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Association of the Viral Oncoprotein STP-C488 with Cellular ras

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The *STP-C488* oncogene of herpesvirus saimiri has transforming activity independent of the rest of the viral genome. We now demonstrate that STP-C488 associates with cellular ras in transformed cells. Mutations that disrupted this association with ras disrupted the transforming ability of the *STP-C488* oncogene. Binding assays showed that STP-C488 was capable of competing with raf-1 for binding to ras. Expression of STP-C488 activated the ras signaling pathway as evidenced by a two- to fourfold increase in the ratio of ras-GTP to ras-GDP and by the constitutive activation of mitogen-activated protein kinase. Consistent with an activation of signaling through ras, STP-C488 expression induced ras-dependent neurite outgrowth in PC12 cells. STP-C488 is the first virus-encoded protein shown to achieve oncogenic transformation via association with cellular ras.

Herpesvirus saimiri (HVS), a member of the γ2 group of herpesviruses, naturally infects squirrel monkeys (*Saimiri sciureus*) of South America. HVS persists in T lymphocytes of the natural host without any apparent disease, but infection of other species of New World primates results in fulminant lymphomas, lymphosarcomas, and leukemias of T-cell origin (19). A pronounced divergence among different strains of HVS has been localized to the left end of viral genomic DNA, and this has led to classification into three subgroups, A, B, and C (11, 30). Strains from subgroups A and C are highly oncogenic and are able to immortalize common marmoset T lymphocytes in vitro to interleukin 2-independent growth (12, 42). Subgroup C strains are further capable of immortalizing human and rhesus monkey lymphocytes into continuously proliferating T-cell lines (3; unpublished data). Human T-cell clones transformed by HVS strain C488 retain the ability to respond to their specific antigens with increased proliferation, cytokine production, and cell killing (2, 6, 32). Recently, sequences with high levels of homology to HVS have been found in AIDS-associated Kaposi's sarcoma (7).

Mutational analyses have demonstrated that the leftmost open reading frame in the genomic DNA of HVS 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, 12, 35). This open reading frame was termed *STP-A11* for saimiri transformation-associated protein of subgroup A strain 11 (35). At a position and orientation equivalent to those of the *STP-A11* reading frame, the highly oncogenic HVS strain C488 was found to contain a distantly related reading frame termed *STP-C488* (4, 22). Both *STP* genes have transforming and tumor-inducing activities independent of the rest of the herpesvirus genome (22). 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 (22). *STP-A11* was less potent than *STP-C488* in transforming ability. Transgenic mice expressing the *STP-C488* oncogene develop extensive epithelial cell tumors but not T-cell lymphomas (34). These results demonstrate a pleiotropic transforming capacity for the *STP-C488* oncogene and suggest that the specificity for T-cell transformation by HVS is likely to be determined by factors other than the *STP-C488* oncogene itself.

A polyclonal anti-STP-C488 antibody was used to identify the protein encoded by the *STP-C488* oncogene (16). The apparent molecular size (20 to 22 kDa) of STP-C488 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is considerably larger than that predicted from the DNA sequence (9.9 kDa). By using indirect immunofluorescence tests and subcellular fractionation, STP-C488 was found to be membrane bound, primarily in perinuclear compartments. These and other results suggested an unusual, membrane-associated, fibrous structure for the transforming STP-C488 oncoprotein (16). In addition, STP-C488 has been shown to be phosphorylated at a serine residue near the amino terminus (17). However, phosphorylation is not absolutely essential for transformation of Rat-1 cells by STP-C488 (17).

The STP-C488 protein is predicted to have three distinct domains. Domain I contains acidic amino acids at the amino terminus (pI = 4.2 for the amino-terminal 17 amino acids). Domain II of STP-C488 contains 18 uninterrupted repeats of collagen motifs, usually of the type Gly-Pro-Pro. Thus, collagen repeats represent 54 of the 102 amino acids of the predicted STP-C488 protein. STP-C488 is sensitive to treatment with collagenase (16), consistent with the 18 uninterrupted collagen-like repeats predicted by the DNA sequence (4). Finally, domain III of STP-C488 has a highly hydrophobic stretch at the carboxyl terminus that is sufficient for a membrane-spanning domain. Mutational analyses have shown that a net negative charge in the 17-amino-acid amino-terminal domain is important for protein structure and transformation (18). Increasing the net negative charge decreased electrophoretic mobility, and decreasing the net negative charge increased electrophoretic mobility. The three glutamic acid residues and overall acidity in this region are necessary to retain potent transforming activity. Interruption of the 18 collagen-like repeats in the central region also interrupted transforming activity. The hydrophobic region at the carboxyl terminus was shown to be important for membrane localization (18).

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Transforming proteins of tumor viruses exert their effects in many cases through specific interactions with cellular regulatory proteins. For example, human papillomavirus E7 and adenovirus E1A associate with Rb (13, 49), human papillomavirus E6 and adenovirus E1B associate with p53 (39, 48), and polyomavirus middle-T antigen associates with c-src (8). To understand the transforming mechanism of STP-C488, we attempted to identify cellular regulatory proteins associated with STP-C488 by using coimmunoprecipitation procedures. We now demonstrate that STP-C488 associates with cellular ras in transformed cells. This association of STP-C488 with ras activates the ras signaling pathway.

MATERIALS AND METHODS

Cell culture and transfection. Rat-1 and COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. PC12 cells were grown in DMEM supplemented with 5% fetal calf serum and 10% horse serum. Sf9 cells were maintained at 27°C in Grace's medium containing 10% fetal calf serum, yeastolate, and lactalbumin hydrolysate. Human and common marmoset T lymphocytes immortalized by HVS were grown in RPMI supplemented with 20% fetal calf serum. A DEAE-dextran transfection procedure was used for transient expression in COS-1 cells. $CaPO_4$ transfection was used to generate stable Rat-1 cell lines.

Antibodies. Affinity-purified Y13-259 and Y13-238 antiras antibodies conjugated with agarose beads were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.) and Oncogene Science (Uniondale, N.Y.). Rabbit polyclonal STP-C488 109 antibody has been described previously (16). Rabbit polyclonal ras, raf-1, and mitogen-activated protein (MAP) kinase antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.), Santa Cruz Biotechnology Inc., and Transduction Laboratories (Lexington, Ky.).

Plasmid constructions. The *STP-C488* gene was cloned into the pFJ vector derived from pSR-α (43) by introducing multicloning restriction enzyme sites at the *Hin*dIII site. To construct plasmid pFJ-GST-STP-C488, the 0.9-kbp *Ssp*I-*Eco*RI DNA fragment from the bacterial expression vector pGEX-2T-STP-C488 was inserted into the *Pvu*II and *Eco*RI sites of the pFJ vector.

Metabolic labeling, immunoprecipitation, and immunoblotting. COS-1 cells at 80 to 90% confluence in a 25-cm^2 dish were rinsed three times with phosphate-buffered saline, washed once with labeling medium (minimum essential medium minus methionine and cysteine and plus 10% dialyzed fetal calf serum), and incubated with 2 ml of the same medium containing 200 μCi of [^{35}S]methionine and [^{35}S]cysteine (New England Nuclear, Boston, Mass.) for 7 h. The cells were harvested and lysed with lysis buffer (0.15 M NaCl, 0.5% Nonidet P-40, and 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] buffer [pH 8.0]) containing 1 mM Na_2VO_3, 1 mM NaF, and protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, pepstatin, and bestatin). Precleared lysates from 10^7 cells were incubated for 2 h at 4°C with affinity-purified Y13-259 or Y13-238 antibody conjugated with agarose beads or with normal rat serum bound to protein A/G agarose beads. Washed immune complexes were separated by SDS–12% PAGE, transferred onto a nitrocellulose membrane, and reacted with anti-STP-C488 109 antibody.

Analysis of ratio of ras-bound GDP to ras-bound GTP. Rat-LXSN and Rat-STP-C488 cells or Sf9 insect cells infected with recombinant baculoviruses were incubated with labeling medium minus phosphate and plus 10% dialyzed fetal calf serum containing 0.5 mCi of $^{32}PO_4$ (New England Nuclear) per ml for 5 h. For thin-layer chromatography, Y13-259 and Y13-238 immunoprecipitates from $^{32}PO_4$-labeled cell lysates were washed with lysis buffer, and nucleotides were eluted with 2 mM EDTA–0.2% SDS–0.5 mM GTP–0.5 mM GDP at 68°C for 20 min. Eluted nucleotides were fractionated on polyethyleneimine-cellulose plates in 1.2 M ammonium formate–0.8 M HCl. Each separated nucleotide from autoradiography was quantitated with a Microtek color/gray scanner or a Bio-Rad Molecular Imager System (model GS-250).

Construction of recombinant baculoviruses. An *Eco*RI-*Bam*HI fragment containing the complete *STP-C488* gene was inserted into the *Eco*RI-*Bam*HI sites of the baculovirus transfer vector pVL1393, and a *Bam*HI fragment containing the N-*ras* gene was inserted into the *Bam*HI site of baculovirus transfer vector pVL1392 (Pharmigen, San Diego, Calif.). Vector plasmids were cotransfected into Sf9 cells with linearized baculovirus DNA. Four days later, virus-containing supernatants were harvested. Cells were infected with recombinant baculovirus to obtain a high-titer stock solution. Recombinant raf-1 baculovirus was kindly provided by Tom Roberts. Sf9 cells infected with baculovirus were assayed for expression of recombinant protein by labeling with [^{35}S]methionine or by immunoblotting. For routine production of recombinant proteins, 10^6 cells were infected with 0.2 ml of each baculovirus supernatant and lysed at 48 h postinfection with lysis buffer, and cleared cell lysates were used for immunoprecipitations.

Cell differentiation assay. Retroviral vectors LXSN and LXSN-STP-C488 were used to generate replication-defective viruses according to procedures previously described (31). PC12 cells were infected with replication-defective retrovirus and selected with 500 μg of G418 per ml. To examine the effect of expression of STP-C488 on the morphology of PC12 cells, cells were seeded at a density of 10^5 cells per 6-cm-diameter tissue culture dish in DMEM supplemented with 10% horse serum and 5% fetal calf serum or with only 5% fetal calf serum. The cells were examined over a 10-day period for cell differentiation.

In vitro kinase assays. For in vitro protein kinase assays, immune complexes prepared as described above were washed once more with kinase buffer (10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM unlabeled ATP, 20 mM Tris [pH 7.0]) and resuspended with 20 μl of the same buffer containing 5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear) and 5 μg of myelin basic protein as a substrate for 15 min at room temperature.

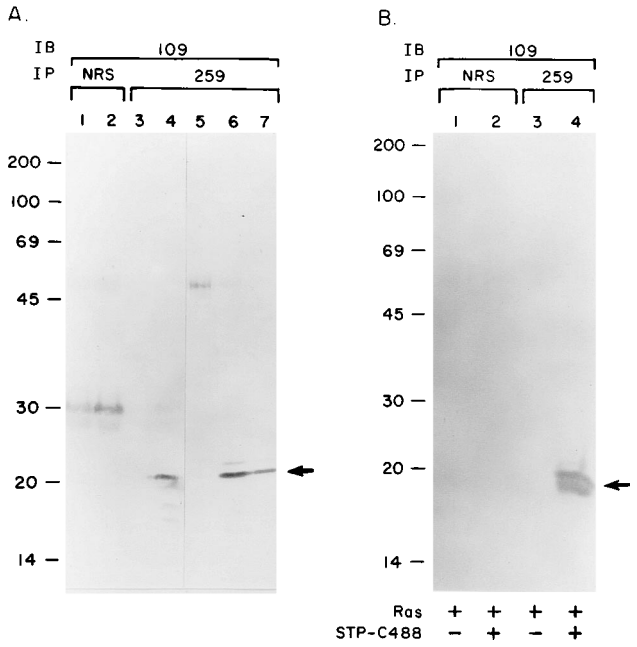

FIG. 1. Association of STP-C488 with cellular ras protein. (A) Coimmunoprecipitation of STP-C488 with cellular ras protein. Immunoprecipitates prepared with agarose beads coupled with purified Y13-259 antibody (lanes 3, 4, 5, 6, and 7) or normal rat serum (NRS) (lanes 1 and 2) were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-STP-C488 109 antibody as described previously (16). Lanes 1 and 3, Rat-LXSN; lanes 2 and 4, Rat-STP-C488; lane 5, Jurkat T cells; lane 6, common marmoset T lymphocytes transformed by HVS C488; lane 7, human T lymphocytes transformed by HVS C488. The 28- and 55-kDa proteins are immunoglobulin light and heavy chains, respectively. (B) Complex formation between ras and STP-C488 in Sf9 insect cells. Sf9 insect cells (10^6) were infected with recombinant baculovirus as indicated at the bottom and lysed at 48 h postinfection with lysis buffer. The same procedures were used for immunoprecipitation with Y13-259 antibody and immunoblot detection with 109 antibody. Arrows indicate STP-C488. IB and IP, antibodies used for immunoblotting and immunoprecipitation, respectively. The numbers on the left of each panel are molecular masses in kilodaltons.

RESULTS

STP-C488 forms complexes with ras. Antiras Y13-259 antibody was found to specifically coprecipitate STP-C488 from common marmoset and human T cells immortalized by HVS strain C488 and Rat-1 cells transformed by STP-C488 (Fig. 1A, lanes 4, 6, and 7). Normal rat serum as a control was negative for coprecipitation of STP-C488. While Y13-259 antibody reacted with denatured ras on immunoblots, it did not react with denatured STP-C488 on immunoblots (data not shown). Additionally, more than 20 different antibodies against cellular proteins, including Shc, Nck, PI-3 kinase, phospholipase C-γ, MAP kinase, epidermal growth factor receptor, platelet-derived growth factor receptor, fibroblast growth factor receptor, GTPase-activating protein (GAP), src, p62, and hsp70, were negative for coprecipitation of STP-C488 in similar assays (data not shown).

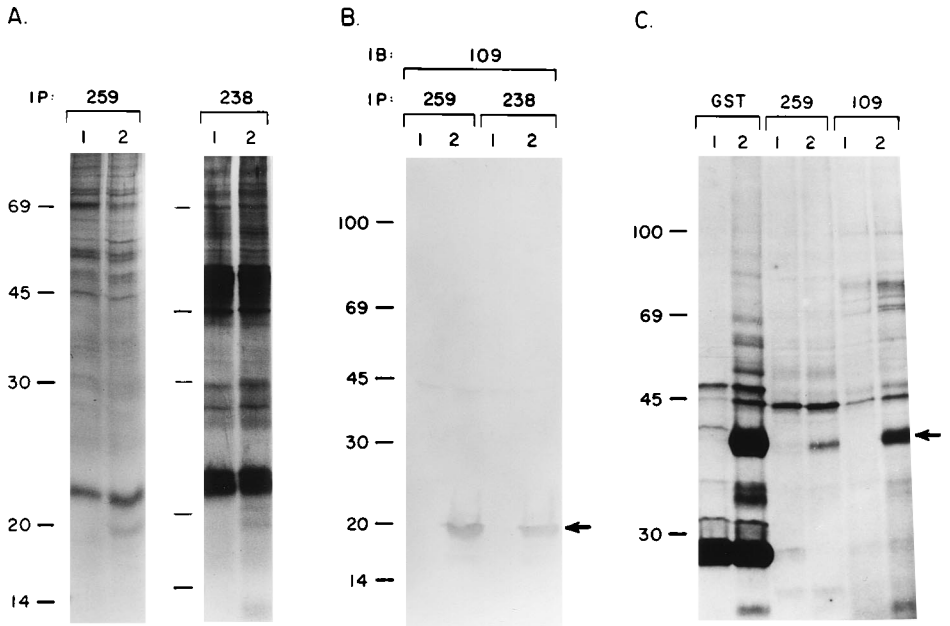

FIG. 2. Association of STP-C488 with cellular ras in COS-1 cells. (A) Immunoprecipitation of ^{35}S-labeled protein with Y13-259 and Y13-238 antiras antibodies. Lane 1, COS-1 cells transfected with pFJ vector; lane 2, COS-1 cells transfected with pFJ-STP-C488. (B) Identification of STP-C488 in anti-ras immune complexes by using anti-STP-C488 109 antibody. Lane 1, COS-1 cells transfected with pFJ vector; lane 2, COS-1 cells transfected with pFJ-STP-C488. (C) Association of ras with GST–STP-C488 fusion protein. Lane 1, COS-1 cells transfected with pFJ vector; lane 2, COS-1 cells transfected with pFJ-GST-STP-C488. STP-C488 expression vectors were constructed and transfected into COS-1 cells as described previously (9). After transfection, cells with (A and C) or without (B) radioactive labeling were lysed with lysis buffer. Proteins for SDS-PAGE were prepared by immunoprecipitation (IP) with the indicated antibody or glutathione-Sepharose column chromatography (GST). Precipitated proteins were detected by autoradiography of the dried gels (A and C) or by immunoblot (IB) analysis with anti-STP-C488 109 antibody (B). Arrows indicate the locations of STP-C488 (B) or GST–STP-C488 (C). The 27-kDa proteins in lanes 1 and 2 of panel C are cellular GST. The numbers on the left of each panel are molecular masses in kilodaltons.

To investigate further the association of STP-C488 with cellular ras, ^{35}S-labeled proteins from transfected COS-1 cells transiently expressing the *STP-C488* gene were incubated with anti-ras Y13-259 and Y13-238 antibodies. Anti-ras antibodies precipitated the 23-kDa ras protein as well as a 20-kDa protein from COS-1 cells expressing STP-C488 (Fig. 2A, lane 2). The 20-kDa protein was precipitated in COS-1 cells transfected with the pFJ-STP-C488 expression vector but not in COS-1 cells transfected with the pFJ vector alone (Fig. 2A, lane 1). The 20-kDa protein coprecipitated with ras by Y13-259 and Y13-238 antibodies reacted with anti-STP-C488 109 antibody, showing that it was indeed STP-C488 (Fig. 2B, lane 2). When transfection was performed with a pFJ vector expressing a glutathione-*S*-transferase (GST)–STP-C488 fusion gene, a major 40-kDa protein was coprecipitated (Fig. 2C); 40 kDa is the size of the GST–STP-C488 fusion protein as determined by immunoblot detection with anti-STP-C488 or anti-GST antibody. Finally, the recombinant baculovirus system was employed to show complex formation between ras and STP-C488. As shown in Fig. 1B, coinfection of Sf9 insect cells with recombinant ras and STP-C488 baculoviruses specifically resulted in the association of STP-C488 with ras. Thus, STP-C488 associates with cellular ras in T cells immortalized by HVS, in Rat-1 cells transformed by the *STP-C488* gene, in transfected COS-1 cells, and in insect cells.

Dependence of STP-C488 transforming activity upon an association with ras. The characterization of 25 different amino acid substitution and deletion modifications has recently identified particular amino acids required for transformation (18). These mutant forms of STP-C488 were examined for complex formation in Rat-1 cells. Transformation-competent mutants, A-2-G, S-3-G, T-14-A, and S-97-A, and transformation-incompetent mutants, I-11-K, E-12-G, and M-1, were employed for these assays. The M-1 mutant contains a 3-amino-acid insertion that interrupts the collagen domain (18). Rat-1 cells stably expressing these mutant forms of the *STP-C488* gene had been established previously by using a retroviral vector (16, 18). Mutant proteins were shown to be expressed at similar levels in Rat-1 cells (18) (Fig. 3C). Precleared lysates of these Rat-1 cells were used for coimmunoprecipitation of STP-C488 by anti-ras Y13-259 and Y13-238 antibodies. The transformation-incompetent STP-C488 mutants, I-11-K, E-12-G, and M-1, were not detected in Y13-238 and Y13-259 anti-ras immune complexes, while wild-type STP-C488 was readily detected under these conditions (Fig. 3A). In contrast, transformation-competent mutants, A-2-G, S-3-G, T-14-A, and S-97-A, were shown to be associated with cellular ras protein (Fig. 3B). Mutants A-2-G and S-3-G showed significant reductions of complex formation with ras (Fig. 3B). The S-3-G mutation eliminates the single serine phosphorylation site within STP-C488 (17). Although this phosphorylation site is not essential for transformation, the S-3-G mutant has reduced transforming activity in Rat-1 cells (17). The foci of cells transformed by the S-3-G mutant did not appear as dense or as deeply piled as those of cells transformed by the parental STP-C488 (17). These results provide strong evidence for the specificity of the vas–STP-C488 association and further suggest that the transforming activity of STP-C488 is dependent upon association with ras.

Competition between STP-C488 and raf-1 for binding to ras. It is known that raf-1 associates with ras in a GTP-dependent manner and functions as a direct downstream effector of ras in mammalian cells (27, 33, 45–47). We therefore examined whether STP-C488 affected the binding of raf-1 to ras protein.

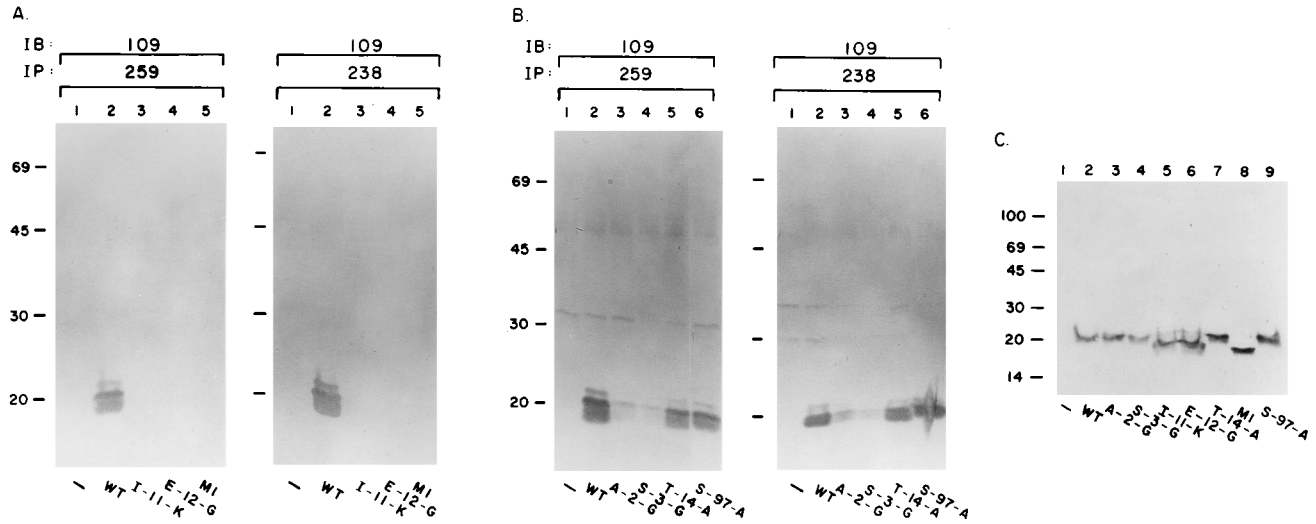

FIG. 3. Dependence of STP-C488 transforming activity on an association with cellular ras protein. (A) Absence of ras association with transformation-incompetent STP-C488 mutants. (B) ras association with transformation-competent STP-C488 mutants. (C) Immunoblot of whole-cell lysates. Rat-1 cells expressing wild-type (WT) or mutant forms of STP-C488 have been described previously (18). Mutant forms of STP-C488 are indicated at the bottom. Lysates of 10^7 cells were used for immunoprecipitation with Y13-259 or Y13-238 antibody. Immune complexes (A and B) or polypeptides in total cell lysates corresponding to 10^5 cells (C) were separated by SDS-PAGE, transferred onto nitrocellulose, and reacted with anti-STP-C488 109 antibody. IB, immunoblotting; IP, immunoprecipitation. The numbers on the left of each panel are molecular masses in kilodaltons.

Recombinant baculoviruses were used to express ras, raf-1, and STP-C488 proteins in insect cells. Insect cell lysates were mixed with affinity-purified Y13-238 antibody conjugated with agarose beads. Washed immune complexes were separated on SDS-PAGE, transferred onto a nitrocellulose membrane, and reacted with anti-STP-C488 109 antibody or anti-raf-1 antibody. Coinfection with recombinant ras and raf-1 or ras and STP-C488 baculoviruses resulted in ras–raf-1 and ras–STP-C488 complex formation (Fig. 4A). A parallel infection with all three recombinant baculoviruses showed that binding of raf-1 to ras was greatly reduced by the expression of STP-C488, while binding of STP-C488 to ras was not altered (Fig. 4A). Immunoblotting showed that similar amounts of raf-1, ras, and STP-C488 proteins were expressed in insect cells coinfected with these three recombinant baculoviruses (Fig. 4B). These results suggest that STP-C488 can compete with raf-1 for binding to ras, and they provide additional evidence for the specificity of the ras–STP-C488 association.

Activation of ras by the expression of STP-C488. ras cycles between an inactive GDP-bound conformation and an active GTP-bound conformation. To study whether cellular ras is activated as a component of signal transduction mediated by STP-C488 transformation, we measured the ratio of ras-bound GTP to ras-bound GDP in vivo in Rat-LXSN and Rat-1 cells transformed by STP-C488, by using polyethyleneimine-cellulose thin-layer chromatography. Quantitation of the indicated regions in Fig. 5A revealed that the relative ratio of ras-GTP to ras-GDP in Rat-1 cells transformed by STP-C488 was two- to fourfold higher than that in Rat-LXSN cells. Furthermore, we measured the ratio of ras-GTP to ras-GDP in insect cells infected with ras baculovirus alone or together with STP-C488 baculovirus. The relative ratio of ras-GTP to ras-GDP was increased greater than threefold by expression of STP-C488 in insect cells (Fig. 5B). Transformation through *erb-2/neu* or v-*src* has been also shown to increase the ratio of ras-GTP to ras-GDP by three- to fourfold (40). Thus, ras protein appears to be activated upon transformation with STP-C488.

Constitutive activation of MAP kinase activity by STP-C488. MAP kinases (Erks) are serine/threonine protein kinases that serve as a convergence point for diverse receptor-initiated signal events at the plasma membrane (36). MAP kinases have been shown to act downstream of ras and to be constitutively active in cells transformed by ras (37, 44, 50). To understand the consequences of ras activation by transformation with STP-C488, the activation of MAP kinase was investigated by $^{32}PO_4$ labeling, by immunoblot reactivity with phosphotyrosine antibody, and by measurement of kinase activity. As shown in Fig. 6, the level of in vivo phosphorylation of the 42-kDa MAP kinase was dramatically elevated in Rat-1 cells transformed by STP-C488; the tyrosine phosphorylation of the 42-kDa MAP kinase was also significantly higher in transformed cells than in Rat-LXSN cells. Since enhanced tyrosine phosphorylation of

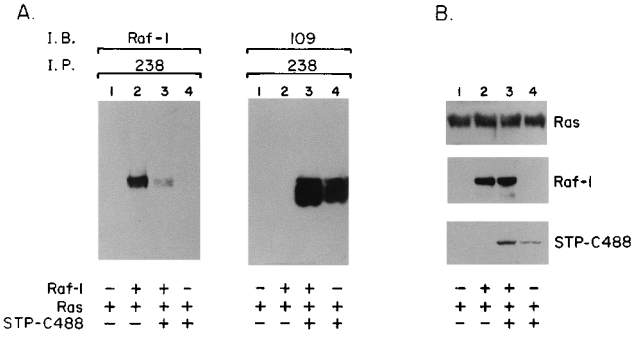

FIG. 4. Competition of STP-C488 with raf-1 for binding to ras. (A) Decrease of complex formation between raf-1 and ras by expression of STP-C488. Sf9 insect cells (10^6) were infected with recombinant baculoviruses as indicated at the bottom and lysed at 48 h postinfection with lysis buffer. ras complexes were precipitated with Y13-238 antibody coupled with agarose beads, resolved by SDS-PAGE, and transferred onto a nitrocellulose membrane. The top half of the membrane was reacted with anti-raf-1 antibody, and the bottom half of the membrane was reacted with anti-STP-C488 109 antibody. (B) Levels of ras, raf-1, and STP-C488 expression in insect cells. Whole-cell lysates of infected insect cells were used to determine the levels of ras, raf-1, and STP-C488 by immunoblotting with polyclonal anti-ras, anti-raf-1, and anti-STP-C488 109 antibodies. I.B., immunoblotting; I.P., immunoprecipitation.

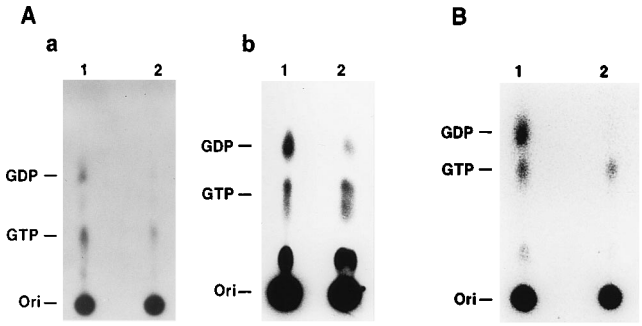

FIG. 5. Activation of ras by expression of STP-C488. (A) Increase in the relative ratio of ras-GTP to ras-GDP in STP-C488-transformed cells. Cell lysates of ^{32}P-labeled Rat-LXSN (lanes 1) and Rat-STP-C488 (lanes 2) were subjected to immunoprecipitation with Y13-259 (a) or Y13-238 (b) antibody. ^{32}P-labeled nucleotides eluted from immunoprecipitates were fractionated by thin-layer chromatography. (B) Increase in the relative ratio of ras-GTP to ras-GDP in insect cells by expression of STP-C488. Insect cells were infected with recombinant ras baculovirus alone (lane 1) or together with recombinant STP-C488 baculovirus (lane 2). ^{32}P-labeled cell lysates were subjected to immunoprecipitation with Y13-238 antibody, and ^{32}P-labeled nucleotides eluted from immunoprecipitates were fractionated by thin-layer chromatography. The origin (Ori) and the positions of GDP and GTP are indicated.

MAP kinase correlates with activated kinase activity, MAP kinase activity was measured with myelin basic protein as a substrate. Indeed, MAP kinase activity was activated greater than fivefold in transformed cells compared with Rat-LXSN cells. Thus, MAP kinase is constitutively activated by transformation with STP-C488.

Induction of ras-dependent cellular differentiation by the expression of STP-C488 in PC12 cells. The association of STP-C488 with ras and the activation of MAP kinase suggest that STP-C488 may be directly involved in controlling the ras signaling pathway. We used the PC12 pheochromocytoma cell line to obtain further evidence for this. Stimulation of PC12 cells with nerve growth factor activates the Trk tyrosine kinase (15, 23, 26) and thereby induces neurite extension; this response is dependent upon the ras pathway (28, 37, 44, 50). A PC12 cell line stably expressing STP-C488 was established with the LXSN retroviral vector. Expression of STP-C488 in PC12 cells did not induce neurite extension during normal culture conditions when extensive cell division was occurring (Fig. 7A). However, reduction of the serum concentration in the culture medium induced extensive neurite outgrowth in PC12 cells expressing STP-C488, while no such neurite outgrowth was observed in parental PC12 cells under the same conditions (Fig. 7B). Thus, STP-C488 is able to induce PC12 cell differentiation similarly to nerve growth factor, which has been shown to act through the ras signaling pathway.

DISCUSSION

In this report we have shown that STP-C488 associates with cellular ras in transformed cells and that the transforming activity of STP-C488 appears to be dependent upon this association with ras. Expression of STP-C488 activated the ras signaling pathway as shown by an increase in the ratio of ras-GTP to ras-GDP and by the constitutive activation of MAP kinase. STP-C488 is the first virus-encoded protein shown to achieve oncogenic transformation via association with cellular ras.

A number of cellular proteins have been found to be directly associated with ras in vivo and/or in vitro; these include raf-1, PI-3 kinase, ralGDS, and rin1 (14, 25, 27, 33, 38, 45–47). ras interacts directly with the catalytic subunit of PI-3 kinase in a GTP-dependent manner through the ras effector site, and this interaction regulates PI-3 kinase activity (38). Additionally, ras physically interacts with the amino-terminal domain of raf-1 in a GTP-dependent manner (27, 33, 45–47). However, the interaction of ras with raf-1 does not result in allosteric regulation of raf-1 kinase activity. Instead, ras functions in the activation of raf-1 by recruiting raf-1 to the plasma membrane, where a separate, ras-independent activation of raf-1 occurs (29, 41). Recently, the yeast two-hybrid system has revealed two additional genes, *ralGDS* and *rin1*, of which the gene products are capable of associating with ras in vivo (14, 25).

Detection of ras-STP complexes by using the STP-C488 antibody for immunoprecipitation followed by a immunoblot with the ras antibody was repeatedly unsuccessful. For this assay, we used anti-STP-C488 antibody that was generated with a synthetic peptide corresponding to the acidic amino terminus. It has been shown that the acidic amino terminus of STP-C488 is likely to be the ligand-binding or active site (18). Thus, anti-STP-C488 antibody may be able to precipitate only those STP-C488 molecules not complexed with ras.

The neutralizing anti-ras antibody, Y13-259, whose reactivity is dependent upon amino acids 63 to 73 in the linear sequence, is not able to precipitate complexes of ras with raf-1 kinase, PI-3 kinase, or ralGDS (25, 27, 33, 38, 45–47). In contrast, the Y13-238 antiras antibody, whose reactivity is dependent upon amino acids 120 to 138 in the linear sequence, successfully coprecipitates complexes of ras with raf-1 kinase, PI-3 kinase, or ralGDS (25, 27, 33, 38, 45–47). Our results with ras–STP-C488 complexes suggest that the region of binding of ras to STP-C488 may be different from that of ras to raf-1 or PI-3 kinase, since both of these antibodies efficiently recognize ras–STP-C488 complexes. However, coinfection of recombinant ras, raf-1, and STP-C488 baculoviruses showed that bind-

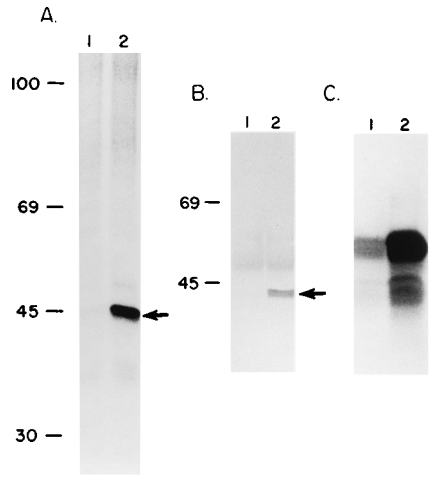

FIG. 6. Constitutive activation of MAP kinase in Rat-1 cells transformed by STP-C488. (A) In vivo phosphorylation of MAP kinase. (B) Tyrosine phosphorylation of MAP kinase. (C) Activation of MAP kinase activity. Rat-LXSN (lanes 1) and Rat-STP-C488 (lanes 2) cells were labeled with $^{32}PO_4$. ^{32}P-labeled 42-kDa MAP kinase protein immunoprecipitated by rabbit anti-MAP kinase antibody was detected by autoradiography of the dried gels. Cell lysates from 10^7 Rat-LXSN and Rat-STP-C488 cells were used for immunoprecipitation with anti-MAP kinase antibody, and anti-MAP kinase immune complexes were used for phosphotyrosine immunoblotting with 4G10 monoclonal antibody (B) or an in vitro kinase reaction with 5 μg of myelin basic protein (C). To quantitate the MAP kinase activity, ^{32}P-labeled myelin basic protein was excised from the gel and the radioactivity was counted. Arrows indicate the 42-kDa MAP kinase protein. The numbers on the left of each panel are molecular masses in kilodaltons.

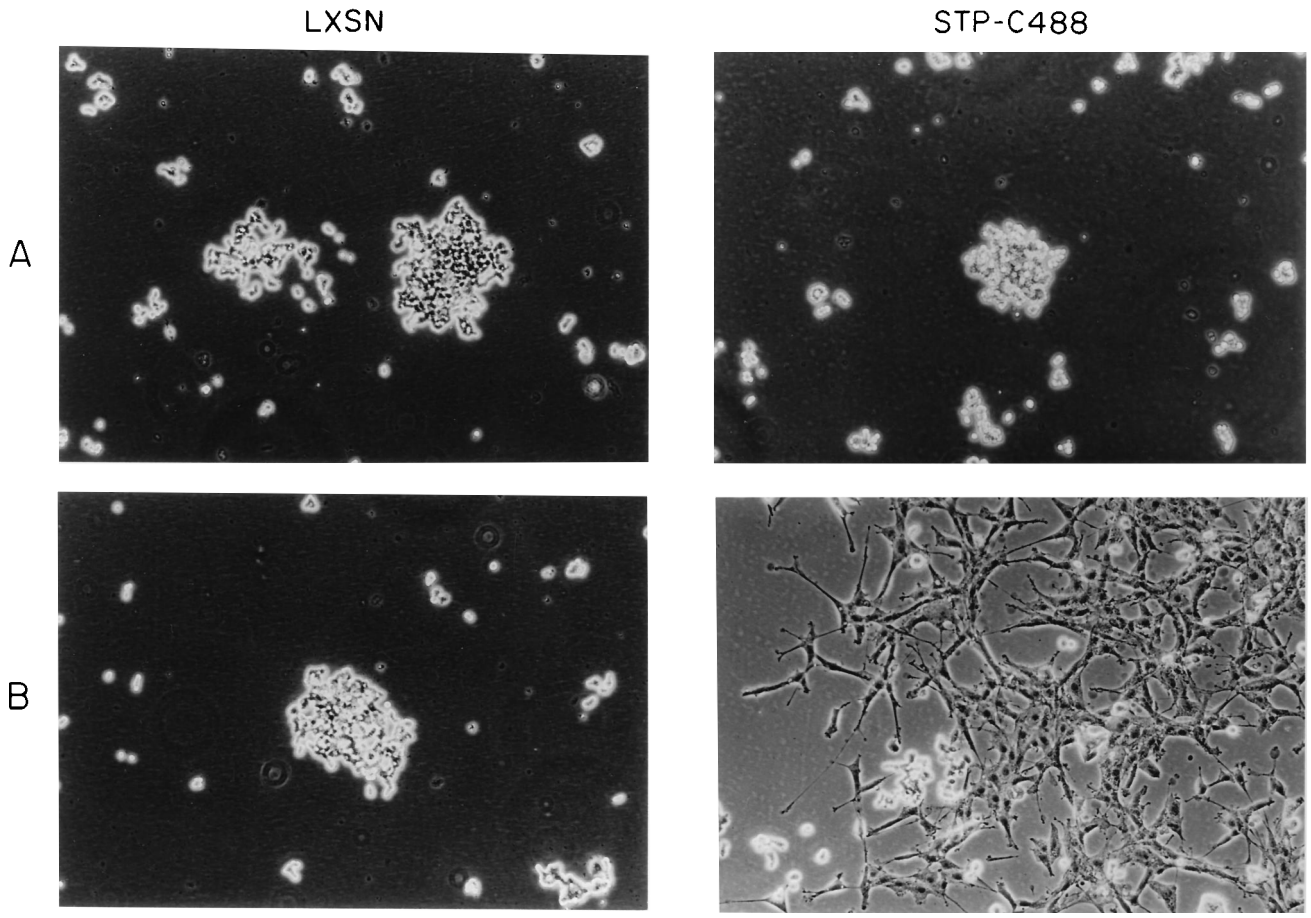

FIG. 7. STP-C488 expression induces neurite outgrowth of PC12 cells. PC12 cells were infected with the retrovirus LXSN or the recombinant retrovirus LXSN-STP-C488; this was followed by selection with 500 μg of G418. G418-resistant PC12-LXSN and PC12-STP-C488 cells were incubated with DMEM containing 5% fetal calf serum and 10% horse serum under normal culture conditions (A) or with DMEM containing only 5% fetal calf serum (B). Photographs were taken at day 10.

ing of raf-1 to ras was greatly reduced by the expression of STP-C488, while binding of STP-C488 to ras was not altered. These observations suggest that ras sequences associating with raf-1 may overlap partially, but not completely, with those that participate in binding to STP-C488. The results further suggest that STP-C488 may have a higher affinity than raf-1 for binding to ras and that the association of STP-C488 with ras thereby stearically hinders the association of raf-1 with the effector domain of ras. Mutagenesis studies of the ras protein will be required to delineate the STP-ras interaction more precisely.

raf-1 is a directly downstream of ras in the ras signal transduction pathway. The displacement of raf-1 from ras by STP-C488 raises intriguing questions as to how STP-C488 association with ras could activate the ras signaling pathway. The relative ratio of ras-GTP to ras-GDP was increased two- to fourfold in cells transformed by STP-C488. Also, coexpression of ras and STP-C488 in insect cells resulted in an increase in the relative amount of GTP-bound ras. Thus, association with STP may directly affect the biochemical activities of ras by suppressing the GTPase activity or enhancing the GTP-binding affinity. Additionally, alteration in complex formation between ras and cellular partners may bypass some of the downstream effectors in the ras signaling pathway or ultimately activate unknown ras signal transduction pathways. Recently, thyrotropin-induced signaling has been shown to be ras dependent but both raf and MEK independent (1), and a wortmannin-sensitive activation pathway in interleukin-2-stimulated T cells regulates MEK and MAP kinase without an alteration of raf activity (24). Thus, ras appears to be able to signal through effectors other than the well-characterized ras-raf-MEK-MAP kinase linear signal cascade.

The STP open reading frame of strain A11 is considerably longer than that of strain C488, and they have little sequence homology (4). Nonetheless, the organization of structural motifs appears to be similar: an acidic amino-terminal domain, centrally located collagen sequences, and C-terminal hydrophobic membrane-spanning sequences. However, in STP-A11, the collagen sequences are not directly repeated as they are in STP-C488; they are dispersed over a considerably longer stretch in the central portion of the molecule (22). Somewhat surprisingly, we have not been able to demonstrate an association between STP-A11 and ras by using the same assays in which the ras–STP-C488 association was readily detected. This appears to be a rare example of a case in which different strains of the same virus have evolved corresponding genes with distinct functional activities. In addition, strain C488 contains a gene adjacent to the *STP-C488* gene, called *tip*, that has no homologous or corresponding gene in strain A11 (4). The protein product of the *tip* gene was recently shown to associate with the major T-cell tyrosine kinase lck (5, 20) and to block its activity (21).

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