Identification and Characterization of the Herpesvirus Saimiri Oncoprotein STP-C488

JAE U. JUNG AND RONALD C. DESROSIERS*

New England Regional Primate Research Center, Harvard Medical School, Southborough, Massachusetts 01772

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The protein encoded by herpesvirus saimiri transforming gene STP-C488 was identified and characterized. Antibodies were produced in rabbits by immunization with keyhole limpet hemocyanin-conjugated synthetic peptides specific for the predicted sequence of STP-C488. STP-C488-encoded protein was detected in recombinant Escherichia coli, transformed Rat-1 cells, transfected COS-1 cells, and in common marmoset T lymphocytes immortalized by herpesvirus saimiri strain 488. STP-C488 protein was sensitive to treatment by bacterial collagenase, consistent with the 18 uninterrupted collagenlike repeats predicted by the DNA sequence. The apparent molecular size of STP-C488 in sodium dodecyl sulfate (SDS)-polyacrylamide gels (20 to 22 kDa) was considerably larger than that predicted from the DNA sequence (9.9 kDa). Using indirect immunofluorescence tests and subcellular fractionation, STP-C488 was found to be membrane bound, primarily in perinuclear compartments. The 18 uninterrupted collagenlike repeats, sensitivity to collagenase, location in the cell, and anomalous migration through SDS-polyacrylamide gels suggest an unusual, membrane-associated, fibrous structure for this transforming herpesvirus oncoprotein.

Unlike other model tumor virus systems, little is known at a molecular level about herpesvirus-induced oncogenic transformations. The greatest amount of information has accumulated with Epstein-Barr virus (EBV) and herpesvirus saimiri, members of the gamma subfamily of herpesviruses. Genetic determinants of EBV immortalization and transformation appear to be complex (for a review, see reference 19); genes for nuclear antigens (EBNA) and latent membrane proteins contribute to the immortalizing and transforming properties of EBV. Herpesvirus saimiri appears to lack homologs of the EBNA genes (11), but it does contain at least one gene (STP) that is important for the transforming properties of the virus (27).

Herpesvirus saimiri strains have been divided into three subgroups (A, B, and C) on the basis of the extent of DNA sequence divergence at the terminal end of L-DNA (24). Subgroups A and C viruses efficiently transform common marmoset peripheral blood T lymphocytes in vitro to interleukin-2 (IL-2)-independent growth (10) and cause rapidly progressing T-cell lymphomas in a variety of New World primate species (12). Mutational analyses have demonstrated that the leftmost open reading frame in genomic DNA of subgroup A strain 11 is required for immortalization of common marmoset T lymphocytes in vitro (27) and for lymphoma induction in vivo (8). This open reading frame is dispensable for virus replication (9, 21) and is termed STP-A11 for saimiri transformation-associated protein of subgroup A strain 11. The sequence of STP-A11 predicts a 164-amino-acid polypeptide with a highly hydrophobic carboxy terminus of 26 amino acids sufficient for a membrane spanning domain, an acidic amino terminus, and a single zinc finger motif (27). At a position and orientation equivalent to those of the STP-A11 reading frame, herpesvirus saimiri strain 488 (subgroup C) was found to contain two novel open reading frames designated ORF-1 and ORF-2 (2). However, ORF-1 and ORF-2 lack significant sequence identity with STP-A11 at the nucleotide level. Except for a hydrophobic carboxy terminus, the predicted amino sequences of ORF-1 and ORF-2 show no obvious similarity to that of STP-A11.

Recent studies demonstrated that ORF-2 of strain C488 and STP-A11 of strain A11 have transforming and tumor-inducing activities independent of the rest of the herpesvirus genome (18). Specifically, ORF-2-containing expression vectors transformed 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. STP-A11 was less potent in its transforming activity. ORF-2 of strain 488 has thus been designated STP-C488 for saimiri transformation-associated protein of subgroup C strain 488 (18).

Close inspection of the STP-A11 and STP-C488 sequences reveals a similar organization in terms of the presence and location of specific structural motifs (18). Both proteins are predicted to have highly acidic amino termini (pI = 3.5 for the amino-terminal 32 amino acids of STP-A11 and pI = 4.4 for the amino-terminal 17 amino acids of STP-C488). The DNA sequence of STP-C488 predicts 18 direct repeats of a 3-amino-acid sequence that is either Gly-Pro-Pro, Gly-Pro-Gln, or Gly-Leu-Pro (2). Thus, 54 of the 102 amino acids of the predicted STP-C488 protein are contained within these collagenlike repeats. The translated amino acid sequence of STP-A11 has nine collagenlike motifs (Gly-X-Y, where X and/or Y is proline) (27). The collagenlike motifs in STP-A11 are not directly repeated as they are in STP-C488, but they seem to be similarly concentrated in the central portion of the protein (35). The putative STP-A11 and STP-C488 proteins also share a hydrophobic stretch at their carboxy termini sufficient for a membrane-spanning domain. These similarities in structural motifs suggest that the two proteins use similar mechanisms to achieve T-lymphocyte transformation, despite a lack of obvious amino acid sequence homology.

We now report the identification of the protein encoded by STP-C488 and describe some of its important characteristics.

* Corresponding author.
MATERIALS AND METHODS

Cell culture and transfections. COS-1 cells and rodent fibroblast Rat-1 cells were grown in Dulbecco’s modified Eagle’s medium with high glucose supplemented with 10% (vol/vol) fetal calf serum (GIBCO Bethesda Research Laboratories, Gaithersburg, Md.) and donor bovine calf serum (Hazelton Laboratories, Lenexa, Kans.), respectively. Immunized common marmoset T lymphocytes were grown in RPMI 1640 plus 20% fetal calf serum. Primary common marmoset peripheral blood lymphocytes were purified by using lymphocyte separation medium (Organan Teknika Corp., Malvern, Pa.), washed, activated with 1 μg of phytohemagglutinin per ml, and then grown in RPMI 1640 plus 20% fetal calf serum supplemented with 10% (vol/vol) human IL-2 (Pharmacia Diagnostic Inc., Fairfield, N.J.). G418-resistant cells Rat-LXSN (18), Rat-STP-C488 (18), and Rat-STP-C488/Lys were maintained in 500 μg of G418 (GIBCO BRL) per ml. DNA was introduced into cells by DEAE-dextran transfection (7) or electroporation (18).

Plasmid construction. DNA containing the STP-C488 open reading frame was amplified from herpesvirus saimiri strain 488 virion DNA by polymerase chain reaction (PCR). STP-C488 includes bp 1051 to 1440 of the published sequence from the left end of L-DNA of herpesvirus saimiri strain 488 (2). PCR cycling was accomplished with a DNA thermal cycler (Perkin-Elmer Cetus Instrument, Norwalk, Conn.) under the following conditions: 3 cycles of 2 min at 53°C for annealing, 2 min at 72°C for polymerization, and 1 min at 94°C for denaturation. The STP-C488 DNA fragment was amplified by PCR by using primers containing BamHI recognition sequences at the ends, purified from an agarose gel, and ligated into the BamHI cloning site of pBSKS+ vector (Stratagene, La Jolla, Calif.). The BamHI DNA fragment containing STP-C488 from pBS-STP-C488 was subcloned into pSV transient eukaryotic expression vector (Pharmacia LKB, Piscataway, N.J.). The STP-C488 DNA fragment was also amplified by PCR using primers containing NdeI and BamHI recognition sequences at the ends, purified from an agarose gel, and ligated into the NdeI and BamHI cloning site of the PET-3a T7 expression vector (a gift from F. W. Studier). STP-C488 DNAs in these clones were completely sequenced to verify 100% agreement with the original sequence. Escherichia coli BC21 was used as the initial host for the T7 expression system (33). Retrovirus vector LXSN containing STP-C488 was constructed previously (18).

A lysine mutation at amino acid 98 was constructed by site-specific mutagenesis using PCR with the above conditions. Oligonucleotide primers from complementary strands representing the 5' and 3' ends of STP-C488 were synthesized with EcoRI and BamHI sites at the 5' ends to facilitate cloning into the EcoRI and BamHI sites of retrovirus vector LXSN, except that the 3' oligonucleotide primer contained a nucleotide change from AAA (Lys) to ATA (Ile). The amplified DNA fragment containing STP-C488/Lys was purified and cloned into retrovirus vector LXSN (25). DNA between the BamHI and EcoRI sites of LXSN-SP-C488/Lys was completely sequenced to verify the presence of the mutation and the absence of any other changes. Following electroporation of Rat-1 cells, resistant cells were selected with 500 μg of G418 per ml.

Antibody production. Two synthetic peptides were used for rabbit antibody production. Synthetic peptide 28, Ala-Ser-Glu-Pro-Asn-Leu-Arg-Tyr-Pro-Ile-Glu-Glu-Thr-Gly, corresponds to residues 2 through 15 of STP-C488, and synthetic peptide 28, Gly-Asp-Arg-Gly-Pro-Pro-Gly-Pro-Gly-Pro-Gly-Pro-Gln, corresponds to residues 15 through 29 of STP-C488. 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 peptide in Freund’s complete adjuvant and two booster injections with the same antigen in incomplete adjuvant at 3-week intervals. The animals were bled 10 days after the last booster injection, and the serum was stored at -70°C. Antiserum was used at a 1:1,000 dilution for the immunoblots and at a 1:100 dilution for the immunoprecipitations.

Protein immunoblots. Approximately 106 cells were harvested by scraping of adherent cells or pelleting of nonadherent cells. Cells were washed with phosphate-buffered saline (PBS) and lysed with 10 mM Tris-HCl (pH 7.5) containing 1% sodium dodecyl sulfate (SDS). Cell suspensions were sonicated twice for 15 s to shear DNA. Polypeptides in cell lysates corresponding to 106 cells were resolved by electrophoresis through SDS-12.5% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose paper by the MilliBlot-SDS Electroblotting system (Hoefer Scientific Instruments, San Francisco, Calif.). Filters were incubated in PBS containing 5% fetal calf serum and 0.4% Tween-20 to reduce background. Immunoblot detections were performed as described previously (20).

Radiolabeling of cells and immunoprecipitation. Cells at 80 to 90% confluence in a 25-cm2 dish were rinsed three times with PBS, washed once with labeling medium (RPMI 1640 minus methionine and leucine plus 5% dialyzed fetal calf serum), and then incubated with 2 ml of the same medium containing 500 μCi of 35S-Trans (specific activity, >1,000 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) and 50 μCi of [14C]Leu (300 mCi/mmol; New England Nuclear Corp., Boston, Mass.) overnight. Cells were incubated in labeling medium without radiisotopes for 30 min before addition of the radiisotopes.

After being labeled, cells were harvested by scraping with a rubber policeman, washed once with PBS, and lysed with 1 ml RIPA buffer (0.15 M NaCl, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS, 50 mM Tris [pH 7.5], 10 μg of aprotinin [United States Biochemical Corp., Cleveland, Ohio] per ml) for 30 min at 4°C with shaking. Lysates were cleared by centrifugation at 13,600 × g for 1 min in a microcentrifuge, and the supernatant was transferred to a clean tube. For immunoprecipitation, 5 μl of rabbit polyclonal antibody and 30 μl of protein A-agarose (Oncogene Science, Manhasset, N.Y.) were added to 500 μl of RIPA buffer containing 100 μl of supernatant and incubated for 4 h at 4°C with shaking. The pellet was washed five times by suspending it in 1 ml of RIPA buffer and by vortexing, centrifuging, and aspirating the supernatant. Finally, the pellet was rinsed twice with 10 mM Tris, pH 7.5, suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and boiled for 5 min. Proteins in the sample were resolved by SDS-12.5% PAGE, and the gel was impregnated with En3Hance (New England Nuclear Corp.) as recommended by the manufacturer, dried, and exposed to Kodak XAR-5 film at -70°C.

Immunofluorescence. Cells on slides were washed with PBS, fixed in acetone at -20°C for 2 min, washed with PBS again, and incubated with rabbit antibody diluted 1:50 in PBS containing 10% goat serum for 1 h at 37°C. After incubation with antibody, cells were washed extensively with PBS, incubated with 1 μg of fluoresceinated goat
F(ab'), anti-rabbit total immunoglobulin (Oncogene Science) for 45 min at 37°C, and then washed three times with PBS. Immunofluorescence was detected with a Bio-Rad confocal microscope MR600.

**Collagenase treatment.** Approximately 10⁷ cells were harvested, washed with PBS, and resuspended in 10 mM Tris-HCl (pH 7.5). Cell suspensions weresonicated twice for 15 s to lyse the cells. Twenty microliters of cell extracts was incubated at 37°C in 50 µl of reaction buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co., St. Louis, Mo.], 10 µg of aprotinin per ml, and 10 mM calcium chloride) with or without 10 µg of collagenase of *Achromobacter* *iophagus* (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. After the reaction, proteins in the cell extracts were resolved by SDS-PAGE and immunoblotted.

**Subcellular localization.** Cells were washed, swollen in hypotonic 10 mM sodium citrate buffer, removed by scraping, and homogenized with 10 strokes of a type B pestle in a Dounce tissue grinder. After confirming total cell breakage by light microscopy, the nuclei and cell debris were completely removed by three cycles of centrifugation at 600 × g for 15 min at 4°C. This supernatant was used for subcellular localization. The supernatant was separated into soluble and insoluble fractions by centrifugation for 1 h at 4°C at 100,000 × g in a Ti 60 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Insoluble fractions were treated with 1 M NaCl or 1% Triton X-100 and then ultracentrifuged again to separate soluble and insoluble fractions.

**RESULTS**

**STP-C488-expressing cells.** To demonstrate the expression of the STP-C488 open reading frame into protein, we first constructed various STP-C488 expression vectors. For bacterial expression, STP-C488 was cloned into the NdeI and *Bam*HI sites of a pET-3a T7 bacterial expression vector. In plasmid pET-STP-C488, STP-C488 was placed under T7 promoter control and the efficient translational initiation signal of gene 10 protein (33). This plasmid was transformed into *E. coli* BC21, a bacterial host strain carrying a chromosomal copy of the gene for T7 RNA polymerase under the control of the inducible lacUV5 promoter (33). Therefore, STP-C488 production was initiated by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were harvested 2 h after IPTG induction.

Previously, the retrovirus vector LXSN derived from murine leukemia virus had been used for stable STP-C488 expression in rodent fibroblast Rat-1 cells (18). G418-resistant Rat-STP-C488 cells stably express STP-C488 mRNA from the murine leukemia virus long terminal repeat in Rat-1 cells. G418-resistant Rat-LXSN cells contain only the LXSN retrovirus vector in Rat-1 cells.

pSVL-STP-C488 was constructed to facilitate transient expression of STP-C488 in COS-1 cells. The coding sequence of STP-C488 in this expression vector is under the control of the simian virus 40 (SV40) enhancer and late promoter. This plasmid also has a functional origin of replication for SV40 and is therefore expected to replicate in COS-1 cells, which express the SV40 T antigen (15). Plasmid pSVL-STP-C488 was introduced into COS-1 cells by DEAE-dextran transfection (7). Transfected COS-1 cells were harvested after 48 h.

Common marmoset peripheral T lymphocytes were previously immortalized in vitro to permanent autonomous growth by herpesvirus saimiri strain 488 virus (10). These T lymphocytes do not require IL-2 for growth and do not produce detectable virus.

**Identification of STP-C488.** To raise antibodies directed against STP-C488, we used synthetic peptides for rabbit immunizations. Peptides 27 and 28, corresponding to amino acid residues 2 through 15 and 15 through 29 of the predicted sequence, were synthesized, coupled via an additional N-terminal cysteine to keyhole limpet hemocyanin, and inoculated into rabbits. Peptide 27, which exhibits no homology with other known proteins, is part of the hydrophilic, acidic domain of STP-C488. Peptide 28 contains four collagene-like motifs of STP-C488 which are homologous to cellular collagen proteins (3). Antibodies induced by peptide 27 were of higher titer and specific for the putative STP-C488 protein. Antibodies induced by peptide 28 had a lower titer and also detected cellular collagens. Therefore, we used anti-STP-C488 rabbit serum 27 for the experiments.

Antibody to peptide 27 reacted with a specific protein having an apparent molecular size of 20 kDa on immunoblots only from STP-C488-expressing cells (Fig. 1A, lanes 2, 4, and 6). Rat-STP-C488 cells, COS-1 cells transfected with plasmid pSVL-STP-C488, and common marmoset T lymphocytes immortalized by herpesvirus saimiri strain 488 all showed the presence of this predominant 20-kDa protein. No such protein was detected in control cells lacking STP-C488 sequences (Fig. 1A, lanes 1, 3, and 5). In addition to the major STP-C488 protein which migrated with an apparent molecular size of 20 kDa, a less-abundant species of slightly slower mobility (22 kDa) was also observed (Fig. 1A, lanes 2, 4, and 6). The 20-kDa protein was also detected in Rat-STP-C488 cells by immunoprecipitation with antibody to peptide 27 (Fig. 1C). This protein was not detected when antibody was preincubated with an excess of synthetic peptide 27 (data not shown). A protein with the same mobility was detected in the *E. coli* expression system (Fig. 1B), suggesting that there are no posttranslational modifications specific for mammalian cells that would affect electrophoretic mobility.

The apparent molecular size of STP-C488 (20 kDa) by SDS-PAGE is approximately double that predicted from the DNA sequence (9.9 kDa) (2). The absence of predicted cysteine residues in STP-C488 essentially excludes the possibility of disulfide linkage. Several reports have shown that other linkages such as metal binding or β-galactose binding could contribute to the stability of dimeric structures and be readily dissociated in the presence of reducing agent in SDS-PAGE (13, 22). However, STP-C488 did not change its migration rate in SDS-PAGE with different concentrations of reducing agent (Fig. 2B). Consensus sequences for N glycosylation (N-X-T/S, where N = asparagine, T = threonine, S = serine, and X = any amino acid) and N-acetylation (N-acetyl, where C = cysteine, a = alphatic amino acid, and X = any amino acid) are absent in the predicted STP-C488 sequence. In addition, attempts to demonstrate myristylation and O glycosylation in mammalian cells yielded negative results (data not shown).

Cellular collagens have a unique type of intra- and intermolecular covalent linkage. Certain lysine and hydroxylysine residues form covalent bonds with each other or with other lysine or hydroxylysine residues (3). In addition, glycosylation at hydroxylysine has frequently been observed in collagens. The specific nature of the glycosylation appears to be characteristic for the collagen type (3). The central region of STP-C488 is predicted to contain 18 uninterrupted repeats of collagen-like motifs. We thus investigated whether STP-C488 may have a similar modification(s) that renders
STP-C488 more slowly migrating in SDS-PAGE. STP-C488 is predicted to contain a single lysine residue at amino acid 98 near the carboxy terminus (2). To test for possible lysine modification in STP-C488, the lysine residue of STP-C488 was changed to an isoleucine residue by site-directed mutagenesis. Mutant STP-C488 containing an isoleucine instead of lysine near the carboxy terminus (STP-C488/Lys) was expressed in Rat-1 cell lines by using retrovirus vector LXS1N for gene transfer. Analysis of G418-resistant Rat-1 STP-C488/Lys cells showed that mutant STP-C488/Lys had the same mobility in SDS-PAGE (Fig. 2A). This suggests that intramolecular linkages and glycosylation are absent at the predicted lysine residue of STP-C488.

Collagenase treatment. To test for the presence of collagenlike sequences in the central region of STP-C488, we examined its susceptibility to collagenase cleavage. Highly purified collagenase from A. iophagus cleaves at the Pro-Gly peptide bond within Gly-X-Pro-Gly sequences in a highly specific manner (6). Collagenase treatment resulted in the complete degradation of the intact STP-C488 molecule in a 1-h incubation, while incubations at 37°C for 1 or 2 h without collagenase had no effect on STP-C488 (Fig. 3A). To demonstrate the specificity of A. iophagus collagenase, half portions of the same reaction mixtures after collagenase treatment were subjected to immunoblot analysis with monoclonal cytoskeleton vimentin antibody. The migration and amounts of cellular vimentin protein were approxi-

mately the same in all reactions with or without collagenase treatment (Fig. 3B). Thus, A. iophagus collagenase cleaved the collagenlike motifs of STP-C488 in a highly specific manner.

Localization of STP-C488 in the cell. The cellular location of STP-C488 was investigated by indirect immunofluorescence tests and subcellular fractionation. Rat-LXSN and Rat-STP-C488 cells grown on glass slides were reacted with anti-STP-C488 antibody to peptide 27 followed by fluoresceinated F(ab')_2, goat anti-rabbit total immunoglobulin. Rat-STP-C488 stained strongly with anti-STP-C488 antibody, exhibiting a very bright cytoplasmic fluorescence (Fig. 4A). At the higher magnification (Fig. 4B and C), the fluorescence clearly visualized a network of tubules in the perinuclear region. This pattern at the periphery of the cellular nucleus is similar to that obtained when the endoplasmic reticulum is visualized with lipophiliic fluorescent dye (26, 28). However, because of the bright staining in the perinuclear region, it was difficult to show whether it was also present in the Golgi complex. Cells were also reacted with antibody 27 without permeabilization followed by fluoresceinated anti-rabbit antibody; fluorescence at cytoplasmic membranes was never detected with such intact cells (data not shown). In addition, cell-free medium did not contain detectable STP-C488 by immunoblot analysis (data not shown).

The subcellular distribution of STP-C488 was investigated by immunoblot analysis of subcellular extracts from Rat-
FIG. 2. No effect of lysine mutation and various reducing conditions on migration of STP-C488 in SDS-PAGE. (A) Immunoblot of Rat-STM-C488/Lys cell extracts. Extracts from Rat-LXSN, Rat-STM-C488, and Rat-STM-C488/Lys cells were electrophoresed, immunoblotted, and reacted with antibody 27. Lanes: 1, Rat-LXSN cells as negative control; 2, Rat-STM-C488 cells; 3, Rat-STM-C488/Lys cells. (B) Various reducing conditions in SDS-PAGE. Equal amounts of cell lysates from Rat-STM-C488 were mixed with SDS sample loading buffers containing different concentrations of β-mercaptoethanol: lane 1, 100 mM; lane 2, 0 mM; lane 3, 200 mM. The molecular markers are the same as those described in the legend to Fig. 1A.

STP-C488 cells. Total cytoplasmic extracts and soluble or particulate fractions on immunoblots were reacted with anti-STP-C488 antibody 27. As shown in Fig. 5, 20-kDa STP-C488 was detected in total cytoplasmic extracts. It was absent from the soluble fraction (Fig. 5, lane S) and found exclusively in the particulate fraction of the cells (Fig. 5, lane P). This STP-C488 could not be extracted from the membranes by washing with high concentrations of salt (Fig. 5, lane N), whereas it was readily solubilized with 1% Triton X-100 (Fig. 5, lane T). Therefore, it appears that STP-C488 is not loosely attached to the membrane, but rather is tightly bound to membrane via hydrophobic interactions, similar to Ras-antagonistic Rap1/Krev-1 protein (1) and a small GTP-binding protein of the Ras superfamily (16).

DISCUSSION

This report describes the identification of STP-C488 protein in transformed cells harboring the STP-C488 gene. Experiments were carried out by using rabbit polyclonal antibodies against synthetic peptides corresponding to STP-C488. Rabbit antibody 27 directed against the amino-terminal 14 amino acids of STP-C488 was shown to be monospecific, exhibiting no cross-reactivities with other cellular proteins on immunoblots. Using anti-STP-C488 antibody, we detected STP-C488 in various STP-C488-expressing cells, including recombinant E. coli, Rat-STM-C488, COS-1 cells transfected with plasmid pSVL-STP-C488, and common marmoset lymphocytes immortalized by herpesvirus saimiri strain 488.

The specific features of cellular collagens include a high content of proline and extensive posttranslational modifications that include hydroxylation of proline and lysine residues, various glycosylations of hydroxyllysine residues, and formation of intra- and intermolecular cross-links through lysine and hydroxylysine (3). The fact that mutant STP-C488/Lys had the same size as wild-type STP-C488 indicates the absence of glycosylation or intramolecular cross-linkages involving this lysine residue. This is the only lysine residue predicted by the STP-C488 sequence. In addition, STP-C488 does not contain consensus motifs for N glycosylation or isoprenylation, and attempts to demonstrate myristylation and O glycosylation yielded negative results. However, we have recently found that STP-C488 is phosphorylated (17). Phosphorylation is responsible for the 20- to 22-kDa doublet in SDS-PAGE (see Fig. 1, 2, and 5). The 22-kDa protein is the phosphorylated form, and the 20-kDa protein is the nonphosphorylated form of STP-C488 (17).

The predominant form of STP-C488 has an apparent molecular size of 20 kDa in SDS-PAGE, which is about double that predicted from its DNA sequence (9.9 kDa). The absence of cysteine in STP-C488 essentially rules out possible disulfide linkages between cysteine residues, as in bovine papillomavirus E5 protein (4). Previous studies with cellular collagens and other fibrous proteins may be helpful in understanding the migration of STP-C488 in SDS-PAGE. Human papillomavirus type 16 oncoprotein E7, composed of 98 amino acids, has been shown to migrate as a protein of 20 kDa in SDS-PAGE rather than the 11 kDa calculated from the sequence (31). The E1A protein of adenovirus type 2 also migrates anomalously through SDS-polyacrylamide gels (32). While these proteins do not contain collagen-like repeats, they are believed to have unusual extended structures (31, 32). The collagen-like repeats in STP-C488 may confer an unusual fibrous structure, as shown previously for cellular collagens (3). Because of the fibrous structure, cellular
collagens have been shown to migrate slowly in SDS-PAGE (3). Additionally, it has been reported that proline richness in protein causes slow migration in SDS-PAGE (34). STP-C488 contains 33 proline residues out of 102 total amino acids (2). Therefore, an unusual fibrous structure and proline richness, not dimerization, are likely to be the main determinants for the slow migration of STP-C488 in SDS-PAGE.

Knowledge of the intracellular location of the STP-C488 gene product should facilitate studies on its mechanism of action. Immunofluorescence tests and subcellular fractionation showed that STP-C488 was localized to the perinuclear region, which includes the endoplasmic reticulum and possibly the Golgi complex. When live cells were reacted with anti-STP-C488 antibody without permeabilization, there was no evidence of cell surface fluorescence, indicating that STP-C488 was not present detectably on the surfaces of transformed Rat-STP-C488 cells. Localization in the Golgi complex was ambiguous because of intense staining in the perinuclear region. However, a few cells had an acentric fluorescence in the perinuclear region (Fig. 4A), suggesting the possible presence of STP-C488 in the Golgi complex. Recently, E5 oncoprotein from bovine papillomavirus (5), v-sis/platelet-derived growth factor (PDGF)-2 transforming gene product (30), Ras-antagonistic Rap1/Krev-1 protein (1), and small GTP-binding proteins (16) have been shown to be associated with the Golgi complex. It has been proposed that E5 protein in the Golgi complex may intercept PDGF receptor molecules on route to the cell surface to activate the PDGF receptor without the addition of PDGF (29), and Rap1/Krev-1 protein could inhibit the transforming activity of Ras protein by competing for a common effector such as cytosolic GTPase-activating protein in the vicinity of the Golgi complex (1). In addition, the endoplasmic reticulum is an important organelle for neoplasia. It is the site of internal Ca\textsuperscript{2+} storage and for inositol triphosphate action, both of which are important intracellular messengers for the initia-

FIG. 4. Immunofluorescence of Rat-STP-C488 cells. Rat-STP-C488 (A, B, and C) and Rat-LXSN (D) cells were reacted first with rabbit antibody 27 and then with fluoresceinated goat F(ab')\textsubscript{2} anti-rabbit total immunoglobulin. The magnifications were 400× (A), 600× (B), 1,000× (C), and 600× (D).
FIG. 5. STP-C488 is tightly associated with membranes. Rat- 
STP-C488 cells were homogenized in hypotonic buffer with a 
Dounce tissue grinder. After the cytoplasmic fraction (lane C) was 
obtained, it was separated into soluble (lane S) and particulate (lane 
P) fractions by ultracentrifugation. The particulate fraction was 
washed with 1 M NaCl (lane N) or Triton X-100 (lane T), and eluted 
proteins were isolated after ultracentrifugation. All proteins were 
electrophoresed, blotted to filter, and reacted with antibody 27. The 
molecular markers are the same as those described in the legend to 
Fig. 1A.

tion of various physiological responses (14). Thus, the perinuclear 
location of STP-C488 as well as its other properties may suggest a possible involvement in signal transduction pathways. However, other possible modes of action, e.g., in 
regulating export of specific RNAs from the nucleus, also 
need to be investigated.

Three distinct domains are likely to contribute to the 
actions of STP-C488. The 18 uninterrupted collagenlike 
repeats in the middle domain can be inferred to form a long 
filibr structure similar to that of cellular collagens (3) and 
may serve as a hinge to extend the active portion of the 
protein to its site of action. The carboxy-terminal hydrophobic 
tail is sufficiently long to anchor STP-C488 to membranes, 
consistent with the localization and cellular subfractionation studies. Although STP-C488 is localized to 
membranes, it has no signal peptide at its amino terminus. 
This aspect is similar to that of polyomavirus middle T 
antigen (23) and mas oncogene (36), which also have no 
signal peptide at their amino termini. Finally, the acidic 
amino terminus may be the active or ligand-binding portion of 
STP-C488. Further studies will be needed to identify the 
amino acids essential for STP-C488 transforming activity, 
the biochemical activities of STP-C488, and the mechanism 
by which STP-C488 brings about altered cell growth.

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