino acid identities varied from 73 to 99% among all two-way comparisons. The highly conserved YAEV/I motif at amino acid residues 115 to 118 was preceded by negatively charged glutamic acid residues and thus matched very well the consensus sequence for binding to SH2 domains of src family kinases. The STPs of these subgroup A strains were shown to associate with cellular src and to be an in vitro substrate for src kinase. Mutational analysis of STP-A11 showed that binding to src kinase required the tyrosine residue at 115, showing that YAEV/I is a likely binding motif for src. Also, tyrosine phosphorylation of STP-A11 by src led to subsequent binding to lck and fyn in vitro. Thus, the association of STP with src is likely to be important for T-cell transformation by subgroup A strains of herpesvirus saimiri.

Herpesvirus saimiri (HVS) belongs to the gamma subfamily of herpesviruses (*Gammaherpesvirinae*). Some of members of this group, e.g., Epstein-Barr virus, HVS, herpesvirus ateles, and herpesvirus sylvilagus, are capable of inducing lymphoproliferative disorders in natural or experimental hosts. Recently, Kaposi's sarcoma-associated herpesvirus has been shown to have high sequence homology to HVS (7, 8, 35, 41). HVS naturally infects squirrel monkeys (*Saimiri sciureus*), a common primate species of the South America rain forests, without any apparent disease association. Infection of marmosets, owl monkeys, and other species of New World primates results in rapidly progressing fulminant lymphomas, lymphosarcomas, and leukemias of T-cell origin (16, 20).

The HVS genome is composed of a linear double-stranded DNA ranging from 145 to 165 kb with an internal segment of unique L-DNA (36% G+C content) flanked by multiple direct repeats of 1.4-kb units of H-DNA (72% G+C content). HVS can be further divided into subgroups A, B, and C on the basis of the extent of DNA sequence divergence at the left end of L-DNA (28). Subgroups A and C are highly oncogenic and are able to immortalize common marmoset T lymphocytes, causing interleukin 2 (IL-2)-independent growth in vitro (13, 37). Subgroup C strains are further capable of immortalizing human, rabbit, and rhesus monkey lymphocytes into continuously proliferating T-cell lines (1, 3).

Mutational analyses have demonstrated that the leftmost open reading frame in the L-DNA of subgroup A strain 11 is not required for viral replication but is required for immortalization of common marmoset T lymphocytes in vitro and for lymphoma induction in vivo (10, 11, 25, 30). This open reading frame is termed *STP-A11*, for saimiri transformation-associated protein of subgroup A strain 11 (30). At a position and in an orientation equivalent to those of the *STP-A11* reading frame, highly oncogenic HVS subgroup C strain 488 was found to contain a distantly related reading frame termed *STP-C488* (4, 23). Although *STP-A11* and *STP-C488* are similarly located and oriented within the genomes of their respective herpesvirus strains, STP-A11 and STP-C488 display only limited sequence similarity (4, 23). Nonetheless, STP-A11 and STP-C488 seem to be organized similarly in terms of the presence and localization of basic structural elements. Both proteins are predicted to have a highly acidic amino terminus and collagen-like repeats in the central region. The primary amino acid sequence of STP-A11 has nine repeats of a collagen-like motif (Gly-X-Y, where X and/or Y is proline), and in STP-C488 it is directly repeated 18 times, comprising more than 50% of the protein (23). These collagen-like repeats are inferred to form a long, fibrous structure similar to cellular collagen, which may serve as a hinge to extend the active domain of STP to its site of action. The STP-A11 and STP-C488 proteins also contain a hydrophobic stretch at their carboxyl termini sufficient for a membrane-spanning domain (17, 23).

Both STPs have transforming and tumor-inducing activities independent of the rest of the herpesvirus genome (23). Specifically, *STP-C488* can transform Rat-1 cells, resulting in apparent loss of contact inhibition, formation of foci, growth at reduced serum concentrations, and formation of invasive tumors in nude mice (23). *STP-A11* has less potent transforming ability than *STP-C488*. Furthermore, transgenic mice expressing STP-A11 developed peripheral pleomorphic T-cell lymphomas, while transgenic mice expressing STP-C488 developed extensive epithelial cell tumors (26, 29). These studies suggested, therefore, that STP of subgroup A may have cellular targets that are distinct from those of STP of subgroup C.

Recently, the protein encoded by *STP-C488* has been identified and dissected in great detail (17–19). Disruption of overall acidity in the amino terminus and interruption of the col-

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lagen-like repeats in the central region induced the loss of transforming activity (19). The hydrophobic region at the carboxyl terminus was shown to be critical for membrane localization of STP-C488 (17, 19). Importantly, STP-C488 was shown to associate with cellular ras in transformed cells (21). This association of STP-C488 with ras activates the ras signaling pathway. Mutations that disrupt the association between STP-C488 and ras disrupt the transforming activity of STP-C488. Thus, STP-C488 is the first virus-encoded protein shown to achieve oncogenic transformation via association with cellular ras.

Despite the fact that STP-A11 was identified initially as an HVS transformation-associated protein, detailed analysis of the functional activity of STP-A11 has not been reported. To understand the structural and functional properties of STP of subgroup A, we analyzed the primary amino acid sequences of six different subgroup A isolates. Based on primary amino acid sequence analysis, we identified structural and functional elements unique to STP of HVS subgroup A, including a putative SH2-binding motif for src family kinases. We now show that STP of subgroup A associates with cellular src kinase by this putative SH2-binding motif and is phosphorylated by the associated src kinase in in vitro kinase assays. These results suggest that STP of subgroup A targets a cellular protein for virus-induced transformation that is different from that used by STP of subgroup C.

MATERIALS AND METHODS

Cell culture and transfection. COS-1 and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO Bethesda Research Laboratories, Gaithersburg, Md.). OMK 637 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. Sf9 insect cells were maintained at 27°C in Grace's medium containing 10% fetal calf serum, yeastolate, and lactalbumin. Puromycin-resistant NIH 3T3 cells were maintained in 5 μg of puromycin (Sigma) per ml. DNAs were introduced into cells by the DEAE-dextran transfection method for transient expression in COS-1 cells or the Ca-phosphate transfection method for stable expression in NIH 3T3 cells.

Virus isolation and molecular cloning of STP genes. To isolate virus, owl monkey kidney cells (OMK 637) were cocultivated with purified peripheral blood mononuclear cells from squirrel monkeys (12, 15). The coculture was kept for 2 to 3 weeks and monitored for the appearance of cytopathic changes. Virus pellets were obtained by centrifugation of 5 ml of cell-free supernatant from infected cell cultures. Virions were suspended in 0.1 ml of 50 mM Tris hydrochloride (pH 7.5)–10 mM EDTA–50 mM NaCl; proteinase K and sodium dodecyl sulfate (SDS) were added to final concentrations of 1 mg/ml and 1%, respectively. After overnight incubation at 65°C, the viral DNA was extracted once with buffer-saturated phenol and once with a chloroform-isoamyl alcohol mixture. Virion DNA was precipitated with 2.5 volumes of ethanol, pelleted by centrifugation, and suspended in Tris-EDTA buffer.

Purified virion DNAs from six different strains of HVS subgroup A were used for PCR amplification with the 5′ primer CGG AAT TCA TGG CAA GAG GTC TAG GTG A, which corresponds to the amino-terminal sequence of *STP-A11* at nucleotides 695 to 714 (30), and 3′ primer CGG AAT TCA TAA TTA CTA GCA TTA AAC C, which corresponds to the carboxyl-terminal sequence of *STP-A11* at nucleotides 251 to 235 (30). The primers used for PCR contain *Eco*RI sites (underlined) used for subsequent cloning. PCR-amplified DNA was digested with restriction enzyme *Eco*RI and subcloned into vector pBS (Stratagene, San Diego, Calif.). Both strands of the inserts were sequenced with Sequenase (United States Biochemicals, Cleveland, Ohio).

Immortalization of common marmoset lymphocytes. Immortalization of lymphocytes from common marmosets (*Callithrix jacchus*) was attempted in 25-cm^2 flasks and in 24-well Linbro tissue culture plates as described previously (13). Media contained 5 mg of β-mercaptoethanol per liter. Normally, immortalized lymphocytes were established in a month after infection and were cultured in the absence of exogenous IL-2.

Antibodies. Two synthetic peptides were used for production of antibodies in rabbits. Synthetic peptide 74, GDPQENDESNGDPP, corresponds to residues 8 through 21 of STP-A11, and synthetic peptide 75, DPPHNTDERSDGDD, corresponds to residues 19 through 32 of STP-A11. These peptides were synthesized with an additional cysteine at the amino terminus, which was used for coupling the peptide to keyhole limpet hemocyanin. Female New Zealand White rabbits were immunized subcutaneously with 100 μg of coupled synthetic peptides in Freund's complete adjuvant and then given two booster injections with the same antigens in incomplete adjuvant at 3-week intervals. Monoclonal antibody AU-1, recognizing the DTYRYI epitope from bovine papillomavirus L1 capsid protein, was purchased from Berkeley Antibody (Richmond, Calif.). Rabbit polyclonal anti-src antibody generated against a synthetic peptide was purchased from Santa Cruz Biotech (Santa Cruz, Calif.), and a monoclonal antibody to src was purchased from UBI (Lake Placid, N.Y.).

Plasmid constructions. Vector pFJ was derived from pSRα-0 (38) by introducing multicloning restriction enzyme sites at the *Hin*dIII site. DNA containing the *STP-A11* open reading frame was amplified from HVS strain A11 virion DNA by PCR with primers containing *Eco*RI and *Bam*HI recognition sequences at the ends. Amplified DNA was ligated into the *Eco*RI and *Bgl*II cloning sites of vector pFJ. For AU-1 tagging, 5′ primer CGC GGA TCC ATG GAC ACC TAT CGC TAT ATA GCA AGA GGT CTA GGT GAA GGA was used for PCR amplification (22). AU-1-tagged *STP-A11* DNAs were completely sequenced to verify 100% agreement with the original sequence. The chicken *src* gene was subcloned into vector pFJ. To generate a pcDNA3 expression vector containing *STP-A11* and *STP-OMI*, an *Eco*RI-*Xho*I fragment containing *STP-A11* or *STP-OMI* was subcloned into the *Eco*RI and *Xho*I sites of pcDNA3.

All mutations in *STP-A11* were generated by PCR with oligonucleotide-directed mutagenesis (14). To facilitate the mutagenesis, the *STP-A11* gene was subcloned into vector pSP72 (Promega Biotech, Madison, Wis.). Oligonucleotide mutant primers from complementary strands of *STP-A11* were synthesized for PCR. PCR cycling for mutagenesis was accomplished with a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, Conn.) under the following conditions: 30 cycles of 2 min at 50°C for annealing, 5 min at 72°C for polymerization, and 1 min at 94°C for denaturation. Each mutant form of *STP-A11* was completely sequenced to verify the presence of the mutation and the absence of any other changes. After confirmation of the sequence, DNA containing the desired *STP-A11* mutation was recloned into the *Eco*RI and *Bgl*II cloning sites of vector pFJ for gene expression.

Immunoprecipitation and immunoblotting. Cells were harvested and lysed with lysis buffer (0.3 M NaCl, 0.1% Nonidet P-40, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] buffer [pH 8.0]) or RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 7.5]) containing 0.1 mM Na_2VO_3, 1 mM NaF, and protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and bestatin). Immunoprecipitated proteins from cleared cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography of the dried gel slabs (22). For protein immunoblots, polypeptides in cell lysates corresponding to 10^5 cells were resolved by SDS-PAGE and transferred to a nitrocellulose membrane filter. Immunoblot detection was performed with a 1:1,000 dilution of primary antibody by enhanced-chemiluminescence assay (Amersham).

Construction of recombinant baculoviruses. A *Bam*HI-*Eco*RI fragment containing the *STP-A11* gene was inserted into the *Bam*HI-*Eco*RI sites of baculovirus transfer vector pVL1393 (Pharmingen, San Diego, Calif.). Vector plasmids were cotransfected into Sf9 cells with linearized baculovirus DNA. Four days later, virus-containing supernatants were harvested. The recombinant baculovirus was amplified to obtain a high-titer stock solution. Sf9 cells infected with baculovirus were assayed for expression of recombinant protein by immunoblotting. Recombinant src baculovirus was kindly provided by Thomas Roberts (Dana Farber Cancer Center). For routine production of recombinant proteins, 10^6 cells were infected with 0.2 ml of each baculovirus supernatant and lysed 48 h postinfection with lysis buffer and cleared cell lysates were used for immunoprecipitations.

In Vitro kinase assays. For in vitro protein kinase assays, complexes prepared as described above were washed once more with kinase buffer and resuspended with 10 μl of the same buffer containing 5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear) for 15 min at room temperature. For some experiments, 5 μg of enolase was added as the substrate.

In vitro binding of GST fusion proteins to STP-A11. Purified glutathione *S*-transferase (GST) fusion proteins (5 μg) noncovalently coupled to glutathione-Sepharose beads were mixed with precleared COS-1 cell lysates, incubated for 2 h at 4°C, and washed four times in lysis buffer. Bound proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with an AU-1 antibody. GST fusion proteins were purchased from Santa Cruz Biotech.

RESULTS

Amino acid sequence analysis of STPs from six different strains of HVS subgroup A. Primary amino acid sequences of STPs from six different subgroup A strains (11, OMI, 254, 483, 197-B, and 494) were determined by sequencing PCR-amplified genes from virion DNA (Fig. 1). Amino acid substitutions, deletions, or insertions in STPs from different strains are shown by comparison with the STP-A11 sequence. Amino acid sequence identity of STP proteins among the subgroup A species varied from 73 to 99% (Table 1). STPs from strains OMI and 254-71 and STPs from strains 494-77 and 197-B showed 98.6 and 92.5% similarity, respectively. The STP from strain 11 showed the most divergence from those of the other five

TABLE 1. Matrix of amino acid identity of STP-A proteins among herpesvirus saimiri subgroup A strains

HVS strain	% Amino acid identity with STP-A from:				
	OMI	483-77	254-71	197-B	494-77
11	74.0	75.3	72.6	76.7	78.8
OMI		82.2	98.6	82.9	82.2
483-77			82.2	81.5	81.5
254-71				81.5	80.8
197-B					92.5

TABLE 2. Structural motifs of STP-A and in vitro immortalization of HVS subgroup A isolates

HVS strain	In vitro immortalization	src SH2-binding motif	Zinc finger motif	No. of G-X-Y motifs[a]	No. of serine residues[b]
11	+	+	+	9	6
OMI	+	+	+	7	11
483-77	NT[c]	+	−	7	10
254-71	+	+	−	7	11
197-B	NT	+	+	7	9
494-77	+	+	+	7	10

[a] Within the first 85 amino acids.
[b] At amino acids 78 to 92.
[c] NT, not tested.

strains. It showed only 72.65 to 78.8% similarity to the STPs of the other strains.

Based on the primary amino acid sequence, the presence of potential structural motifs was assessed (Table 2). Three basic structural motifs (an acidic amino terminus, a collagen-like repeat, and a hydrophobic carboxyl terminus) were identified on the basis of previous analysis of STP-C488 (19, 23). The acidic amino terminus from amino acids 1 to 34 and the hydrophobic stretch at the carboxyl terminus from amino acids 136 to 164 appeared to be highly conserved in all of the six strains that were examined. The collagen-like repeats (G-X-Y, where X and/or Y is proline) are located within the first 85 amino acids, but they are not directly repeated as they are in STP-C488. However, seven to nine repeats were consistently found in all of the strains. In addition, close inspection of the amino acid sequence revealed other structural motifs which are not present in the STP of subgroup C. First, a serine-rich region was found over a 15-amino-acid stretch from amino acid positions 78 to 92. The number of serine residues in this region varied from 6 to 11 among the strains. Secondly, a potential Zn finger motif was found at amino acid positions 120 to 135. However, this putative Zn finger motif, His-X_2-His-X_6-Cys-X_2-Cys, was not conserved in strains 254-71 and 483-77. Finally, a possible SH2-binding motif for src-related kinases at amino acid positions 111 to 118 was found to be highly conserved in all of the strains. SH2 domains generally contain about 150 amino acids and directly recognize phosphotyrosine. Systematic searches for optimal sequences for SH2 domains have found that individual members of SH2-containing proteins select unique tyrosine-containing sequences for binding (36). As shown in Fig. 1, the STPs of all of the strains contained the highly conserved YAEV/I sequence at amino acids 115 to 118, preceded by two negatively charged glutamic acid residues. This motif matches very well the consensus sequence for SH2 binding (EExxYEEV/I) to src family kinases (36).

To investigate the relevance of conserved and variant sequences for transforming activity, HVS subgroup A strains 11, OMI, 254-71, and 494-77 were examined for in vitro primary T-cell immortalization. All four strains were capable of immortalizing common marmoset T cells, causing IL-2-independent cell growth (Table 2). This suggests that sequence variations in the STP of these strains have no effect on HVS transforming ability.

Identification of STP of subgroup A strain 11. To demonstrate expression of the *STP-A11* open reading frame, an expression vector containing *STP-A11* was constructed in plasmid pFJ containing the SRα-0 promoter (38) and expressed in COS-1 cells. To generate a specific antibody against STP-A11, two peptides corresponding to amino acid residues 8 through 21 and 19 through 32 of the predicted sequence were synthesized, coupled via an additional amino-terminal cysteine to keyhole limpet hemocyanin, and used to inoculate rabbits. As shown in Fig. 2A, a mixture of both peptide antibodies immunoprecipitated a protein having an apparent molecular size of

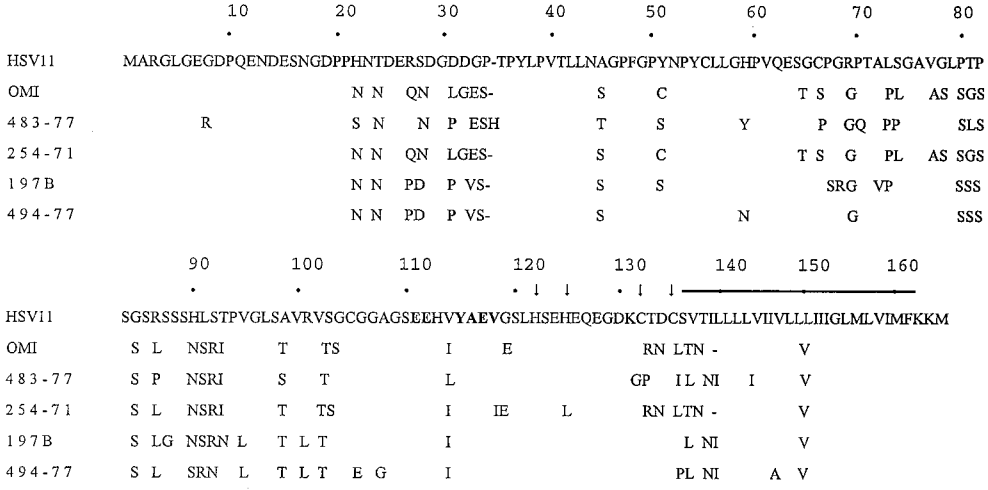

FIG. 1. Primary amino acid sequence analysis of STPs from HVS subgroup A strains. DNAs representing STPs from five different species of HVS subgroup A (OMI, 483-77, 254-71, 197-B, and 494-77) were amplified from viral DNAs by using PCR primers specific for the first 7 amino acids and the last 11 carboxyl-terminal amino acids. Individual PCR products were sequenced and aligned with the STP sequence of subgroup A strain 11 as described previously (30). Boldfaced amino acids indicate the putative SH2-binding motif, and arrows indicate the putative Zn finger motif. The bar above the sequence shows the hydrophobic region.

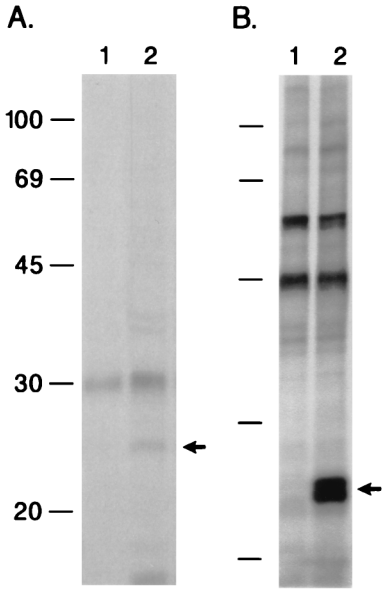

FIG. 2. Identification of STP-A11 protein. Immunoprecipitation of ^{35}S-labeled STP-A11 protein by rabbit antibodies against synthetic peptides 74 and 75 (A) or AU-1 antibody (B). After immunoprecipitation, proteins were separated in SDS-PAGE. Lanes: 1, COS-1 cells transfected with pFJ; 2, COS-1 cells transfected with pFJ-STP-A11 (A) or pFJ-AU1-STP-A11 (B). The arrows indicate the STP-A11 protein. The molecular size markers used were lysozyme (14 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (69 kDa), phosphorylase *b* (97.4 kDa), and myosin (200 kDa).

26 kDa only from COS-1 cell lysates expressing STP-A11. To facilitate the immunoprecipitation, the *STP-A11* gene was tagged with an AU-1 epitope at the amino terminus. The AU-1 antibody also reacted with radioactive protein having an apparent molecular size of 26 kDa upon immunoprecipitation from COS-1 cells transfected with the AU1-tagged *STP-A11* expression vector (Fig. 2B). No such protein was detected in control cells lacking the *STP-A11* or *AU1-STP-A11* gene in COS-1 cells. While the molecular mass of STP-A11 predicted by the DNA sequence is 16 kDa, the protein migrated at 26 kDa on SDS-PAGE. This abnormal migration is similar to that previously described for STP-C488 (17).

Association of STP-A11 with src. As described above, the STPs of all of the strains contained a highly conserved putative SH2-binding motif for src family kinases. To investigate the possible association of STP-A11 with src family kinases, COS-1 cells were cotransfected with an expression vector for STP-A11 and tyrosine kinase src, lck, or fyn. To facilitate immunoprecipitation, AU-1-tagged STP-A11 was used for expression in COS-1 cells. After cotransfection, cell lysates were used for immunoprecipitation with an anti-AU-1 antibody. The immunoprecipitates were then resolved by SDS-PAGE after in vitro kinase reaction with [γ-^{32}P]ATP as described in Materials and Methods. This analysis showed that src specifically associated with and phosphorylated STP-A11 in vitro, while lck or fyn did not do so detectably (Fig. 3A). Also, a 60-kDa phosphorylated protein which comigrated with the src protein on SDS-PAGE was detected in STP-A11 complexes from cell lysates containing STP-A11 and src. To show that the 60-kDa phosphorylated protein associated with STP-A11 was indeed src, polypeptides

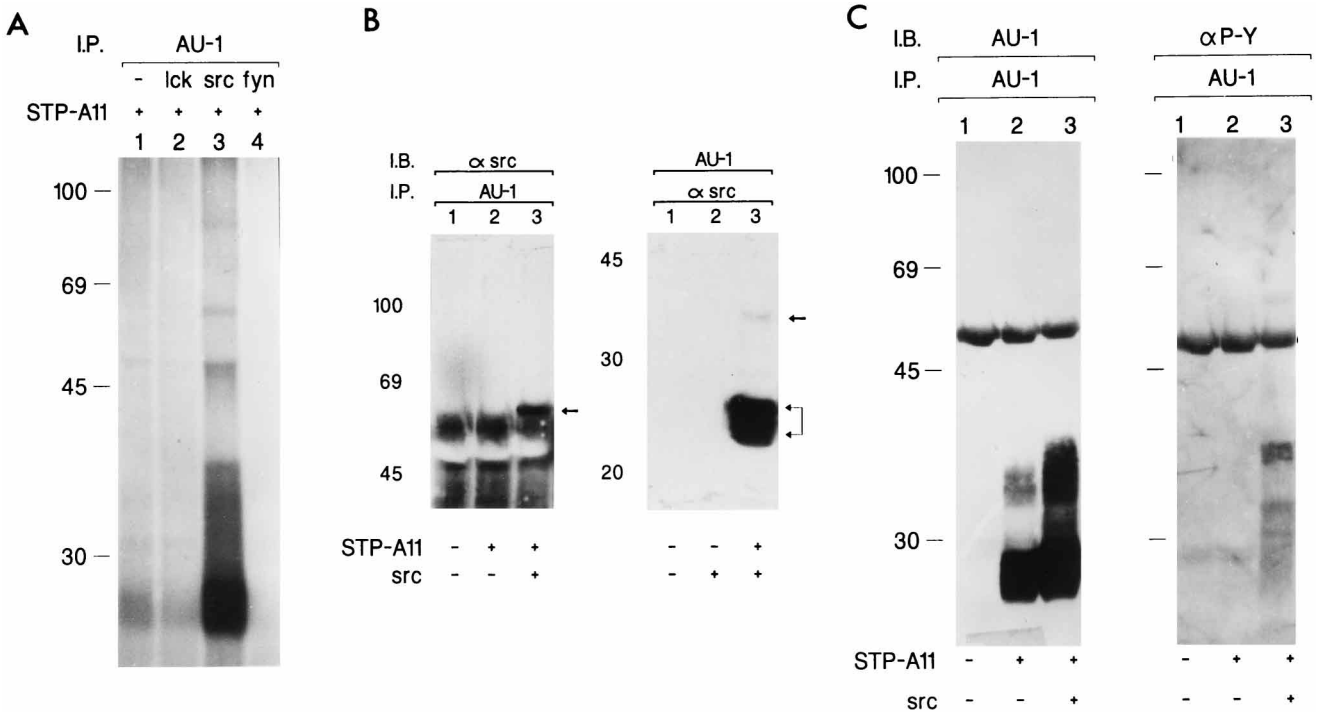

FIG. 3. Association of STP-A11 with src in COS-1 cells. (A) Formation of complexes between STP-A11 and src in COS-1 cells. COS-1 cells were transfected with pFJ-AU1-STP-A11 along with pFJ (lane 1), pFJ-lck (lane 2), pFJ-src (lane 3), or pFJ-fyn (lane 4). Cell lysates were used for immunoprecipitation (I.P.) with an AU-1 antibody. AU-1 immune complexes were used for in vitro kinase reaction with [γ-^{32}P]ATP. ^{32}P-labeled products were separated by SDS-PAGE, and the gel was exposed to X-ray film. (B) Identification of association between STP-A11 and src in COS-1 cells. I.B., immunoblot. (C) Slow migration of STP-A11 on SDS-PAGE induced by tyrosine phosphorylations. Conditions of transfection into COS-1 cells are indicated at the bottom. After transfection, cells were lysed with lysis buffer. Proteins were prepared for SDS-PAGE by immunoprecipitation with the indicated antibody. Precipitated proteins were detected by immunoblot analysis with an AU-1 or antiphosphotyrosine (P-Y) antibody. The 55-kDa band is the heavy chain of immunoglobulin.

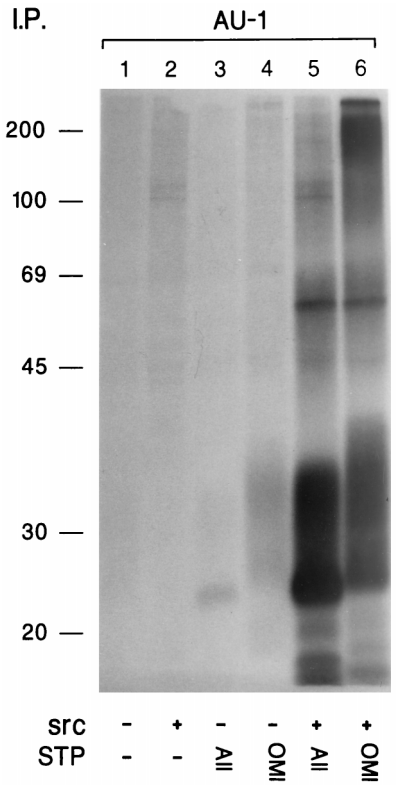

FIG. 4. Association of src with STP-OMI in COS-1 cells. COS-1 cell lysates were used for immunoprecipitation (I.P.) with an AU-1 antibody. AU-1 immune complexes were used for an in vitro kinase reaction with [γ-^{32}P]ATP. ^{32}P-labeled products were separated by SDS-PAGE, and the gel was exposed to X-ray film. Lanes: 1, COS-1 cells transfected with pFJ; 2, COS-1 cells transfected with pFJ-src; 3, COS-1 cells transfected with pFJ-AU1-STP-A11; 4, COS-1 cells transfected with pFJ-AU1-STP-OMI; 5, COS-1 cells transfected with pFJ-AU1-STP-A11 and pFJ-src; 6, COS-1 cells transfected with pFJ-AU1-STP-OMI and pFJ-src.

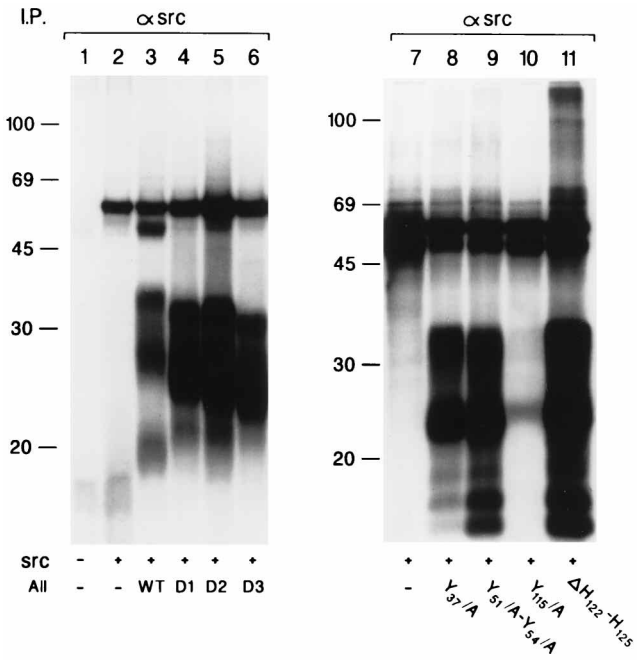

FIG. 5. Identification of the binding element of STP-A11 for src association. COS-1 cell lysates were recovered from transfections as indicated at the bottom. Anti-src antibody immunoprecipitates were subjected to an in vitro kinase reaction with ^{32}P-[γ-^{32}P]ATP and resolved by SDS-PAGE.

present in AU-1 immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with an anti-src antibody. The 60-kDa protein associated with STP-A11 was indeed detected by the anti-src antibody (Fig. 3B). Conversely, polypeptides present in src immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with the AU-1 antibody. The 26-kDa protein associated with src was detected by the AU-1 antibody (Fig. 3B). These results demonstrate that STP-A11 specifically associates with src in COS-1 cells.

Interestingly, the STP-A11 protein after an in vitro kinase reaction, migrated with several different species on SDS-PAGE which ranged from 26 to 35 kDa. This suggested that tyrosine phosphorylation of STP-A11 by src kinase changed the SDS-PAGE migration rates. To investigate this, *STP-A11* was expressed alone or together with the *src* gene in COS-1 cells. Immunoprecipitates recovered by the AU-1 antibody from these cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with the AU-1 or antiphosphotyrosine antibody. When *STP-A11* was expressed alone in COS-1 cells, STP-A11 migrated mainly with an apparent molecular weight of 26 kDa on SDS-PAGE (Fig. 3C). When *STP-A11* was coexpressed with *src* in COS-1 cells, the appearance of slow-migrating species of STP-A11 which had an apparent molecular mass of 35 kDa was detected (Fig. 3C). These species with slow mobility were further identified as highly tyrosine-phosphorylated forms of STP-A11 by immunoblot analysis with an antiphosphotyrosine antibody (Fig. 3C). Thus, these tests demonstrated that STP-A11 associated with src and was phosphorylated in vitro by the associated src, and slow migration on SDS-PAGE was associated with tyrosine phosphorylation.

Association of STP-OMI with src. Next, we investigated whether src kinase was capable of associating with other strains of HVS subgroup A. To address this question, we examined complex formation of STP-OMI with src under conditions identical to those used with STP-A11 in COS-1 cells. The full-length *STP-OMI* gene was also modified to encode an *AU-1* epitope tag at the amino terminus and cloned into expression vector pFJ. After cotransfection, cell lysates were used for immunoprecipitation with an AU-1 antibody. The immunoprecipitates were then resolved by SDS-PAGE after an in vitro kinase reaction with [γ-^{32}P]ATP (Fig. 4). STP-OMI, like STP-A11, was found to associate with src and was phosphorylated in vitro by src. Autophosphorylated 60-kDa src was detected in immune complexes of both STPs (Fig. 4, lanes 5 and 6). Similar to what was seen with STP-A11 (Fig. 3A), slow-migrating species of STP-OMI were also detected when src kinase was coexpressed. Also, the weak phosphorylation of both STPs which was detected in Fig. 4, lanes 3 and 4, was likely induced by endogenous src. STP from HVS subgroup C strain 488 was not associated with src under the same conditions (data not shown). Taken together, these results demonstrate that the STP of subgroup A strain OMI, as well as that of strain 11, is capable of associating with src.

Identification of elements in STP-A11 required for binding to src. The SH2 and SH3 domains of src have been shown to be responsible for direct protein-protein interaction and to modulate cytoplasmic signaling downstream of src (2, 5, 32, 33, 36). The SH2 domain recognizes specific amino acid sequences containing phosphotyrosine (36), while the SH3 domain rec-

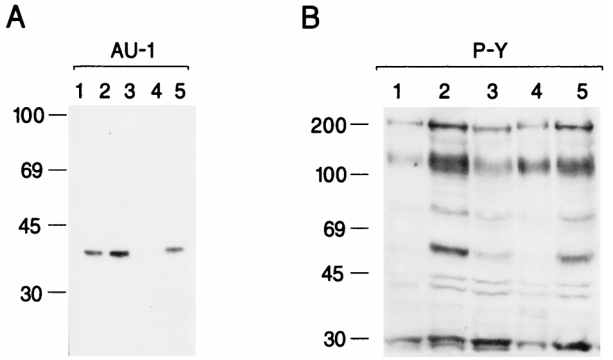

FIG. 6. Stable expression of STP-A11 and overall tyrosine phosphorylations in NIH 3T3 cells. Immunoblot of cell lysates from four cloned NIH 3T3 cells stably expressing STP-A11 with an AU-1 antibody (A) or an antiphosphotyrosine (P-Y) antibody (B). NIH 3T3 cells were transfected with pBabe-AU1-STP-A11 and cloned with puromycin at 5 μg/ml. Four stable clones were selected, and these cell lysates were resolved by SDS-PAGE. The expression of STP-A11 was determined by immunoblotting with the AU-1 antibody, and overall tyrosine phosphorylation was determined by immunoblotting with the antiphosphotyrosine antibody. Lanes: 1, untransfected NIH 3T3 cells; 2, NIH 3T3/STP-A11 clone 1; 3, NIH 3T3/STP-A11 clone 2; 4, NIH 3T3/STP-A11 clone 3; 5, NIH 3T3/STP-A11 clone 4.

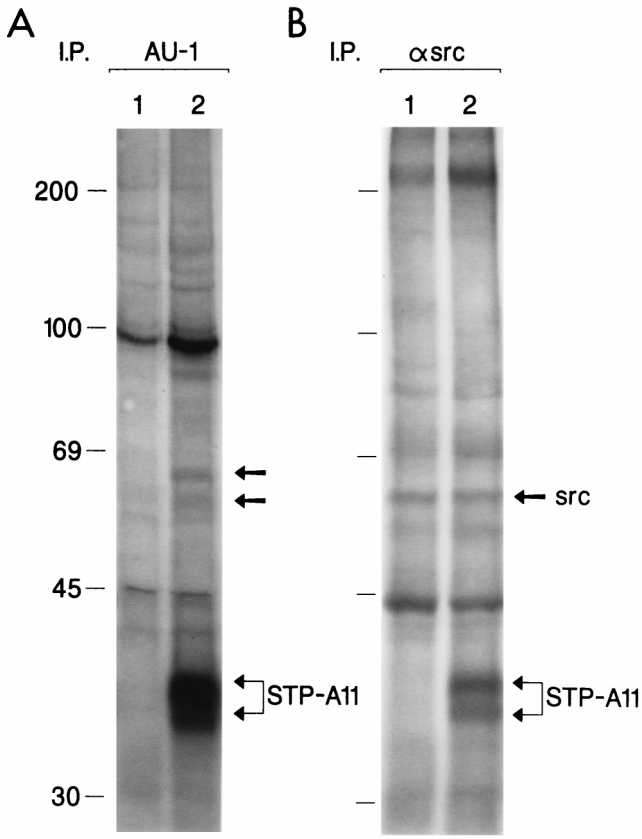

FIG. 7. Association of STP-A11 with cellular src in NIH 3T3 cells. Lysates from NIH 3T3 (lanes 1) and NIH 3T3/STP-A11 clone 2 (lanes 2) cells were used for immunoprecipitation (I.P.) with an AU-1 antibody (A) or an anti-src antibody (B). Immune complexes were subjected to an in vitro kinase reaction, and ^{32}P-labeled proteins were visualized by autoradiography.

ognizes a 9- or 10-amino-acid proline-rich sequence (34, 40). STP-A11 contains four tyrosine residues, at amino acid position 37, 51, 54, and 115 in its central region. The tyrosines at 37, 54, and 115 are completely conserved in all of the strains examined in this study. The amino acid stretch including Y_{115} features an SH2-binding motif for src family kinases, as described in Fig. 1 (36). To identify specific binding elements of STP-A11 required for association with src, each tyrosine residue was replaced with alanine by PCR-derived mutagenesis. Additionally, three serial deletion mutations in the amino-terminal acidic domain and a deletion mutation from H_{122} to H_{125} of the putative Zn finger motif were included for the binding assays. Complex formation of mutant forms of STP-A11 with src was assessed by coimmunoprecipitation. COS-1 cell lysates were used for immunoprecipitation with an anti-src antibody after cotransfection with mutant forms of STP-A11 and src. Immunoprecipitates were resolved by SDS-PAGE after an in vitro kinase reaction as described in Fig. 3A. The substitution of A for Y_{115} specifically disrupted the association between STP-A11 and src, while Y_{37}-to-A, Y_{51}-to-A and Y_{54}-to-A mutations had no effect on binding of STP-A11 to src (Fig. 5). Also, mutations D1, D2, and D3, containing 5-, 10-, and 15-amino-acid deletions from the amino-terminal acidic domain, respectively, and mutation ΔH_{122}-H_{125} at the putative Zn finger motif did not interfere with the ability of STP-A11 to bind to src (Fig. 5). All of these STP-A11 mutant proteins were expressed at equivalent levels in COS-1 cells (data not shown). Thus, in all of the mutant proteins studied, disruption of src binding was specific for the Y_{115} mutation. These data demonstrate that the amino acid sequence surrounding residue Y_{115} is critical for binding to src.

Association of STP-A11 with cellular src in stably transfected NIH 3T3 cells. To understand the role of STP-A11 association with cellular src, NIH 3T3 cells were used to establish stable cell lines expressing the *STP-A11* gene. After $CaPO_4$ transfection with recombinant expression vector pBabe-STP-A11, cells were selected by growth in medium containing puromycin at 5 μg/ml and, furthermore, puromycin-resistant cells were cloned into single-cell populations. To demonstrate expression of STP-A11 in these cells, cell lysates of four cloned cell lines were used for immunoblot analysis with AU-1 antibody. Three of four cloned cells expressed high levels of STP-A11, while clone 3 cells did not express STP-A11 (Fig. 6A). Expression of STP-A11 by the parental NIH 3T3 cells used as a negative control was not detected (Fig. 6A, lane 1). Interestingly, STP-A11 in stable NIH 3T3 cell lines had an apparent molecular mass of 35 kDa, which was shown to be the tyrosine-phosphorylated form of STP-A11 in Fig. 3C. The same cell lysates from these clones were examined for the level of overall tyrosine phosphorylation by using an antiphosphotyrosine antibody (Fig. 6B). Increased tyrosine phosphorylation of 60- and 85-kDa cellular proteins was detected only in cells expressing STP-A11. In contrast, the level of tyrosine phosphorylation of 60- and 85-kDa proteins was not increased in clone 3 cells, which did not express STP-A11 (Fig. 6B, lane 4). These results suggested that expression of STP-A11 induced the tyrosine phosphorylation of cellular 60- and 85-kDa proteins. To verify an association of STP-A11 with src, the src protein was precipitated with a rabbit polyclonal anti-src antibody from NIH 3T3 and NIH 3T3/STP-A11 clone 2 cell lysates prepared with 0.5% Nonidet P-40 detergent. Immunoprecipitates were incubated with [γ-^{32}P]ATP and analyzed by SDS-PAGE. Strong phosphorylations of 60-kDa src were observed from NIH 3T3 and NIH 3T3/STP-A11 cells, while a 35- to 37-kDa phosphorylated protein was detected only from NIH 3T3/STP-A11 cells (Fig. 7B). In parallel, immunoprecipitates derived from the same cell lysates by the AU-1 antibody were

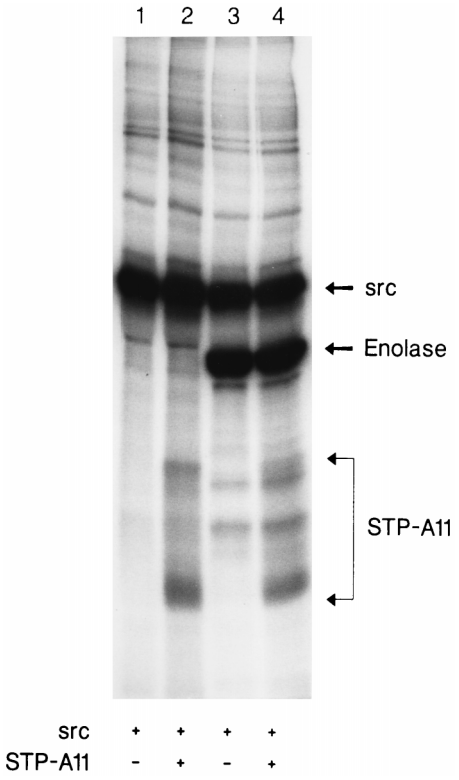

FIG. 8. No alteration of src enzymatic activity by association with STP-A11 in insect cells. Insect cells were infected with recombinant baculoviruses as indicated at the bottom. At 48 h after infection, cell lysates were used for immunoprecipitation with an anti-src antibody. src immune complexes from each cell lysate were assayed for kinase activity with (lanes 3 and 4) or without (lanes 1 and 2) 5 μg of enolase protein as the substrate. ^{32}P-labeled products were separated by SDS-PAGE, and the gel was exposed to X-ray film.

used for an in vitro kinase assay with [γ-^{32}P]ATP. Again, 35- to 37-kDa STP-A11 and 60-kDa src were detected only from NIH 3T3/STP-A11 cells (Fig. 7A). An additional 65-kDa phosphorylated cellular protein was detected in STP-A11 immune complexes (Fig. 7A, lane 2). Thus, STP-A11 associates with src not only upon transient expression in COS-1 cells but also upon stable expression in NIH 3T3 cells.

No alteration of src kinase activity caused by association with STP. It has been previously shown that one of the early region-encoded proteins of polyomavirus, middle-T antigen, forms a complex with and is phosphorylated by c-src. In addition, the src protein associated with middle-T antigen exhibits stimulated kinase activity (9). However, we did not detect increased autophosphorylation activity of src in NIH 3T3 cells expressing STP-A11 (Fig. 7). To examine further whether association of STP-A11 with src induces src kinase activity, a recombinant baculovirus system was employed. Insect cells were infected with recombinant src baculovirus alone or together with recombinant STP-A11 baculovirus. At 2 days after infection, cell lysates were used for immunoprecipitation with an anti-src antibody. In vitro kinase reactions were then carried out with 5 μg of enolase as the substrate (Fig. 8). Again, STP-A11 was associated with and phosphorylated by src in insect cells infected with recombinant STP-A11 and src baculoviruses (Fig. 8, lanes 2 and 4). However, association of STP with src did not induce the src autophosphorylation activity as well as its kinase activity toward enolase as the substrate (Fig. 8, lanes 3 and 4). Therefore, unlike polyomavirus middle-T antigen, STP-A11 does not appear to induce kinase activity of src by complex formation.

Consequences of STP-A11 phosphorylation for binding to lck and fyn. While the consensus sequence for binding to the SH2 domains of lck and fyn is very similar to that of src (36), STP-A11 was specifically associated with src but not detectably associated with lck or fyn in COS-1 cells (Fig. 3). However, modified forms of STP-A11 could still conceivably bind to lck or fyn. To test whether tyrosine phosphorylation of STP-A11 by src allows interaction with lck or fyn in vitro, *AU-1*-tagged *STP-A11* was expressed with or without *src* in COS-1 cells. Precleared cell lysates from COS-1 cells were mixed with GST fusion proteins containing the SH2-SH3 domain of lck or fyn. Polypeptides bound to GST fusion protein were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with an AU1 antibody. While the GST-SH2-SH3 fusion protein of lck or fyn did not bind to STP-A11 from COS-1

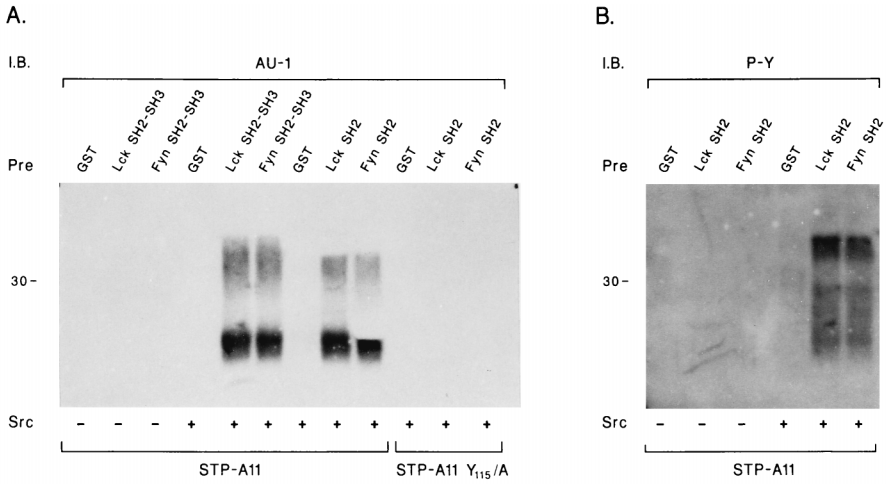

FIG. 9. Tyrosine phosphorylation of STP-A11 by src is sufficient to form a stable complex with SH2 of lck and fyn in vitro. COS-1 cell lysates were recovered from transfections as indicated at the bottom. Glutathione-Sepharose beads containing 5 μg of the GST fusion proteins indicated above the lanes were mixed with cell lysates and washed with lysis buffer. Associated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with an AU-1 antibody (A) or an anti-phosphotyrosine (P-Y) antibody (B). Pre and I.B. indicate precipitation and immunoblotting, respectively.

cells in the absence of *src* expression, it did bind to STP-A11 from COS-1 cells in which *src* was coexpressed (Fig. 9A). The same result was obtained with fusion proteins containing GST and the SH2 domains of both lck and fyn, showing that the SH2 domains of lck and fyn were responsible for binding to modified STP-A11. Mutant STP-A11 Y_{115}/A, which did not associate with src and was not phosphorylated by src, was also tested in in vitro binding assays with GST-SH2 made with lck or fyn. The GST-SH2 fusion protein made with lck or fyn did not bind to STP-A11 Y_{115}/A under the same conditions in which it bound to STP-A11 (Fig. 9A). Finally, immunoblotting with an antiphosphotyrosine antibody showed that the STP-A11 bound to the GST-SH2 made with lck or fyn was tyrosine phosphorylated (Fig. 9B). These results indicate that tyrosine phosphorylation of STP-A11 by src allowed the subsequent binding to lck and fyn in vitro.

DISCUSSION

STP-A11, an oncogene of the prototypic strain of HVS subgroup A, was identified initially by investigating natural deletion mutants which did not induce lymphoma in New World primates (10, 12). In this study, we examined the primary amino acid sequence of the STP proteins of six different isolates in subgroup A. The six STPs of subgroup A displayed conserved structural features that include an acidic amino terminus, a hydrophobic carboxyl terminus, and collagen-like repeats, which have been also found in STP from HVS subgroup C (Table 2). These basic structural elements of STP-C488 were shown previously to contribute to its transforming activity and its tightly membrane-anchored fibrous structure, which results in unusual mobility on SDS-PAGE (17, 19).

STP of subgroup A was found to contain three additional structural elements which are not found in STP of subgroup C strains (Table 2). First, a putative Zn finger motif is found in most of the strains of subgroup A examined. However, this Zn finger motif was not required for src binding. The serine-rich motif was found to be highly conserved in the central region of the STPs of all of the strains. The physiological function of this serine-rich motif remains to be identified. Recently, serine-rich motifs have been implicated in certain protein-protein interactions. Several signaling molecules, including glycoprotein Ib-IX, Raf, and Bcr kinase, interact directly with 14-3-3 protein through serine-rich motifs (31, 39). Glycoprotein Ib-IX, Raf, and Bcr kinase retain the short stretch of serine-rich segments, and their serine-rich domains are similarly clustered in a helical structure. The serine-rich motif of STP of subgroup A strains, therefore, may also be involved in interaction with yet-to-be-identified cellular proteins.

A possible binding motif for the SH2 domain of src kinase was identified around amino acid position 115 of STP of subgroup A, and this motif was highly conserved in all six of the strains examined in this study (Fig. 1). The mutation of Y_{115} to A specifically disrupted the association between STP-A11 and src. Thus, mutational analysis supports the putative SH2-binding motif of STP-A11 as the specific binding element for src. In addition, association with src seems to be a common characteristic of STPs from HVS subgroup A strains, as evidenced by complex formation of STP-A11 and STP-OMI with src.

src kinase phosphorylates STP of subgroup A in vitro in a manner that generates species with slower mobility on SDS-PAGE (Fig. 3). Recently, Mayer et al. showed that the SH2 domain of nonreceptor tyrosine kinases binds with high affinity to tyrosine-phosphorylated target, leading to processive phosphorylation of other tyrosine residues in the target protein (27). This event may explain the appearance of several phosphorylated species of STP with slow mobility, representing multiple tyrosine-phosphorylated forms of STP. However, other types of modification may also be involved in the slow migration of STPs of subgroup A on SDS-PAGE.

Previously, we have shown that STP of subgroup C strain 488 associates with cellular ras and its association with ras activates the cellular ras signaling pathway (21). In contrast, we have found that STP-A11 does not associate with cellular ras (unpublished results). Instead, we show that STP of subgroup A associates with src via a YxxV/I sequence with the characteristic of an SH2-binding motif. Thus, the STPs from HVS subgroup A and C strains appear to employ different cellular targets for virus-induced transformation. These differences in cellular targets are associated with different pathological activities in transgenic mice. STP-A11 induces peripheral pleomorphic T-cell lymphomas in transgenic mice, while STP-C488 induces epithelial tumors. Thus, the *STP-A* and *STP-C* oncogenes show a marked difference in the cellular partners used for oncogenic transformation despite a clear similarity at the genetic level. However, STPs of both subgroups A and C target signaling molecules proximal to the membrane, which can amplify signals to downstream molecules.

src, the first retroviral oncogene to be discovered, encodes one of the membrane-bound nonreceptor tyrosine kinases (9). Since its identification, numerous proteins have been identified as downstream targets and substrates for src (9, 24). Middle-T antigen, one of the polyomavirus gene products, initiates and maintains transformation of established cell lines by association with cellular src (9). In addition, complex formation of middle-T antigen with src stimulates kinase activity by stabilizing a form of src that is not phosphorylated at tyrosine 527, which is thought to regulate src kinase activity negatively. Stimulation of src kinase by complex formation has been shown to be directly involved in the oncogenicity of middle-T antigen (6, 9). However, unlike polyomavirus middle-T antigen, association of STP-A11 with src did not increase src enzymatic activity. This suggests that mechanisms other than activation of src kinase enzymatic activity are involved in the transforming activity of group A STPs. The association between STP-A11 and src may, however, enhance sequential interactions of STP-A11 with other cellular signaling molecules. The tyrosine phosphorylation of STP-A11 by src could allow subsequent binding to the SH2 domain of lck or fyn and the resultant downstream signals to trigger cell division. Our demonstration of lck and fyn association with src-phosphorylated STP-A is consistent with this sequential model for the transforming activity of STP-A.

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