Simian Virus 40 large Tumor antigen modulates the Raf signaling pathway

1Grammatikakis, N., 2Jaronczyk, K., 1Grammatikakis, A., 2Vultur, A., 2,3Brownell, H.L., 2Benzaquen, M., 2Rausch, C., 2Lapointe, R., 4Gjoerup, O., 4Roberts, T.M. and 2Raptis, L.

1Dept. of Physiology, Tufts University School of Medicine, Boston, MA. 2Departments of Microbiology and Immunology and Pathology, Queen's University, Kingston, Ont. Canada, K7L 3N6. 3Present address: Joslin Diabetes Center, Boston, MA. 4Department of Cancer Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

*Corresponding author

Address: Dept. of Microbiology and Immunology, Botterell Hall, Rm. 715, Queen's University, Kingston, Ontario, Canada K7L3N6.
Phone number: (613) 533-2462
FAX number: (613) 533-6796
e-mail: RAPTISL@POST.QUEENSU.CA

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Abstract
The large Tumor antigen of SV40 (SVLT) is a potent oncogene. Although inactivation of the p53 and pRb tumour suppressors has been causally linked to SVLT's transforming properties, its exact mechanism of action remains undefined. Previous data indicated that Ras is activated in SVLT expressing cells. In this report we show that SVLT also increases Raf kinase activity in both insect and mammalian cells, thus identifying the Raf kinase as an additional target of SVLT. Our results further show that SVLT was still able to activate Raf in cells where Ras levels had been drastically reduced through expression of an antisense construct, indicating that SVLT may activate Raf at least partly by a mechanism which is independent of its stimulatory effect on Ras.
Introduction

The DNA tumour virus Simian virus 40 (SV40) has been associated with human malignancies, particularly malignant mesothelioma (1,2). Neoplastic transformation by this virus is mediated mainly by its Large Tumor antigen (SVLT), a nuclear oncoprotein capable of transforming a variety of mammalian cell types (3,4). Deletion mutagenesis studies revealed that this ability may result from its interaction with a number of cellular gene products, including the tumor-suppressor proteins, p53 and members of the retinoblastoma-susceptibility gene product (pRb) family [pRb, p107, p130, reviewed in (5,6)]. SVLT binds to pRb family members and inactivates their ability to restrain cell proliferation.

We have previously demonstrated that neoplastic transformation by SVLT requires the activity of the cellular Ras protooncogene product (c-Ras or Ras), which is a key player in a pathway that relays signals from membrane tyrosine kinases to the nucleus (7,8). SVLT was unable to fully transform cells where endogenous Ras levels were reduced through the introduction of a ras-antisense construct or the dominant-negative mutant, Ras$^{\text{N17}}$ (9). Later work (10) also showed that pRb inactivation in cells from pRb-null mice causes a dramatic increase in Ras activity. To investigate whether SVLT might be activating c-Raf (Raf), the most prominent Ras downstream target, recently shown to be important in opposing apoptosis (11), we examined the effects of SVLT upon Raf catalytic activity. To examine whether activation of this pathway by SVLT requires extensive protein synthesis, we reconstituted the system in baculovirus/Sf9 insect cells,
which is currently the most widely used model for measuring the activity of putative Raf regulators (12). In conjunction with insect cell systems, we examined these relationships in mammalian cells, where these components were either stably or transiently expressed through transfection. The results show that aside from Ras, SVLT can also activate Raf. Interestingly, this effect may be, at least in part, Ras-independent since SVLT-mediated Raf activation can take place in cell lines where Ras levels have been reduced through antisense Ras expression.
Experimental procedures

Cell culture and gene expression

SVLT-expressing, rat F111 cells were prepared through infection with a pBabeHygro-based retroviral vector and Hygromycin resistance selection (9). For the production of Ras-deficient cells, mouse 10T½ fibroblasts were transfected with a ras-antisense construct expressing the EJ-ras gene downstream and in the opposite orientation from the mouse metallothionein promotor. These cells express approximately 30% the Ras levels present in the parental line. SVLT was expressed in these cells using the same retroviral vector and Hygromycin resistance selection. A number of lines were produced which permanently express the ras-antisense and SVLT (9). c-Ras levels were restored to the baseline in these cells as follows: A 2.3 kb fragment from the human c-H-ras-1 gene, coding for the four ras exons and three introns as well as a poly-A addition signal sequence, was introduced into the retroviral vector pDOL at the unique BamHI cloning site. The ψ2 packaging line was transfected with this plasmid and the culture supernatant from a mixture of G418-resistant stable transfectants was used to infect the 10T½ cells and their derivatives. Due to aberrant splicing of the message in the packaging line caused by the ras introns, more than 95% of the infected target cells contain various combinations of ras gene exons, while a very small proportion of them expresses a correctly spliced c-Ha-ras gene (7). This would ensure that the antisense ras RNA is inactivated, while a functional, transforming ras-sense message is not being produced (7). Therefore, approximately one thousand Ras-deficient 25B8, 25SV7t or control 10T½ cells, growing
in a 6 cm petri, were infected with 3 mls of filtered culture supernatant from \( \psi^2 \) cells transfected with this plasmid and the Ras levels, as well as the phenotypic characteristics of the cells examined.

Rat F111 and mouse 10T½ fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Freshly plated cells were transfected at 70-80% confluence using superfect (Qiagen). Cell cultures were harvested and lysed 48 hours post transfection.

**Production of baculovirus vectors**

Sf9 cells were grown at 27.5°C, in TC-100 medium supplemented with 10% fetal calf serum (Gibco). The wt SVLT or the K1 SVLT mutant insert was excised from the original pSG5 vector [a gift of J. DeCaprio (13)] and subcloned in the BamH1 site of the pVL1393 vector (Pharmingen). This plasmid was transfected into insect Sf9 cells together with the baculo-Gold vector, according to the manufacturer's instructions (Pharmingen). Plaques were picked and recombinant virus propagated for use in infection experiments. The baculovirus encoding GST-SVLT(1-147) or GST-SVLT(1-147 D44N) mutant (14) was constructed by first performing PCR amplification from pGEX-4T-2 containing either the SVLT(1-147) or SVLT(1-147 D44N) cDNA insert. Subsequently, the amplification products were cloned into pVL 1392 and recombinant baculoviruses produced using BaculoGold (BD Pharmingen).

Baculovirus infections were performed essentially as described (12,15). The amounts of infecting viruses were adjusted to keep the amounts of the proteins being studied the same when protein production in a single infection was compared with the one
in a double or triple infection. To accomplish this, preliminary experiments were
performed, using different ratios of the infecting viruses over a range of multiplicities of
infection at each ratio (16,17). Cells were harvested 48 hours later. Protein production was
monitored by immunoblotting.

**Antibody reagents**

Anti-active Mek antibodies (No 9120) were obtained from QCB/Biosource and
NEB. The anti-Ras antibody pan-Ras Ab2 was from Oncogene Science and anti-SVLT
antibodies (Pab 101 and Pab 108) were from Pharmingen.

**Kinase assays**

For Raf kinase assays, cells were lysed 48 hrs after transfection of mammalian
cells or infection of Sf9 cells with the indicated combinations of expression vectors and
Raf was immunoprecipitated with a C-terminal specific Raf antibody (a gift of R.
Narsimhan). Following two washes in kinase buffer (25 mM Hepes, pH 7.5, 10 mM
MgCl2, 1 mM DTT), [γ³²P]ATP and protein substrate (kinase-dead Mek, K97M) were
added to the immunoprecipitate and incubated for 30 min at room temperature (12,15).
Labeled proteins were resolved by polyacrylamide gel electrophoresis and visualized by
exposing the dried gels to X-Ray film. Quantitation was performed by phosphorimager
analysis using the ImageQuant program.

**Neoplastic transformation assays**

For anchorage-independent growth measurements, approximately 10⁴ cells were
suspended in 2 ml of 0.33% Agarose (Sigma) containing Dulbecco-modified Eagle's
medium supplemented with 15% fetal calf serum, on top of a feeder layer of the same
medium containing 0.7% agarose, in 6 cm petri dishes (18). Growth was recorded and photographs taken 10 days later under brightfield illumination. Foci formation assays were performed by plating approximately 200 SVLT expressing cells together with $10^4$ normal cells in a 6 cm petri as described before (19).
**Results**

**SVLT stimulates Raf catalytic activity in insect and mammalian cells**

Previous results showed that SVLT expression results in Ras activation (9). SVLT is also well known to interact *in vivo* with uncharacterized kinases [(20) and references therein]. To examine whether SVLT might be activating Raf, a serine/threonine kinase and the most prominent Ras downstream target, we examined the effect of SVLT upon the Raf catalytic activity. To this end, we reconstituted the system in baculovirus infected, insect Sf9 cells, a model which has been widely used in the past in assessing the effect of potential Raf regulators (12). Raf, alone or in conjunction with SVLT, was expressed in Sf9 cells using baculovirus vectors and its activity examined by *in vitro* kinase assay (Fig. 1). Detergent cell extracts were precipitated with an anti-Raf antibody and the Mek substrate added to washed immunoprecipitates together with $[^{32}\text{P}]$ATP as described in Experimental procedures. Assays were terminated by the addition of SDS-loading buffer, the boiled samples resolved by SDS-PAGE and phosphorylated proteins visualised by autoradiography of dried gels. In accordance with the well established notion that Raf's capacity for autophosphorylation is a direct reflection of its kinase activity assayed *in vitro* (17), the ability of Raf to phosphorylate itself as well as the exogenously added Mek substrate was increased by SVLT expression (Fig. 1A, lane 2 vs lane 3). As a control, we examined the effect of SVLT upon coexpressed kinase-dead Raf (Raf-K375M). As shown in Fig. 1B, lanes 5 and 6, SVLT coexpression had no effect upon the background kinase activity associated with the mutated Raf. Similarly, coinfection with SVLT and an empty
baculovirus vector lacking an insert did not reveal any Raf labelling (Fig. 1B, lanes 7 and 8). These results taken together indicate that SVLT stimulates the catalytic activity of the Raf kinase in insect cells.

To further quantitate the effect of SVLT upon Raf activity, we determined the degree of Raf stimulation by different amounts of co-expressed SVLT. Sf9 cells were infected with recombinant baculovirus vectors expressing either (i) Raf and Ras, or (ii) Raf, Ras and SVLT. As shown in Fig. 2, the catalytic activity of Raf immunoprecipitated from cells infected with a 1/20 dilution of the Raf-expressing vector was low (lane 2), but progressively increased upon coinfection with the SVLT-expressing vector, in proportion to the amount of SVLT in the cell, as assessed by Western blotting (lanes 3-5). This increase was evident even in the absence of Ras coexpression (lanes 10 and 12). Moreover, SVLT coinfection dramatically increased Raf kinase when the Raf vector was used at an 1/100 dilution (lane 6 vs 7-9), although, as expected, the overall signal was weaker, due to the lower amounts of Raf protein expressed. The above results taken together suggest that, same as the membrane-bound tyrosine-kinases (17), SVLT can stimulate the catalytic activity of Raf in this system.

Extensive genetic and biochemical evidence has shown that full neoplastic transformation by SVLT requires binding to proteins of the retinoblastoma family (5). To examine the importance of pRb binding by SVLT for Raf activation, we tested the effect of the transformation-defective mutant K1 which is altered at the pRb binding site [glu107 to lys, (4)]. Sf9 cells were infected with different amounts of recombinant baculovirus vectors expressing wt SVLT or the K1 mutant, and their effect upon the co-expressed Raf
kinase measured as above. As shown in Fig. 1B, coexpression of the K1 mutant stimulated Raf activity but to a lesser extent than the wild type (compare lanes 1 and 2 with 3 and 4). These results indicate that, although the pRb binding site is required for full Raf activation by SVLT in Sf9 cells, some activation is present even in the absence of this sequence.

The structure of SVLT as well as all other oncogenes of the polyoma viruses has evolved to include a conserved N-terminal DnaJ domain which has been shown to be essential for their individual functions (21). DnaJ proteins normally regulate the folding of protein substrates by the heat-shock protein 70 (Hsp70) chaperone family (22). Both the critical importance of the J domain in SVLT function and the absolute requirement for the Hsc70 binding site in cis with the known pRb and p53 binding motifs for SVLT-mediated transformation have been established (5,6,23). To examine the role of the DnaJ domain for Raf activation by SVLT, the effect of a mutant known to disrupt the function of this domain [asp44 to asn, 1-147, see Experimental Procedures (4)] was tested. Sf9 cells were infected with different amounts of recombinant baculovirus vectors expressing wt SVLT or the D44N mutant, and their effect upon the co-expressed Raf kinase measured as above. As shown in Fig. 3, coexpression of this mutant stimulated Raf activity but to a lesser extent than the wild type (compare lanes 3 and 9), indicating that full Raf activation by SVLT requires an intact DnaJ domain.

To further investigate whether the observed Raf activation by SVLT is also occurring in mammalian cells, in the absence of viral infection and protein overexpression, we examined the stimulation of Raf kinase by SVLT in rat F111 fibroblasts. SVLT was expressed in these cells through the retroviral vector pBabeHygro and a typical clone,
FSV1a, was chosen for further study (9). The kinase activity of endogenous Raf was measured in these cells before or after stable SVLT expression using the kinase-dead Mek as a substrate, as described above. As shown in Fig. 4A, phosphorylation of the Raf substrate is approximately four times as high in SVLT-expressing, FSV1a cells compared to their normal F111 counterparts. To test whether recombinant Raf can also be activated by SVLT after transient expression, we also measured the in vitro catalytic activity of exogenous GST-tagged Raf, produced through transfection of plasmid pEBG-Raf (15) in the two lines. As shown in Fig. 4B (lanes 2 and 3), phosphorylation of the Raf substrate was twice as high in SVLT-expressing FSV1a cells. Furthermore, in addition to cell lines permanently expressing SVLT, we transiently coexpressed SVLT along with Raf by transfection in F111 fibroblasts and compared Raf activation with cells transfected with Raf alone. Detergent cell lysates were precipitated with GSH-sepharose and Raf activity determined using the inactive Mek substrate as above. As shown in Fig. 4B (lanes 4 and 5), Raf activity was approximately three fold higher in cells transiently transfected with SVLT and Raf (lane 5), compared to cells transfected with Raf alone (lane 4), while the SVLT-K1 mutant activated Raf to approximately 60% of the wild-type (lane 6). Similar results were obtained with SVLT-expressing mouse 10T½ cells (not shown). The above data taken together indicate that, aside from Sf9 cells, SVLT can also activate the Raf kinase in rodent fibroblasts.

**SVLT effects are partly Ras independent**

The Raf protooncogene product has been shown to be physically and functionally
associated with Ras, which is known to transmit signals from tyrosine kinase receptors to Raf and other downstream effectors (24). Therefore, we investigated the possibility whether Ras might be mediating the transmission of SVLT signals to Raf and its downstream effectors, or whether SVLT's effects on Raf activity might be, at least partly, independent of Ras. To this end, we expressed SVLT through retroviral infection in 10T½-derived cell lines where Ras expression was downregulated through transfection with a ras-antisense construct (line 25B8, see Experimental procedures) (9). Individual colonies were picked, expanded into clones, tested for SVLT levels by Western blotting and clones with high SVLT levels (e.g. 25SV7t) were examined further. Detergent cell lysates were prepared and Raf kinase activity measured using the kinase-dead Mek as substrate as described above (15). As shown in Fig. 5A, SVLT caused a dramatic increase in phosphorylation of the kinase-dead Mek Raf substrate, added to anti-Raf immunoprecipitates from the Ras-deficient 25B8 cells, indicating that Raf activation by SVLT is not substantially inhibited by Ras downregulation. In addition, as shown in Fig. 5B, activation of endogenous Mek by SVLT, as detected with an antibody specific for its activated form, can occur in the Ras-deficient cells to the same extent as in the parental line indicating that, in addition to Raf, Mek activation by SVLT can occur in the face of low endogenous Ras levels.

pRb inactivation by SVLT has been demonstrated to lead to Ras activation (10). Since, as shown above, SVLT is able to activate Raf in mammalian cells even after Ras downregulation, it is possible that the presence of the pRb binding site might not be an
absolute requirement for SVLT transformation in this system. Therefore, we examined the phenotypic effect of the K1 mutant which is unable to bind pRb and can activate Raf to approximately 60% the levels of the wt (Fig. 1B, lanes 3 and 4 vs lanes 1 and 2 and Fig. 4B, lanes 5 and 6). Rat F111 clones stably expressing the K1 mutant were produced through transfection and examined for their transformation-related parameters (19). As shown in Fig. 6, K1-expressing F111 cells (FK1) displayed a transformed morphology on plastic (A), although they were unable to grow in agar (C), indicating that this mutant is partially transforming in this system (25). In addition, K1-expressing F111 cells produced approximately 50% the number of discernible foci on plastic as formed by the wt.

We have previously shown that full neoplastic transformation by SVLT requires the function of c-Ras (9). However, as shown in Fig. 6, SVLT expression in the Ras-deficient, 10T½ fibroblasts (e.g. clone 25SV7t) did bring about morphological transformation (panel E vs F), although not the ability to grow under anchorage-independence conditions (H). These results are consistent with the interpretation that the Raf activation seen under conditions of low Ras levels may be sufficient to bring about a partial transformation.

To ensure that the observed phenotype is indeed due to c-Ras downregulation, c-Ras levels were restored to the baseline by infection with the culture supernatant from ψ2 packaging cells transfected with a retroviral vector carrying an intron-containing, Ras gene insert [see Experimental procedures (7)]. Infected cells displayed normal c-Ras levels by Western blotting and a near-normal growth rate within twenty days (9), while their uninfected counterparts maintained the slow growth characteristics for at least a year in
culture (7,9). Moreover, infected 25SV7t cells were able to grow in agar in a manner indistinguishable from their counterparts with normal Ras levels (9). Presumably, the high expression levels of a combination of ras exons in the target lines under control of the strong murine leukemia virus long terminal repeat promotor are responsible for inactivation of the ras antisense, thus restoration of normal growth rate and transformation by SVLT.
Discussion

Results from a number of laboratories have indicated that activation of Raf normally requires binding to Ras which brings Raf to the plasma membrane for further activation through tyrosine phosphorylation (26,27). However, Ras-independent mechanisms for the activation of Raf and its downstream effectors have also been described in both insect and mammalian cells. For example, in baculovirus-infected insect cells v-Src has been shown to activate Raf in a Ras-independent manner (16), while in mammalian cells (28,29) integrin or TPA-mediated Erk activation can occur via a Ras-independent pathway (30-32). In the context of SVLT signalling, it was recently shown that SVLT transfection in NIH3T3 cells bypasses the requirement for Ras or Src function during PDGF-stimulated DNA synthesis (33).

Several lines of evidence point to the possibility that Raf activation by SVLT might be partly independent of Ras:

1. SVLT expression in Sf9 cells does increase the activity of co-expressed Raf, although to lower levels than the combination of SVLT plus Ras. Although the endogenous Ras might be able to compensate to some extent, its levels would be very low, compared to the virally expressed SVLT and Raf.

2. Albeit to lower levels than wt-SVLT, the K1 mutant which cannot activate Ras (9), is able to activate Raf.

3. Raf and Mek are activated by SVLT in mammalian cells where endogenous Ras expression is downregulated through the introduction of an anti-sense ras construct, to
similar levels as the parental line.

How SVLT activates Raf is only speculative at the moment. Clues pointing towards the involvement of the cellular chaperone machinery are provided by our finding (Fig. 3) that the DnaJ homology domain SVLT mutant (D44N), which is unable to bind to and stimulate the activity of Hsp70 (5), has also a severely compromised ability to activate Raf. In support of this hypothesis, recent work has established that Raf is bound to and modulated \textit{in vivo} by the chaperone machinery including the Hsp90 and Hsp70 chaperone families (15,34-36). Therefore, although it appears possible that SVLT might activate Raf partly through Ras stimulation, under conditions of low Ras levels SVLT might be still activating Raf by sequestering hsp70, which was recently shown to inactivate Raf through Bag1 binding (36). The latter could also operate in the presence of Ras. The precise mechanism through which chaperone proteins might be mediating Raf activation will be addressed in future experiments.

Examination of different transformation-related parameters in response to graded levels of oncogene expression in rat F111 cells previously revealed the existence of a hierarchy, with morphological transformation, focus formation, agar growth and tumorigenicity requiring progressively higher levels of oncogene expression (37). In fact, the K1 mutant which is able to only partially activate Raf, \textit{is} able to partially transform rat F111 cells as judged by morphology on plastic, but not to anchorage-independence, indicating that the observed Raf activation by this mutant might be sufficient for morphological transformation. This is in keeping with our results indicating that although SVLT is not able to transform Ras-deficient cells to agar growth and tumorigenicity, it is
able to induce morphological transformation, suggesting that this property may be exquisitely sensitive to weak SVLT signals, or that morphological changes may be independent of Ras (9). In any event, the high Raf activity levels in the Ras-deficient, SVLT-expressing 25SV7t cells indicate that Raf activation does not equal full neoplastic transformation in this system. Additional, Ras-mediated pathways involving other Ras effectors such as PI-3 kinase could play a significant role.

In conclusion, the present work demonstrates that SVLT can activate Raf, at least partly in a Ras-independent manner. Future experiments will examine whether this effect is mediated by SVLT’s ability to modulate Raf’s interaction with chaperone proteins, and in turn its catalytic activity.

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Figure legends

Figure 1

SVLT expression increases Raf kinase activity in insect cells

A. In vitro kinase assays were performed with Raf immunoprecipitated from extracts of Sf9 cells infected with vectors encoding Raf alone (1 and 2), or Raf and SVLT (3 and 4) with (2 and 3) or without (1 and 4) added kinase-dead Mek, Raf substrate. Aliquots of the same extracts were resolved by SDS/PAGE and transferred to a nitrocellulose membrane which was probed with anti-SVLT, or anti-Raf antibodies, as indicated. Arrows point to the positions of SVLT, Raf and Mek. Horizontal bars point to the positions of molecular weight standards.

B. In vitro kinase assays were performed with Raf immunoprecipitated from extracts of Sf9 cells infected with vectors encoding wt-SVLT (1-2 and 5-8) or the SVLT-K1 mutant (3-4), at a 1/100 (1,3,5,7) or 1/400 (2,4,6,8) dilution, respectively, together with wt Raf (1-4), kinase-dead Raf (5-6), or empty vector, lacking an insert (7-8). As a control, levels of Raf and SVLT were examined by Western blotting using anti-Raf or anti-SVLT antibodies as indicated. Phosphorimager analysis indicated that, in repeated experiments, Raf activation by the K1 mutant was approximately 60% of the wt.
Quantitative effect of SVLT upon Raf activation in insect cells

*In vitro* kinase assays were performed with Raf immunoprecipitated from extracts of Sf9 cells infected with vectors encoding Ras alone (1/10 dilution, lane 1), Ras and a 1/20 or 1/100 dilution of Raf (lanes 2-5 and 6-9, respectively), without (lanes 2 and 6) or with increasing amounts of SVLT, as indicated. Lanes 10 to 12, cells infected with Raf alone (lane 10), or Raf, Ras and SVLT (lane 11) or Raf and SVLT, without Ras coinfection (lane 12). Note the dramatic increase in Raf kinase activity upon SVLT expression especially under conditions of relatively low Raf levels (lane 6 vs 9); this increase occurs even in the absence of coexpressed Ras (lane 10 vs 12).

Aliquots of the same extracts were resolved by SDS/PAGE and transferred to a nitrocellulose membrane which was probed with anti-SVLT, anti-Ras or anti-Raf antibodies, as indicated. Arrows point to the positions of SVLT, Ras or Raf. Numbers at the right refer to the positions of molecular weight markers.
**Figure 3**

**DnaJ domain SVLT mutant exhibits a reduced ability to activate Raf**

*In vitro* kinase assays were performed with Raf immunoprecipitated from extracts of Sf9 cells infected with 1/10 dilutions of Raf and Ras vector stocks, without (lanes 2, 8, 14 and 17), or with coinfection with decreasing concentrations of vectors expressing the SVLT-wt (dilutions of 1/100, 1/500, 1/1,000, 1/2,000 and 1/4,000, lanes 3-7) or the SVLT-D44N (dilutions of 1/200 and 1/500, lanes 9-11) mutant, as indicated. Note the reduced activation level of Raf by the D44N mutant, compared to wt (lane 3 vs 9). Phosphorimager analysis indicated that, in repeated experiments, Raf activation by D44N was approximately 40% of the wt.

Aliquots of the same extracts as above were resolved by SDS/PAGE and transferred to a nitrocellulose membrane which was probed with anti-SVLT, anti-Ras, or anti-Raf antibodies, as indicated. Numbers at the right refer to the positions of molecular weight markers.

**Figure 4**

**SVLT expression increases Raf kinase activity in mammalian cells**

**A.** SVLT enhances Raf activity in SVLT-transformed cells. *In vitro* kinase assays were performed with Raf immunoprecipitated from extracts of SVLT-expressing, FSV1a cells (lane 2) or the parental F111 line (lane 1), with added kinase-dead Mek as Raf substrate.
Aliquots of the same extracts were resolved by SDS/PAGE and transferred to a nitrocellulose membrane which was probed with anti-Raf antibodies, as indicated. Arrows point to the positions of Raf and Mek.

B. SVLT enhances Raf activity in transiently transfected cells. pEBG-Raf either alone (lanes 2-4) or in combination with pCMV-SVLT (lane 5) or pCMV-K1 (lane 6) were transfected into normal F111 (lanes 2 and 4-6) or SVLT-transformed FSV1a cells (lane 3), as indicated. Cultures were then serum-starved for 48 hours and detergent cell lysates precipitated with GSH-sepharose. Raf activity was determined using a non-active recombinant Mek substrate. Lane 1: control, untransfected cells.

**Figure 5**

**Raf and Mek activation by SVLT in Ras-deficient cells**

A. Extracts from Ras deficient, 10T½ cells without (25B8, lane 1) or with SVLT expression (25SV7t, lane 2) were immunoprecipitated with an antibody against Raf and *in vitro* kinase assays performed with kinase-dead Mek as substrate. Lower panel: Protein cell extract aliquots were blotted and probed for Raf.

B. Extracts from normal (lanes 1 and 2) or Ras deficient (lanes 3 and 4), 10T½ cells without (lanes 1 and 3) or with overexpressed SVLT (lanes 2 and 4) were blotted with an anti-active Mek antibody. Lower panel: Protein cell extract aliquots were blotted and
probed for Ras using the pan-Ras Ab2 antibody. Fluorimeter analysis revealed that lines 25B8 and 25SV7t contained approximately 30% the Ras levels of the parental 10T½ [(9), see Experimental procedures].

Figure 6

Phenotypic effects of SVLT under conditions of low Ras activity.

F111 cells expressing wt-SVLT (line FSV1a, B and D) or the K1-SVLT mutant (line FK1, A and C) were plated on plastic, on top of a monolayer of normal F111 cells (A and B), or in agar (C and D). Photographs were taken ten days later. Note the partially transformed morphology of FK1 cells (A, arrow) compared to the normal F111 (arrowhead in A and B) and their inability to grow in agar (C) compared to FSV1a (D).

E to H: Ras-deficient, 10T½ cells (25B8), or their SVLT-expressing counterparts (25SV7t) were grown on plastic and photographed under phase-contrast illumination (E and F), or placed in soft agar medium (G and H). Note the transformed morphology of the SVLT-expressing, Ras deficient 25SV7t cells (F), although they are unable to grow in agar (H).
Figure 1
Figure 3
Figure 4
Figure 5

A

25B8  25SV7t

46- [image of 32P-Mek(kd)]

69- [image of Raf]

1  2

ip: Raf kinase vs Mek(kd)

Western α-Raf

B

10T1/2  10SV2b  25B8  25SV7t

46- [image of Phospho-Mek]

69- [image of Ras]

1  2  3  4

Western α-active Mek

Western α-Ras