Substitution of *ras* for the Herpesvirus Saimiri STP Oncogene in Lymphocyte Transformation

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STP-C488 (STP of herpesvirus saimiri [HVS] group C strain 488 [C488]) is the only virus-encoded protein found to associate with cellular *ras* and activate *ras* signal transduction pathways. To investigate an important role for *ras* signal transduction in STP-dependent growth transformation, we constructed recombinant strains of HVS C488 in which the STP-C488 oncogene was replaced with cellular normal ras (c-*ras*) or viral oncogenic ras (v-*ras*). Recombinant HVSΔSTP/v-*ras* immortalized primary common marmoset T lymphocytes to interleukin-2-independent growth as efficiently as wild-type HVS C488 (wt HVS), while recombinant HVSΔSTP/c-*ras* did so with low efficiency. Whereas wt HVS immortalized CD4$^-$ CD8$^+$ single-positive T lymphocytes, HVSΔSTP/c-*ras*- and HVSΔSTP/v-*ras*-immortalized cells were principally CD4$^+$ CD8$^+$ double-positive T lymphocytes. In addition, HVSΔSTP/v-*ras*-immortalized T cells showed a high level of *ras* expression and exhibited an adherent macrophage-like morphology. These phenotypes were likely caused by the drastic activation of AP-1 transcriptional factor activity. Finally, HVSΔSTP/v-*ras* and HVSΔSTP/c-*ras* each induced lymphoma in one of two common marmosets, although onset of disease was more rapid with the v-*ras* virus. These results demonstrate that *ras* can substitute for the STP oncogene of HVS C488 to allow immortalized growth of primary lymphoid cells and that an activated form of *ras* does so more efficiently than the normal cellular form of *ras*.

Herpesvirus saimiri (HVS) subgroup C strains contain a divergent form of the STP oncogene at the left end of the coding portion of the genome (5, 20). STP of group C strains (STP-C) and STP from group A strains (STP-A) are each sufficient for the transformation of rodent fibroblast cells in vitro, but STP-C is considerably more potent (22, 25). Transgenic mice expressing STP-C488 (STP from group C strain 488 [C488]) developed invasive epithelial cell tumors (32), while STP-A11 (STP from group A strain 11 [A11]) transgenic mice developed peripheral pleomorphic T-cell lymphomas (28). Deletion of either form of STP yields virus no longer capable of immortalizing lymphocytes in vitro or of inducing fatal lymphomas in common marmosets (12–15, 27, 33). Since HVS lacking STP can be repeatedly isolated from peripheral blood of common marmosets for months and years, STP is not required for viral replication or persistence in vivo but it is essential for transformation in cell culture and for lymphoma induction in common marmosets (12, 15).

Transforming proteins of tumor viruses exert their effects in many cases through specific interactions with cellular regulatory proteins (9, 18, 35, 41, 42). We have demonstrated that STP-C488 associates with cellular *ras* in transformed cells (24). Mutations that disrupt this association with *ras* disrupt the transforming ability of the STP-C488 oncogene (24). Binding assays show that STP-C488 is capable of competing with *raf-1* for binding to *ras*. Expression of STP-C488 activates 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 induces *ras*-dependent neurite outgrowth in PC12 cells (24). Unlike STP-C, STP-A binds to the SH2 domain of Src kinase and is phosphorylated by the associated Src kinase in in vitro kinase assays (29). Mutational analysis of STP-A11 shows that binding to Src kinase requires the tyrosine residue at 115, showing that YAE(V/I) is a binding motif for the SH2 domain of Src. Also, tyrosine phosphorylation of STP-A by Src leads to subsequent binding to Lck and Fyn in vitro (29). These results suggest that STP of subgroup A targets a cellular protein for virus-induced transformation that is different from that used by STP-C.

In this report, we demonstrate that *ras* is capable of substituting for the HVS STP-C488 function in lymphocyte transformation. Recombinant HVSΔSTP/c-*ras* and HVSΔSTP/v-*ras* in which the STP-C488 gene was replaced with a normal cellular *ras* (c-*ras*) or an oncogenic viral *ras* (v-*ras*) gene were isolated. Recombinant HVSΔSTP/c-*ras* and HVSΔSTP/v-*ras* virus immortalized primary T lymphocytes to interleukin-2 (IL-2)-independent growth and induced lymphoma in common marmosets. These results suggest that activation of *ras* signal transduction pathways is important for T-cell growth transformation by HVS.

MATERIALS AND METHODS

Cell culture and virus propagation. Owl monkey kidney cells (OMK 637) were cultivated in minimal essential medium supplemented with penicillin, streptomycin, L-glutamine, and 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO BRL, Grand Island, N.Y.) were used for the propagation of HVS C488. Low-passage OMK cells (<30 passages) were used for the transfections. Primary common marmoset peripheral blood mononuclear cells (PBMCs) were purified by using lymphocyte separation medium (Organon Teknika Corp., Malvern, Pa.). Cultures of common marmoset PBMCs in immortalization assays with HVS recombinants were performed in RPMI 1640 medium supplemented with penicillin, streptomycin, amphotericin B (Fungizone), L-glutamine, 20%

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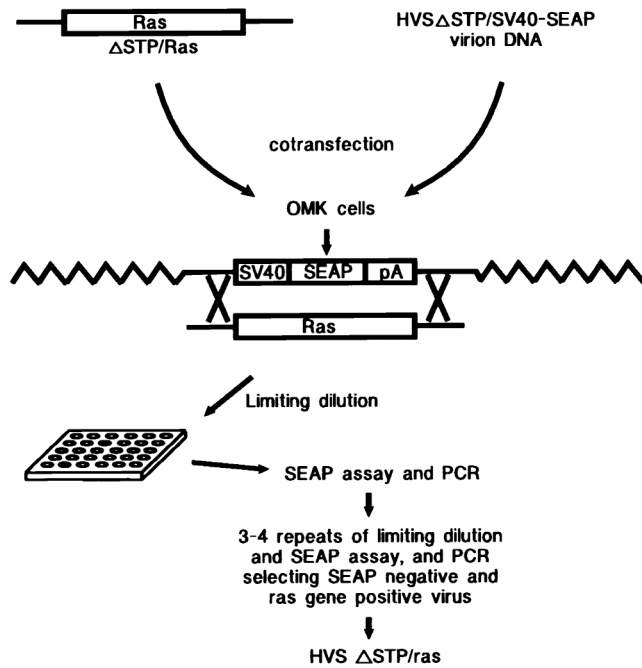
FIG. 1. Schematic diagram to construct the recombinant HVS containing c-*ras* or v-*ras*. The detailed procedure has been described previously (16). pA, polyadenylation site.

(vol/vol) heat-inactivated fetal bovine serum, and 5 mg of β-mercaptoethanol per liter.

Virion DNA isolation. HVS virion preparations were obtained from culture medium of infected OMK cells after removal of cell debris by low-speed centrifugation, followed by pelleting of the virus at 18,000 rpm for 2 h in an SS-34 rotor. To purify intact virion DNA, the virus was disrupted at 60°C for 2 h in lysis buffer containing 10 mM Tris (pH 8.5), 1 mM EDTA, 1% (vol/vol) Sarkosyl, and 0.1 mg of proteinase K per ml. Extraction of the aqueous solution first with an equal volume of phenol and then twice with chloroform was sufficient to purify the virion DNA for use in transfections. Sterile cut pipette tips were used for manipulating virion DNA without shearing.

Construction of recombinant HVS. The complete STP coding sequence was deleted from 3.6 kb of the left end of L-DNA of HVS C488 by PCR, and the multicloning sites were inserted into the STP locus. Human cellular H-*ras* (2) or oncogenic viral H-*ras* (2) was cloned into the multicloning sites of 3.1 kb of L-DNA. Linearized plasmid DNA containing 3.5 kb of L-DNA with the *ras* gene was cotransfected to OMK cells with HVSΔSTP/SV40-SEAP virion DNA by the calcium phosphate protocol. A pure form of recombinant virus with the SEAP reporter replaced with c-*ras* or v-*ras* was isolated by limiting dilution and repeated selection of SEAP-negative virus to OMK cell monolayers in 48-well tissue culture plates performed as described previously (16) (Fig. 1). SEAP production was detected by a liquid scintillation counter measurement of the chemiluminescence produced in assays of cell culture medium by using Phospha-Light reagents (Tropix Inc., Bedford, Mass.) according to the manufacturer's recommendations.

In vitro immortalization of common marmoset lymphocytes. Assays of lymphocyte immortalization in vitro have been described previously (14). PBMCs were isolated from 3-ml heparinized blood specimens from common marmosets (*Callithrix jacchus*) by centrifugation through lymphocyte separation medium (Organon Teknika) followed by washing in RPMI 1640 culture medium. PBMCs from each animal were individually washed, resuspended in RPMI 1640 medium, and then distributed in 1-ml volumes containing approximately 10^6 cells into 12-well tissue culture plates. Cells were then infected at a multiplicity of infection ranging from 1 to 5 with 1 ml of purified HVS stocks. Cells were maintained in RPMI 1640, with the growth medium changed every 3 to 4 days. Immortalization or cell death was assessed microscopically.

Experimental infection of common marmosets. In vivo oncogenicity of the HVS C488 recombinants was assessed by experimental infection of common marmosets (*C. jacchus*). Marmosets were injected intramuscularly with 10^5 50% tissue culture infective doses ($TCID_{50}$) of virus in a volume of 1 ml. Sera and blood cell pellets were collected and frozen at −70°C weekly during the first 4 weeks and every 2 weeks thereafter. Viral loads in PBMC specimens were assessed periodically by duplicate plating of 10^6 PBMCs and serial threefold dilutions of PBMCs on OMK cells in 24-well tissue culture plates (15). Animals

that became moribund were euthanized and received complete necropsies. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Immunoblotting and antibody. Cells were harvested and lysed with lysis buffer (0.15 M NaCl, 0.5% Nonidet P-40, 50 mM HEPES 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 10^5 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel, transferred onto nitrocellulose membranes, and reacted with antibody. Rabbit polyclonal STP-C488 antibody 109 used in these experiments has been described previously (22). Ras antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.) and Transduction Laboratories (Lexington, Ky.).

Isolation of genomic DNA and PCR analysis. Genomic DNA was isolated with a Qiagen genomic isolation kit according to the manufacturer's protocol. Five micrograms of purified genomic DNA was used for PCR amplication using 5′ and 3′ primers, which correspond to the upstream and downstream sequences, respectively, of the STP-C488 gene. Amplified DNA was cloned into the TA cloning vector (Invitrogen, San Diego, Calif.). Both strands of five independent clones were subsequently sequenced by using an ABI PRISM 377 automatic DNA sequencer.

FACS analysis. For fluorescence-activated cell sorting (FACS) 5×10^5 to 1×10^6 immortalized marmoset lymphocytes were washed twice in phosphate-buffered saline (PBS) and incubated with the appropriate monoclonal antibody for 30 min at 4°C. Thereafter, cells were washed twice with PBS, fixed with 2% paraformaldehyde, and analyzed with a FACscan (Becton Dickinson and Co., Mountain View, Calif.). Antibodies used in this study that react with common marmoset lymphocyte antigens included CD2-phycoerythrin (PE) (Becton Dickinson), CD3-fluorescein isothiocyanate (FITC) (PharMingen, San Diego, Calif.), CD4 (Olympus clone; Chromoprobe, Mountain View, Calif.), CD8-FITC (Coulter, Hialeah, Fla.), CD25-PE, and -FITC (Becton Dickinson), CD56-PE (Becton Dickinson), and HLA-DR PercP (Becton Dickinson).

Reporter assays. Approximately 10^7 cells were electroporated at 960 μF and 200 V. All transfections included the construct pGKβgal, which expresses β-galactosidase from a phosphoglucokinase promoter, together with construct OCT-SEAP, NFAT-SEAP, 3X-κB-luc, or TRE-luc. TRE-luc contains the AP-1 binding site upstream of a minimal *fos* promoter (21). As controls, cells were treated with 10% IL-2 or tetradecanoyl phorbol acetate (TPA). At 24 h posttransfection, cells were washed once in PBS and lysed in 200 μl of reporter lysis buffer (Promega, Madison, Wis.). Assays for luciferase or alkaline phosphatase activity were performed with a luciferase assay (Promega) or with the Phospha-Light chemiluminescence assay (Tropix) in a Luminometer. Values were normalized to β-galactosidase activity.

RESULTS

Isolation of HVSΔSTP/c-*ras* and HVSΔSTP/v-*ras* recombinants. To examine whether *ras* is capable of substituting for the STP oncogene in lymphocyte transformation, STP-C488 of HVS was replaced with a cellular normal H-*ras* (c-*ras*) or an oncogenic viral H-*ras* (v-*ras*). We have recently described construction of HVSΔSTP/SV40-SEAP, in which the STP gene was replaced with a gene for simian virus 40 (SV40)-secreted engineered alkaline phosphatase (SEAP) as a reporter for isolation of the recombinant HVS (16). As shown in Fig. 1, nononcogenic HVSΔSTP/SV40-SEAP virion DNA was used for isolating recombinant HVSΔSTP variants containing either the c-*ras* or v-*ras* gene via homologous recombination. The complete STP coding sequence was deleted from 3.6 kb of the left end of L-DNA of HVS C488 by PCR, and multicloning sites were inserted into the STP locus. Human cellular H-*ras* or oncogenic viral H-*ras* was cloned into the multicloning sites of 3.1 kb of L-DNA. In these constructs, c-*ras* and v-*ras* genes were under control of the STP promoter (19). Linearized plasmid DNA containing 3.5 kb of L-DNA with the *ras* gene insert was cotransfected into OMK cells with HVSΔSTP/SV40-SEAP virion DNA, using calcium phosphate. To isolate recombinant HVSΔSTP/c-*ras* or HVSΔSTP/v-*ras*, SEAP-negative virus was recovered from limiting dilutions (Fig. 1). To confirm the correct genetic structure of the recombinant virus, virion DNA from recombinant HVSΔSTP/c-*ras* or HVSΔSTP/v-*ras* was used for PCR using 5′ and 3′ primers (see Materials and Methods). Wild-type HVS C488 (wt HVS) virion DNA was used as a control for PCR analysis. Five of five plasmid clones

TABLE 1. In vitro and in vivo oncogenicity of HVSΔSTP/c-*ras*
or HVSΔSTP/v-*ras*

Virus	No. positive/no. tested			Survival (days)
	In vitro immortalization[a]	Virus recovery[b]	In vivo lymphoma	
None	0/10			
HVS C488	10/10	2/2	2/2	19 and 20
HVSΔSTP		2/2	0/2	>300
HVSΔSTP/SV40-SEAP	0/10	2/2	0/2	>300
HVSΔSTP/c-*ras*	2/10	2/2	1/2	38, >300
HVSΔSTP/v-*ras*	10/10	2/2	1/2	28, >300

[a] Primary T-lymphocyte immortalization.
[b] Virus was recovered from PBMCs of infected marmoset as described previously (15).

derived from virion DNA of this recombinant virus were shown
to contain the presence of c-*ras* or v-*ras*, the absence of unde-
sired aberrant mutations, and the absence of wild-type STP
sequence.

In vitro immortalization assay. Common marmoset T lym-
phocytes are immortalized efficiently to IL-2-independent
growth by infection with wt HVS (12, 15). To examine the
transforming activity of *ras* in the context of HVS, assays of in
vitro immortalization of primary marmoset T lymphocytes
were performed with HVSΔSTP/c-*ras* and HVSΔSTP/v-*ras*. wt
HVS and HVSΔSTP/SV40-SEAP were used as controls for
these assays. HVSΔSTP/c-*ras*, HVSΔSTP/v-*ras*, HVSΔSTP/
SV40-SEAP, and wt HVS at equivalent viral titers were added
to unstimulated PBMCs from 10 common marmoset donors.
The recombinant HVSΔSTP/v-*ras* uniformly immortalized
lymphocytes from all 10 of the common marmosets to IL-2-
independent growth as was seen with the wt HVS (Table 1). In
contrast, the recombinant HVSΔSTP/c-*ras* immortalized only 2
of the 10 primary cultures of common marmoset lymphocytes
(Table 1). These HVSΔSTP/c-*ras*-immortalized T cells were
highly sensitive for growth at low cell density. Additionally, the
growth of HVSΔSTP/c-*ras*-immortalized cells was significantly
retarded even after 6 months of culture. As described previ-
ously (17), HVSΔSTP/SV40-SEAP did not immortalized any
of these primary PBMCs (Table 1). These results demonstrate
the transforming capacity of the *ras* gene within the context of
the HVS genome. Additionally, they show that v-*ras* substitutes
for the STP oncogene much more efficiently than c-*ras*.

In vivo lymphoma induction. We have shown previously that
experimental infection of common marmosets with wt HVS
consistently induces lymphomas within 19 to 25 days of infec-
tion (12, 15). To further investigate the oncogenic potential of
HVSΔSTP/c-*ras* and HVSΔSTP/v-*ras*, two common marmosets
were injected intramuscularly with 10^5 TCID$_{50}$ of each virus in
a volume of 1 ml. Two marmosets were inoculated with wt
HVS as positive controls, whereas two marmosets were inoc-
ulated with each of HVSΔSTP or HVSΔSTP/SV40-SEAP as
negative controls. Consistent with prior experiments, infection
with wt HVS induced lymphomas in common marmosets. Mar-
mosets infected with wt HVS were sacrificed on days 19 and 20
postinfection, when death appeared imminent (Table 1). One
marmoset infected with HVSΔSTP/c-*ras* and one infected with
HVSΔSTP/v-*ras* also developed lymphoma. The onset of ter-
minal illness with HVSΔSTP/v-*ras* was slightly delayed com-
pared to wt HVS, while it was further delayed with HVSΔSTP/
c-*ras* (Table 1). The animals infected with HVSΔSTP/v-*ras*
or HVSΔSTP/c-*ras* were sacrificed on day 28 or 38 post-
inoculation, respectively. The two marmosets infected with
HVSΔSTP/c-*ras* or HVSΔSTP/v-*ras* remain alive 10 months

postinoculation. Additionally, four of four marmosets infected
with HVSΔSTP/SV40-SEAP or HVSΔSTP remained healthy
for over 10 months after infection (Table 1). Viruses were
isolated from all of the infected animals at week 2 postinocu-
lation and from the remaining live animals after 20 weeks
postinoculation (data not shown). To confirm the presence of
the *ras* gene and the absence of the STP gene after infection,
virus was isolated from PMBCs of infected animals by cocul-
tivation with OMK cells. One microliter of isolated virus was
directly used for PCR to amplify the *ras* gene. Additionally,
genomic DNA of lymph nodes of sacrificed animals was used
for PCR and sequence analysis to amplify the *ras* gene. DNA
sequencing confirmed that the amplified fragment contained
the c-*ras* or v-*ras* gene, with no sign of contamination of wt
HVS and with no sequence variation.

Necropsy of animals infected with HVSΔSTP/c-*ras*, HVSΔ
STP/v-*ras*, and wt HVS revealed multicentric lymphoma consis-
tent with HVS-induced pathology as previously described (12,
15). The most extensive neoplastic infiltrates were observed in
the kidneys of wt HVS-, HVSΔSTP/c-*ras*-, or HVSΔSTP/v-*ras*-
infected marmosets (data not shown). No differences were
noted in the nature of lymphomas induced by wt HVS,
HVSΔSTP/c-*ras*, or HVSΔSTP/v-*ras*. Thus, these results dem-
onstrated that recombinant HVSΔSTP/c-*ras* and HVSΔSTP/v-
ras induced lymphomas in common marmoset.

**Surface expression of lymphocyte antigens in transformed
cells.** Common marmoset cells immortalized by wt HVS have
been shown to be CD3$^+$ CD4$^-$ CD8$^+$ CD56$^+$ T cells which are
most likely derived from a population of natural killer cells
(14). Common marmoset cells transformed by wt HVS,
HVSΔSTP/c-*ras*, or HVSΔSTP/v-*ras* were used to examine the
surface expression of lymphocyte antigens by FACS analysis.
We immortalized PBMCs from the same marmoset donor with
wt HVS, HVSΔSTP/c-*ras*, or HVSΔSTP/v-*ras* for comparative
analyses. Common marmoset cells transformed by wt HVS
were CD3$^+$ T lymphocytes which were CD4$^-$ CD8$^+$ single-
positive cells as found previously, whereas common marmoset
cells transformed by HVSΔSTP/c-*ras* or HVSΔSTP/v-*ras* were
CD3$^+$ T lymphocytes which were mainly CD4$^+$ CD8$^+$ double-
positive cells (Fig. 2). Additional common marmoset cell lines
independently transformed by HVSΔSTP/c-*ras* or HVSΔSTP/
v-*ras* showed the same phenotypes as those described above
(data not shown).

Surface expression of HLA-DR and CD25, which have been
shown to be associated with the activation of T cells (34, 36),
was measured on common marmoset T cells immortalized by
wt HVS, HVSΔSTP/c-*ras*, or HVSΔSTP/v-*ras*. Surface expres-
sion of CD25 and HLA-DR on HVSΔSTP/c-*ras*-transformed T
cells was approximately 10- and 100-fold less than that on wt
HVS- or HVSΔSTP/v-*ras*-transformed T cells (Fig. 2). Thus,
results demonstrated that transformation of common marmo-
set PBMCs with HVSΔSTP/v-*ras* leads to a highly activated,
fully transformed phenotype, as seen with wt HVS infection,
that is not completely mimicked with HVSΔSTP/c-*ras*.

**Morphologic changes in HVSΔSTP/v-*ras*-transformed T
cells.** Numerous adherent cells were observed among common
marmoset T cells immortalized by HVSΔSTP/v-*ras* (Fig. 3).
Over 60% of the common marmoset T cells immortalized by
HVSΔSTP/v-*ras* exhibited an adherent phenotype with an ap-
pearance of the mature macrophages (Fig. 3). In contrast, cells
immortalized by wt HVS and HVSΔSTP/c-*ras* were not adher-
ent (Fig. 3). To investigate the origin of these adherent cells,
we used FACS analysis to examine the surface expression of
lymphocyte markers. These experiments showed that the ad-
herent cells had the same surface phenotypes as the floating
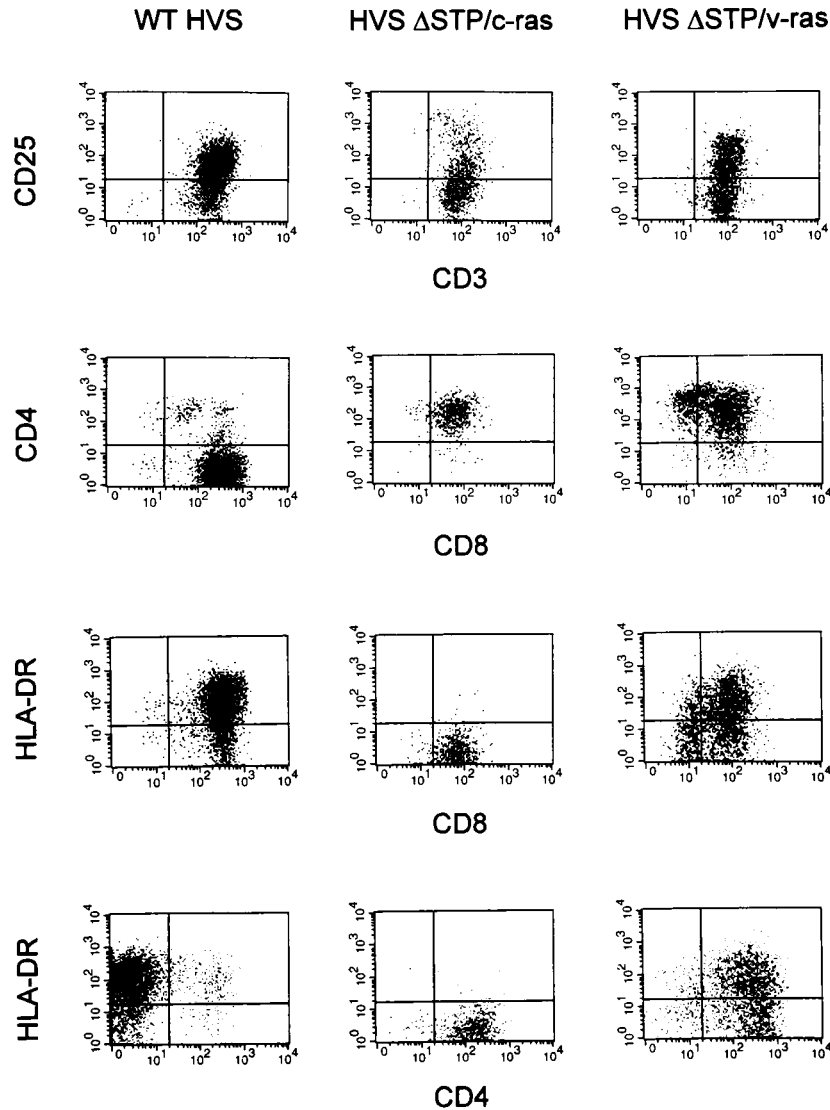HVSΔSTP/v-*ras*-transformed T cells, which were CD3$^+$ CD4$^+$

FIG. 2. Analysis of surface expression of lymphocyte antigens on wt HVS-, HVSΔSTP/c-*ras*-, or HVSΔSTP/v-*ras*-transformed cells. Surface phenotypes were determined by flow cytometry. Positive gates were established by using an isotype-matched control antibody.

CD8^{+}. Additionally, both adherent and floating HVSΔSTP/v-*ras*-transformed T cells were negative for surface expression of CD11b, a marker for macrophages (data not shown).

ras expression in transformed cells. Expression of STP-C488 and *ras* in common marmoset T cells immortalized by wt HVS, HVSΔSTP/c-*ras*, or HVSΔSTP/v-*ras* was examined by immunoblot analyses. STP-C488 expression was not detected in common marmoset T cells immortalized by recombinant HVSΔSTP/c-*ras* or HVSΔSTP/v-*ras*, while it was readily detected in common marmoset T cells immortalized by wt HVS (Fig. 4). Surprisingly, *ras* expression was much higher in common marmoset T cells immortalized by recombinant HVSΔSTP/v-*ras* than in cells immortalized by HVSΔSTP/c-*ras* (Fig. 4). Also, cellular *ras* expression in wt HVS-transformed cells was very low (Fig. 4). Since the *tip* gene is downstream of the STP gene in a bicistronic transcriptional unit, we also investigated whether insertion of the *ras* gene at the STP locus affected the expression of *tip*. Lysates of wt HVS-, HVSΔSTP/c-*ras*-, or HVSΔSTP/v-*ras*-immortalized T cells were used for

immunoprecipitation with anti-Tip antibody followed by in vitro kinase reaction. This analysis showed the equivalent levels of tyrosine kinase activity associated with *tip* in different cells (data not shown). Thus, the replacement of STP-C488 with *ras* did not alter the expression of *tip* detectably.

Activation of AP-1 transcription factor activity in HVSΔSTP/v-*ras*-transformed cells. Activation of cellular *ras* signal transduction leads to activation of AP-1 transcription factor activity, which ultimately induces the expression of a number of cellular genes (21). We measured AP-1 transcriptional factor activity by using a reporter construct. The TRE-luc reporter construct containing the AP-1 binding site upstream of a minimal *fos* promoter and the pGKβgal construct containing the β-galactosidase gene downstream of a phosphoglucokinase promoter were electroporated into wt HVS- and HVSΔSTP/v-*ras*-transformed cells. Since the growth of common marmoset T cells immortalized by HVSΔSTP/c-*ras* was greatly retarded, these cells were not used in these assays. Twenty-four hours after electroporation, luciferase activity was measured
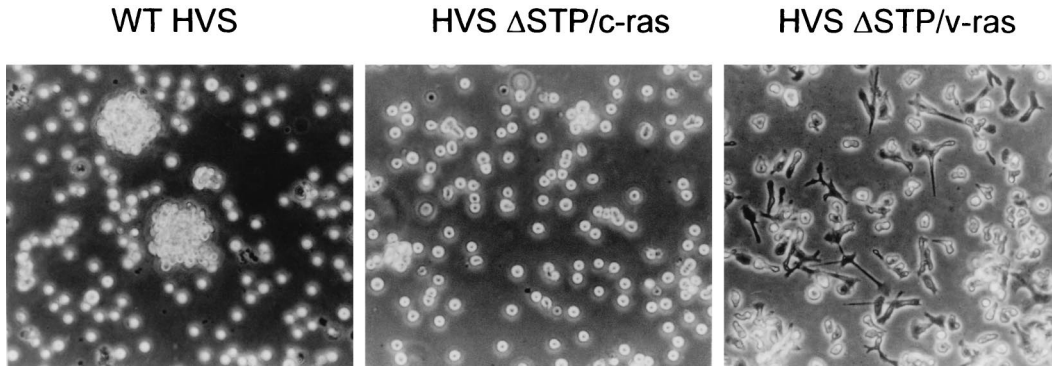
WT HVS   HVS ΔSTP/c-ras   HVS ΔSTP/v-ras

FIG. 3. Morphological changes in common marmoset T cells transformed by HVSΔSTP/v-*ras*. Cells were photographed with a phase microscope at a magnification of ×100.

and normalized to β-galactosidase activity. We observed that AP-1 transcription factor activity was approximately 20-fold higher in HVSΔSTP/v-*ras*-transformed cells than in wt HVS-transformed cells (Fig. 5). As controls, wt HVS- or HVSΔSTP/v-*ras*-transformed cells were treated with TPA or IL-2 for 48 h. This analysis showed dramatically increased AP-1 activity in these cells (data not shown).

We similarly measured NF-κB, NFAT, and OCT-1 activity in these cells by using reporter constructs. wt HVS- or HVSΔSTP/v-*ras*-transformed T cells were transfected with NF-κB-driven reporter plasmid 3X-κB-luc, NFAT-SEAP, or OCT-SEAP and control β-galactosidase plasmid pGKβgal. Unlike AP-1 activity, NF-κB, NFAT, and OCT-1 transcriptional activities were not altered in these cell lines (Fig. 5). Thus, the results demonstrated that HVSΔSTP/v-*ras*-transformed cells showed a specific increase of AP-1 transcriptional factor activity compared to wt HVS-transformed T cells.

DISCUSSION

The results described here demonstrate that *ras* is capable of substituting for the STP oncogene of HVS in in vitro T-lymphocyte transformation and in lymphoma induction in vivo. Recombinant HVSΔSTP/v-*ras* induced transformation much more efficiently than did HVSΔSTP/c-*ras*. Additionally, the activation of *ras* signal transduction in HVSΔSTP/v-*ras*-immor-

talized T cells resulted in a significant increase in AP-1 transcriptional factor activity as well as an adherent macrophage-like morphology. These data support an important role for *ras* signal transduction in STP-dependent cell growth transformation of HVS subgroup C strains.

Ras plays a pivotal role in a variety of signal transduction and differentiation processes. Ras is a plasma membrane-associated guanine nucleotide-binding protein that cycles between a GDP-bound form and a GTP-bound form (2, 7). Oncogenic mutant forms of Ras display an increased proportion of Ras in the GTP-bound state relative to the GDP-bound state, which activates the biological functions of Ras. Ras-GTP exerts its biological effects by interacting with cellular effector molecules such as Raf and phosphatidylinositol 3-kinase (26, 31, 37, 39, 40). We have demonstrated that the association of STP-C488 with *ras* activates *ras* signaling pathways, as shown by an increase in the ratio of Ras-GTP to Ras-GDP and by the constitutive activation of mitogen-activated protein kinase (24). Additionally, STP-C488 is able to induce PC12 cell differentiation similarly to nerve growth factor, which has been
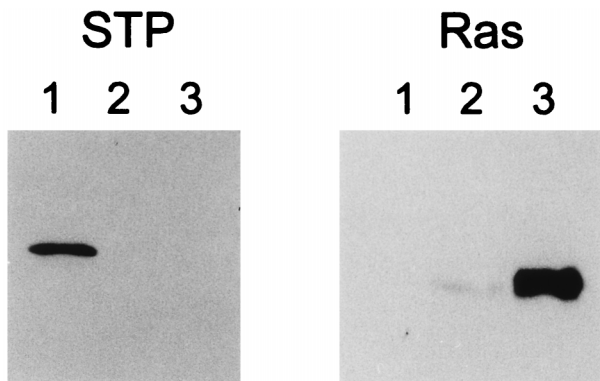

FIG. 4. Immunoblot analysis of HVSΔSTP/c-*ras*- or HVSΔSTP/v-*ras*-transformed cells. The same amounts of precleared lysates of 5×10^6 cells were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with antibodies against STP-C488 or Ras. Reactivity was detected by enhanced chemiluminescence. Lane 1, wt HVS-transformed cells; lane 2, HVSΔSTP/c-*ras*-transformed cells; lane 3, HVSΔSTP/v-*ras*-transformed cells.
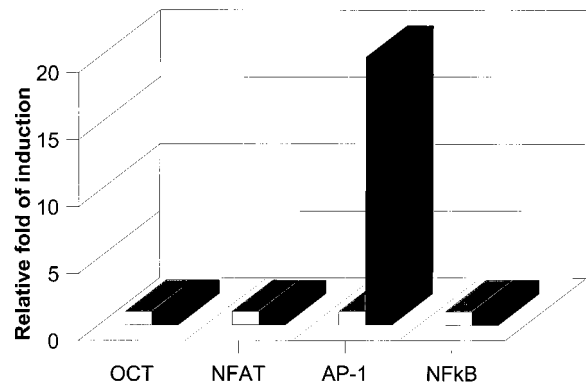

FIG. 5. Activation of AP-1 transcription factor activity in HVSΔSTP/v-*ras*-transformed cells. A total of 10^7 cells transformed by wt HVS (□) or HVSΔSTP/v-*ras* (■) were electroporated at 960 μF and 200 V with 30 μg of pGKβgal and 30 μg of reporter plasmid TRE-luc, NF-κB-driven 3X-κB-luc, NFAT-SEAP, or OCT-SEAP. At 24 h after transfection, cell supernatants and lysates were used for luciferase, alkaline phosphatase, and β-galactosidase assays. Luciferase and alkaline phosphatase activities were determined and normalized on the basis of β-galactosidase activities. Fold activation represents normalized luciferase and alkaline phosphatase activity relative to that of the wt HVS-transformed cells 24 h after transfection. Values represent averages of three independent experiments.
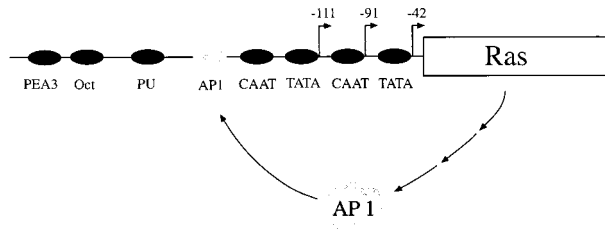
FIG. 6. Schematic diagram of upregulation of *ras* expression from STP promoter. The −91 basal transcriptional initiation site and −42 and −111 TPA-inducible transcriptional initiation sites are indicated with arrows as described previously (19).

shown to act through the *ras* signal transduction (24). Thus, the transforming activity of STP-C488 appears to be dependent on the activation of *ras* signal transduction pathways.

While recombinant *ras* viruses are capable of inducing in vitro T-lymphocyte transformation, cells immortalized by these viruses are phenotypically different from those immortalized by wt HVS. wt HVS-immortalized cells are CD4− CD8+ single-positive T cells, whereas HVSΔSTP/c-*ras*- and HVSΔSTP/v-*ras*-immortalized cells are CD4+ CD8+ double-positive T cells. While human and rhesus T cells immortalized by HVS are often CD4+ CD8+ double positive, common marmoset T cells immortalized by HVS have always been CD4− CD8+ single positive (1, 3–5, 8, 23, 30). Lymphocytes coexpressing CD4 and CD8 antigens have been shown in normal human PBMCs; however, these cells constitute less than 3% of the total T-cell population (6). Lectin treatment in vitro has been shown to stimulate the generation of CD4+ CD8+ cells in human PBMCs, although the functional role of these cells is unclear (6). Thus, it seems likely that HVSΔSTP/c-*ras* and HVSΔSTP/v-*ras* can alter the expression profile of lymphocyte surface antigens upon immortalization instead of selectively targeting this cell type for immortalization.

It is intriguing that the level of *ras* expression is significantly higher in cells transformed by HVSΔSTP/v-*ras* than in those transformed by HVSΔSTP/c-*ras* (Fig. 4). Expression of *ras* in the recombinant HVSΔSTP/v-*ras* is driven by the STP-C488 promoter. In fact, STP-C488 transcription has been shown to be highly inducible upon phytohemagglutinin or TPA stimulation (19), and the STP promoter has been shown to contain a putative AP-1 transcription factor binding site at position −138 relative to the translational initiation site (19). These features suggest that activation of the cellular AP-1 transcriptional factor by upregulation of the *ras* signal transduction pathway in HVSΔSTP/v-*ras*-transformed cells contributes to activation of the STP promoter by positive feedback, which may ultimately lead to the high level of v-*ras* gene expression (Fig. 6). Thus, the high level of *ras* expression as well as oncogenic *ras* activity may enhance the transforming efficiency of HVSΔSTP/v-*ras* compared to HVSΔSTP/c-*ras*.

Over 60% of common marmoset T cells immortalized by HVSΔSTP/v-*ras* displayed an adherent phenotype. FACS analysis showed that these adherent cells were not derived from macrophage lineage. Several changes in cell morphology take place during lymphocyte activation (11). Spherical resting T cells become polarized during activation, developing a well-defined cytoplasmic projection which has been called the cellular uropod (11). It can be hypothesized that activation of the cellular AP-1 transcriptional factor by upregulation of the *ras* signal transduction pathway in HVSΔSTP/v-*ras*-transformed cells induces the surface expression of adhesion molecules, which in turn alters cellular morphology. In fact, expression of

cellular adhesion molecules including intercellular cell adhesion molecule 1 and vascular cell adhesion molecule 1 is regulated by the AP-1 transcription factor (10, 38). However, because of the lack of antibodies which cross-react with the surface antigens of New World monkey lymphocytes, expression of these adhesion molecules could not be assessed on the immortalized marmoset cell lines.

In this report, we have shown that an activated form of *ras* can efficiently substitute for the STP oncogene in the context of HVS to induce lymphocyte transformation. However, only one of two common marmosets infected with recombinant HVSΔSTP/v-*ras* or HVSΔSTP/c-*ras* developed lymphoma, while two of two marmosets infected with wt HVS developed lymphoma. Additionally, the onset of disease induction with HVSΔSTP/v-*ras* was delayed slightly compared to that with wt HVS, whereas it was even further delayed with HVSΔSTP/c-*ras*. Thus, while activated *ras* appears sufficient to substitute for the STP oncogene in transformation in the context of viral infection, certain factors may influence the efficiency with which it can do so. For example, the level or timing of *ras* expression or activation could influence a number of critical variables such as cell survival, susceptibility to immune attack, or per-cell production of virus. Further, additional cellular genes other than *ras* may be involved in STP function. Recently, we have found that STP binds to tumor necrosis factor receptor-associated factors, and this interaction induces NF-κB activation (unpublished results). Thus, these factors may influence the efficiency of lymphoma induction by the recombinant *ras* viruses. Nevertheless, results in this report support an important role for *ras* signal transduction in STP-dependent cell growth transformation by group C strains of HVS. Also, this is the first demonstration at least among gamma herpesviruses that the activated form of a cellular partner can substitute for the activity of a viral gene in vitro and in vivo.

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