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Simian Virus 40 Small Tumor Antigen Activates AKT and Telomerase and Induces Anchorage-Independent Growth of Human Epithelial Cells

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Human keratinocytes immortalized by full-length or early-region simian virus 40 (SV40) DNA grow in agarose and form tumors in nude mice, in contrast to keratinocytes immortalized by the E6/E7 genes of human papillomaviruses. To determine the molecular basis for this biological difference in growth, we have used the individual SV40 oncogenes (large T antigen [LT] and small t antigen [st]) and human papillomavirus oncogenes (E6/E7) to study the progression of human epithelial cells from the nonimmortal to the immortal state as well as from the immortal to the anchorage-independent state. Transfection of primary human foreskin keratinocytes with LT did not immortalize cells but did extend the in vitro life span and produced cells that were resistant to calcium- and serum-induced terminal differentiation. Cells transfected with st alone did not passage beyond vector-transfected keratinocytes. The simultaneous expression of LT- and st-immortalized keratinocytes occurred without evidence of crisis and, as anticipated, these immortal cells were anchorage-independent for growth. Interestingly, we found that keratinocytes expressing both LT and st, but not keratinocytes with LT alone, exhibited increased phosphorylation of the protein kinase AKT. In addition, AKT activation was paralleled by an increase in telomerase activity. Addition of st to anchorage-dependent keratinocytes, expressing either LT (nonimmortal) or E6/E7 (immortal), converted the cells to anchorage independence, with similar accompanying increases in AKT phosphorylation and telomerase activity. However, it was not possible to induce keratinocyte growth in agarose with activated AKT and/or overexpressed hTERT, indicating that these newly defined st-induced activities are not sufficient for progression to the anchorage-independent state.

The simian virus 40 (SV40) small-t antigen (st) is a 174-amino-acid protein that enhances rodent cell transformation mediated by the SV40 large-T antigen (LT) (7) and permits the transformation of growth-arrested cells (43). st is essential for reentry of density-arrested fibroblasts into the cell cycle through its downregulation of the cyclin kinase inhibitor p27 (37) and also for the transformation of human fibroblasts and mesothelial cells (8, 36, 51). Serum and phorbol ester tumor promoters can substitute for defective st mutants, suggesting that st may modulate growth factor signaling pathways (33). st binds the cellular protein phosphatase 2A (PP2A) (39) and inhibits PP2A activity in vitro (50) and in vivo (45). The inhibition of PP2A by st activates phosphoinositide 3-kinase (PI 3-kinase)-dependent protein kinase C ζ (PKC ζ) signaling pathways and leads to the activation of NF-κB-dependent transcription and the mitogen-activated protein kinase cascade (45). The end result of these activities is the induction of cell proliferation.

The PI 3-kinase/c-Akt kinase cascade plays an important role in cell survival (reviewed in reference 15). c-Akt, the cellular homologue of the transforming viral oncogene v-Akt, is a serine/threonine protein kinase related to PKC (5). Phosphorylation of Akt at Thr308 and Ser473, which is mediated by upstream kinases regulated by phospholipid products of PI 3-kinase (4, 22), is required for Akt kinase activation (6, 13, 46). Akt kinase activity is necessary and sufficient to block apoptosis (19, 20) and can induce cell cycle progression (1, 10). The pro-apoptotic proteins identified as substrates of Akt include Bad, caspase-9, forkhead family members, and IKK (reviewed in reference 15), and all of these targets contain consensus phosphorylation sites (RXRXXS/T-bulky hydrophobic) (49).

Activation of the Akt pathway through PI- 3-kinase can contribute to enhanced activity of telomerase, a necessary but insufficient step in the oncogenic progression of human cells. The control of telemere length by telomerase is important for extending the life span of the cell (17, 23), and telomerase is activated in most human cancers and immortal cell lines (28, 41). The transcriptional activation of telomerase by papillomavirus E6 protein is an important step in the eventual immortalization of these cells (29) and the long-term survival of transformed cells that emerge in E6/E7-expressing cells. In addition to activation by transcriptional mechanisms, the activity of telomerase can be regulated posttranslationally. Telomerase contains two putative Akt kinase phosphorylation consensus sequences that are phosphorylated by Akt in vitro, resulting in activation of telomerase (26). Furthermore, PP2A inhibits telomerase activity in lysates of human breast cancer cells (32), and this effect may be mediated through downregulation of Akt.

In this study, we explored the effects of SV40 st on some of

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these activities in epithelial cells to determine the basis for its ability to activate cell survival and longevity pathways as well as to promote transformation, as evidenced by growth in agarose. Given the critical role of st in human cell transformation, analysis of st-dependent pathways may lead to a fundamental understanding of the requirements for progression to the tumorigenic state.

MATERIALS AND METHODS

Plasmids. The plasmids pPVU0 (25), pdl2005 (43), and pdl536 (44) have been described previously. Respectively, these plasmids encode LT plus st, LT alone, and st alone. Plasmids encoding wild-type Akt (c-Akt) and the myristylated, constitutively active form of Akt (ca-Akt) were graciously supplied by Alfonso Bellacosa (FoxChase). The full-length st, c-Akt, ca-Akt (4), and human telomerase reverse transcriptase (hTERT) genes were cloned into retroviral plasmid pBABE-puro (34).

Cell culture. Primary human keratinocytes were derived from neonatal foreskins as described previously (40) and were grown in Keratinocyte-SFM medium (Invitrogen). The primary HFK cells (passage 0) were transfected with the plasmids pPVU0, pdl2005, and pdl536, using FuGene 6 transfection reagent (Boehringer Mannhem) as specified by the manufacturer. Colonies surviving the terminal differentiation assay (see below) were pooled and designated HFK/Tt and HFK/T.

NCO cells were grown in 3+1 medium (3 volumes of KSF medium to 1 volume of Dulbecco Modified Eagle Medium [DMEM]). NCO and HFK/T cells (at passage 18) were infected at a multiplicity of 10 PFU/cell with retrovirus expressing st. Retrovirus-infected cells were selected in puromycin (400 ng/ml) for 10 days and designated NCO/t and HFK/T+t, respectively. NCO cells were infected with retrovirus expressing wild-type Akt, constitutive active Akt, or hTERT. Those cells were selected in puromycin (400 ng/ml) for 10 days. SD3443 and NIH 3T3 cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

Retrovirus. SD3443 cells were transfected with pBABE-puro-st using Lipofect Amine Plus reagent (Life Technologies, Inc.) as specified by the manufacturer. Culture supernatants containing retrovirus were collected 24 h posttransfection. Viral titers of the supernatants were determined using NIH 3T3 cells.

Differentiation assay. The differentiation assay has been described previously (40). Briefly, HFK cells (passage 0) were plated at 50% confluency in 10-cm-diameter dishes and transfected with plasmids containing viral genes. After 24 h, the cells were transferred into 175-cm ^{2} flasks and maintained in KSF medium until confluent. The medium was replaced by a selection medium consisting of DMEM supplemented with 10% fetal bovine serum and 1 μg of hydrocortisone (Sigma) per ml. Only the keratinocytes that resist calcium- and serum-induced differentiation signals are able to proliferate and form nonstratifying colonies. Colonies were pooled and grown in 3+1 medium.

Preparation of cell extracts and Western blot analysis. Cells (10-mm dishes) were washed once with phosphate-buffered saline, lysed in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and passed through a 21-gauge needle. The protein concentration of clarified lysates was determined using the Bio-Rad DC protein assay with bovine immunoglobulin G (IgG) as the standard. Proteins were separated on a 4 to 20% Tris-glycine gradient gel (Novex) and then were electrophoretically transferred to an Immobilon-P polyvinylidane difluorid (PVDF) membrane (Millipore). The primary antibody, mouse anti-SV40 T-antigen monoclonal antibody (Pab 108; Santa Cruz) was used at a dilution of 1:500. Mouse anti-p27 antigen monoclonal antibody (F-8; Santa Cruz), rabbit anti-phospho-Akt (Ser473), and anti-Akt polyclonal antibodies (both from New England Biolab) were used at a dilution of 1:1,000. The secondary antibodies, alkaline phosphatase-conjugated goat anti-mouse IgG and anti-rabbit IgG (Tropix) antibodies, were used at a dilution of 1:5,000. Western blots were visualized by using CDP-Star chemiluminescent substrate (Tropix).

Soft agar assay. The growth of keratinocytes in soft agar was assayed as described previously (48).

Telomeric repeat amplification protocol. Cells were grown in 10-cm dishes to 80% confluence and were lysed in 400 μl of TRAP buffer (0.5% CHAPS, 10 mM Tris [pH 7.5], 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 10% glycerol, 0.1 mM AEBSF). A modified telomeric repeat amplification protocol (TRAP) was performed as described by Kim et al. (28). Briefly, 3 μg of lysates was incubated at room temperature for 30 min in a 50-μl reaction volume containing 1× PCR buffer (20 mM Tris [pH 8.4], 50 mM KCl), 100 ng of telomerase substrate (TS primer, 5′-AATCCGTCGAGCAGAGTT-3′), 50 μM each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 1.5 mM $MgCl_2$, and 0.5 μM T4 gene protein (Boehringer Mannheim). Then 100 ng of the downstream primer (CX, 5′-CCCTTACCCTTACCCTTACCCTAA-3′) and 2.5 U of *Taq* DNA polymerase were added to each reaction mixture. This was followed by 31 cycles of PCR amplification (94°C for 30 s, 50°C for 30 s, and 72°C for 45 s) after an initial denaturation step at 94°C for 4 min. The products of the PCR amplification were separated on a 10% nondenaturing polyacrylamide gel and visualized using the Gelcode color silver-staining kit (Pierce).

RESULTS

LT, but not st, induces resistance to signals for terminal differentiation and extends the life span of keratinocytes. Primary HFK cells can be passaged 8 to 10 times in culture before the cells cease dividing and become senescent. In addition, when early-passage keratinocytes are exposed to 2.0 mM calcium and 10% serum, they undergo terminal differentiation. These physiological responses have been adapted in a quantitative colony-forming assay to identify papillomavirus genes that interfere with cell differentiation and promote keratinocyte immortalization (40). In the present study, we used this assay to determine whether both LT and st are necessary for altering keratinocyte growth and differentiation. HFK cells (passage 0) were transfected with plasmids encoding both LT and st, or LT or st alone as described above. Differentiation was induced with serum-calcium, and the number of proliferating colonies was determined after 5 weeks. Vector-transfected and st-transfected keratinocytes failed to induce colonies. However, both LT- and LT-st-transfected keratinocytes formed approximately 60 colonies (data not shown), suggesting that resistance to differentiation and increased cellular proliferation was the consequence of LT expression. Colonies from LT-st- and LT-transfected keratinocytes, designated HFK/Tt and HFK/T, respectively, were pooled and evaluated for T antigen expression (Fig. 1A). LT was present in both cell types, although its amount was significantly larger in the HFK/T cells. st was present only in the HFK/Tt cells.

st allows HFK/T cells to progress to the immortalized state. While the life span of HFK/T cells was greater than that of normal or vector-transfected HFKs, they entered crisis, characterized by cell rounding and extensive cell death, at passage 25. HFK/T cells never survived beyond passage 30. This was in distinct contrast to HFK/Tt cells, which grew without evidence of crisis for more than 50 passages, suggesting that st was responsible for this phenotype. To evaluate whether st was directly responsible for allowing precrisis HFK/T cells to become immortal, we infected HFK/T cells at passage 18 with a retrovirus encoding st (see Materials and Methods) and monitored their growth. Similar to keratinocytes simultaneously transfected with LT and st (HFK/Tt), the HFK/T cells transduced with st (HFK/T+t) established efficiently into a cell line, although there was some evidence of cell death from passages 27 to 30. The HFK/T+t cells expressed both LT and st, as shown in Fig. 1A. Thus, st enabled HFK/T keratinocytes to transit the crisis phase and become immortal.

st alters cell morphology, enhances anchorage-dependent growth, and induces anchorage-independent growth. Since st clearly facilitates the immortalization of HFK/T cells, we investigated whether st also affects the growth properties of HFK/T cells. At late passages (after passage 20), when grown on plastic substratum, HFK/Tt cells exhibited a uniform, com-

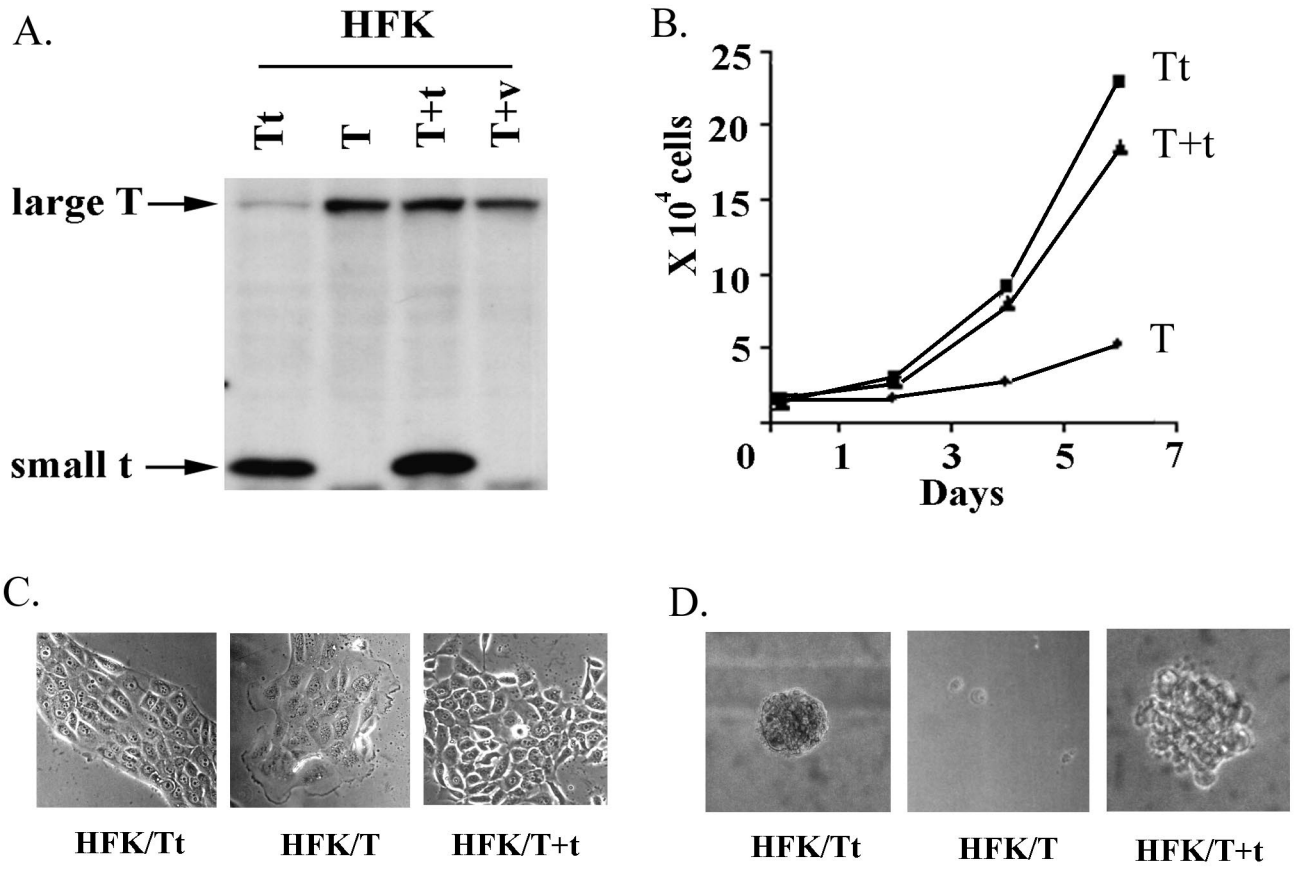

FIG. 1. Expression of st in LT-expressing HFK cells leads to immortalization and anchorage-independent growth. Primary HFK cells (passage 0) were transfected with plasmid pPVU0 (encoding SV40 LT and st) or pdl2005 (encoding LT). Colonies surviving terminal differentiation assay were pooled and designated HFK/Tt and HFK/T. At passage 18, HFK/T cells were infected with a retrovirus encoding st (HFK/T+t) or the empty vector (HFK/T+v) as a control. (A) Lysates were prepared from indicated cells at passage 20. Proteins were separated on a 4 to 20% gradient gel and then analyzed by Western blotting using anti-SV40 T-antigen monoclonal antibody. (B) HFK/Tt (squares), HFK/T+t (triangles), and HFK/T (circles) cells at passage 20 were seeded at a density of 2×10^4 cells per 10-cm dish. Cell growth was measured by counting cells every 48 h. (C) Phase-contrast photographs of cell cultures at passage 20 are shown. (D) HFK/Tt, HFK/T, and HFK/T+t cells (passage 19) were photographed after growth in soft agar for 5 weeks.

pact appearance, in contrast to the more flattened appearance of the HFK/T cells (Fig. 1C). To determine whether the basal cell-like morphology of HFK/Tt cells was due to the effects of st, we also evaluated the morphology of the st-transduced HFK/T cells, HFK/T+t. The morphology of HFK/T+t cells closely resembled that of HFK/Tt cells, indicating that st was responsible for this observed phenotype. The HFK/T+t cells also grew more rapidly than HFK/T cells, similar to HFK/Tt cells (Fig. 1B). Finally, when HFK/T+t cells were evaluated for their ability to grow in agarose, they formed larger and more numerous colonies than those observed with HFK/Tt cells (Fig. 1D). HFK/T cells did not grow in agarose at either early or late stages. The induction of anchorage independence by st was nearly immediate. When HFK/T cells were infected with st-encoding retrovirus at passage 18 and assayed for growth at passage 19, they formed colonies in agarose (Table 1). Thus, the addition of st to HFK/T cells induces rapid alterations in cell morphology and growth; most importantly, st induces an anchorage-independent phenotype.

st increases the level of phosphorylated Akt. Previous studies have shown that st binds the cellular PP2A (39) and inhibits PP2A activity (50). Phosphorylation of Akt, which is required for activation of its kinase activity, is negatively regulated by PP2A, raising the possibility that the level of phosphorylated Akt in st-expressing cells might be increased. As shown in Fig. 2A, the amount of phosphorylated Akt in the HFK/Tt cell line was significantly larger than in the HFK/T line. Furthermore, the introduction of st into HFK/T cells (HFK/T+t) increased

TABLE 1. HFK, HFK/Tt, HFK/T, HFK/T+v, and HFK/T+t cells in soft agar[a]

Cell line	No. of colonies at:	
	Passage 18	Passage 19
HFK	0/0/0/0	0/0/0/0
HFK/Tt	12/10/9/10	18/19/22/26
HFK/T	0/3/0/0	0/0/2/0
HFK/T+v		0/0/3/4
HFK/T+t		48/51/62/54

[a] Petri dishes (35 mm) were seeded with 2×10^3 cells in 0.3% agarose. Experiments were performed twice, and each time in duplicate. Colonies were counted after 5 weeks.

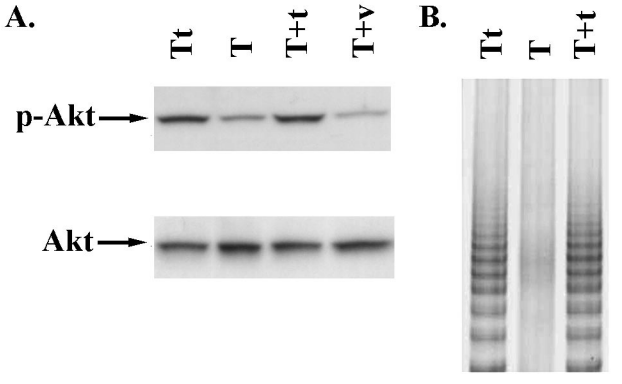

FIG. 2. (A) Expression of st in LT-expressing HFKs induces phosphorylation of Akt. Cell lysates were prepared from the indicated cells at passage 19. Proteins were separated on a 4 to 20% Tris-glycine gradient gel and analyzed by Western blotting for the level of phosphorylated Akt (P-Akt) and Akt by using phospho-specific and phosphorylation-independent antibodies, respectively. (B) Expression of st in LT-expressing HFKs increases telomerase activity. A TRAP assay was used to measure the activity of telomerase in extracts of the indicated cells at passage 22.

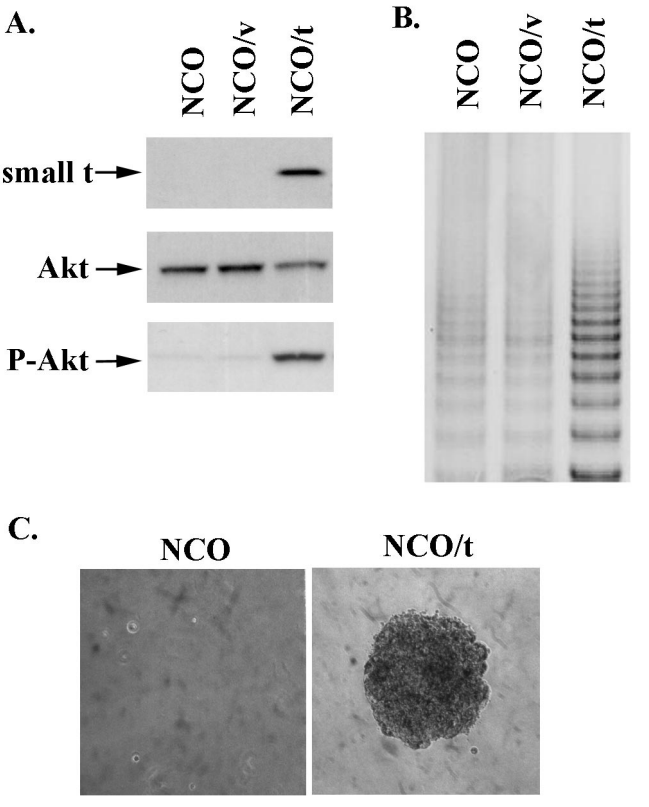

FIG. 3. Expression of st in HPV-immortalized keratinocytes (NCO cells) induces alterations in Akt phosphorylation, telomerase activity, and anchorage-independent growth. NCO cells were infected with a retrovirus encoding st (NCO/t) or empty vector (NCO/v) as a control and were selected for puromycin-resistant cells. (A) Cell lysates were prepared from NCO, NCO/v, and NCO/t cells and separated on a 4 to 20% Tris-glycine gradient gel. The separated proteins were transferred to a PVDF membrane and used for Western blotting with anti-SV40 T-antigen monoclonal antibody or anti-phospho-specific or phosphorylation-independent Akt antibodies. (B) The indicated cell lines were lysed in TRAP buffer, and 3 μg of protein was used in a TRAP assay (see Materials and Methods). The products of the TRAP reaction were separated on a 10% nondenaturing polyacrylamide gel and visualized by silver staining. (C) NCO, NCO/v, and NCO/t cells were photographed after growth in soft agar for 4 weeks.

the amount of phosphorylated Akt, demonstrating the role of st in this alteration. As measured by densitometry, the level of phosphorylated Akt in HFK/Tt and HFK/T+t cells was 2.3-fold higher than in HFK/T cells. Since there was no change in the total amount of Akt in these cell lines (Fig. 2A), st increases the amount of phosphorylated Akt only.

st increases the activity of telomerase. Li et al. have demonstrated that PP2A is able to inhibit telomerase activity in the lysates from human breast cancer cells (32). In addition, hTERT, a subunit of the telomerase complex, contains two consensus Akt kinase phosphorylation sites and can be activated through Akt-mediated phosphorylation in vitro (26). These results suggest that telomerase potentially may be regulated by PP2A and Akt in vivo. Since we demonstrated that the expression of st in HFK/T cells increased the level of phosphorylated Akt, we reasoned that the activity of telomerase might also be up-regulated. Using TRAP, we measured the activity of telomerase in the cell lines described in Fig. 2A. As shown in Fig. 2B, the activity of telomerase in HFK/T was undetectable whereas the activity in HFK/Tt was very high. When st was expressed in HFK/T (HFK/T+t), the activity of telomerase increased significantly and was similar to the level seen in HFK/Tt. These results demonstrated that the expression of st in HFK/T leads to activation of telomerase as well as phosphorylation of Akt.

st induces alterations in Akt, telomerase, and the growth of papillomavirus-immortalized keratinocytes. To determine whether st could induce anchorage-independent growth in other nontumorigenic keratinocytes, we initiated studies with the NCO cell line. NCO is an HFK cell line immortalized by human papillomavirus 18 (HPV-18) DNA and, similar to nearly all HPV-immortalized keratinocytes, is incapable of growing in agarose or forming tumors in nude mice. Analogous to our generation of the HFK/T+t cell line, we used retroviral infection to express st in NCO cells. st was readily detected by immunoblotting in the st-transduced cells (NCO/t) but not in the parent cell line or a cell line infected with control retrovirus (NCO/v) (Fig. 3A). The NCO/t cells, in contrast to the parent NCO cells or the NCO/v cells, grew in agarose after only two passages (Fig. 3C and Table 2). There was a further increase in colony-forming efficiency up to passage 6 (Table 2). Thus, st rapidly converts immortal NCO cells to the anchorage-independent state.

To determine if the altered growth of NCO/t cells was accompanied by changes in Akt phosphorylation (as observed with the HFK/T cells), we measured the amount of phosphorylated Akt by immunoblotting with a phospho-Akt-specific antibody (Fig. 3A). The level of phosphorylated Akt was 4.2-fold higher (by densitometry) in NCO/t cells than in the NCO and NCO/v cells, while identical amounts of total Akt were present in all of these cell lines (Fig. 3A). Furthermore, the expression of st in NCO cells also induced an increase in telomerase activity (Fig. 3B). While NCO cells exhibited greater telomerase activity than did HFK/T cells (Fig. 2B), it is clear that the

TABLE 2. Growth of NCO cells in soft agar[a]

Cell line	No. of colonies at:	
	Passage 2	Passage 6
NCO	0/0/0/0	0/0/0/0
NCO/v	0/0/0/0	0/0/0/0
NCO/t	60/55/50/71	>200
NCO/wt-Akt	0/1/0/2	0/1/3/0
NCO/ca-Akt	0/1/0/0	0/0/0/0
NCO/hTERT	1/0/1/0	0/0/0/0
NCO/wt-Akt+hTERT	0/0/0/0	0/1/0/0
NCO/ca-Akt+hTERT	0/1/0/1	1/0/0/0

[a] Petri dishes (35 mm) initially were seeded with 2×10^3 cells. Experiments were performed twice, each time in duplicate. Colonies were counted after 4 weeks.

NCO/t cells have much higher telomerase activity than either the parent NCO cell line or the parallel control NCO/v cell line. Thus, st can induce Akt phosphorylation and telomerase activity in cells which are immortalized by viral proteins other than LT.

Overexpression of Akt and the telomerase subunit hTERT does not induce anchorage-independent growth in papillomavirus-immortalized keratinocytes. The new biochemical activities of st described above, activation of Akt and telomerase, might contribute (either individually or cooperatively) to the progression of st-expressing epithelial cells to the anchorage-independent state. To evaluate this possibility, we examined whether overexpression of activated Akt and/or telomerase could induce the anchorage-independent growth of NCO cells. Ectopic expression of hTERT, the rate-limiting catalytic subunit of telomerase, can induce telomerase activity in telomerase-negative cells (9, 14), and we therefore engineered a retrovirus to express this telomerase subunit. Overexpression of constitutively active Akt (ca-Akt), but not wild-type Akt (c-Akt), transduces a survival signal in a differentiating neuronal cell line (4) and inhibits anoikis (27). Akt, ca-Akt, or hTERT (a subunit of telomerase) was introduced into NCO cells by retrovirus infection. HA-tagged Akt protein was readily detected by immunoblotting in the Akt-transduced cells (NCO/c-Akt and NCO/ca-Akt) but not in the parental cell line or a cell line infected with control retrovirus (NCO/v) (Fig. 4, four left lanes). However, neither NCO/c-Akt nor NCO/ca-Akt grew in agarose (Table 2). Overexpression of hTERT in NCO, NCO/c-Akt, or NCO/ca-Akt cells (Fig. 4, three right lanes) did not induce anchorage-independent growth either. Therefore, activation of Akt or telomerase (or both) is not sufficient to induce the full transformation of immortalized cells. Parenthetically, the separate and combined expression of activated Akt and hTERT was also unable to immortalize primary keratinocytes (data not shown).

DISCUSSION

Papillomavirus E6 and E7 proteins readily immortalize human keratinocytes, but additional alterations are necessary for the cells to become fully transformed (35). In contrast, SV40 LT and st both immortalize and transform these cells. This report describes initial efforts to dissect the effects of these individual viral proteins on cell differentiation, immortalization, and transformation.

Contributions of SV40 to the immortalization of human cells have been studied most frequently in fibroblasts, for which it is well established that LT extends the life span of primary cells beyond the normal senescence point (M1) but cannot prevent the subsequent apoptotic crisis that occurs at M2. Although most cells fail to bypass M2, rare immortal clones do arise but only when LT is present (42). The st protein has never been shown to play a significant role in the immortalization of fibroblasts. HFK cells show considerable differences from this paradigm, and st allows LT-expressing cells to bypass M2 efficiently. At late passages, HFK cells expressing LT only (HFK/T cells) entered a crisis stage during which they rounded up and detached from the substratum. Morphologically, they appeared similar to keratinocytes undergoing apoptosis (47). None of the HFK/T cells survived this crisis. However, the introduction of st into HFK/T allowed these cells (HFK/Tt and HFK/T+t) to bypass cellular crisis. Although limited transient apoptosis has been observed in our cultures (data not shown), the majority of the cells continued to proliferate and have not been found to encounter any later restrictions to permanent growth. Therefore, st plays an important role in facilitating the immortalization of HFK cells.

Our studies with HFKs are consistent with the results of previous studies with rat embryo fibroblast cells showing that LT induces apoptosis (12) and that st inhibits apoptotic cell death induced by large T (31). While the mechanism of survival from apoptotic signals may be complex, our results suggest that Akt activation may be one critical component of st-induced bypass of cellular crisis. Akt is generally known to have a key

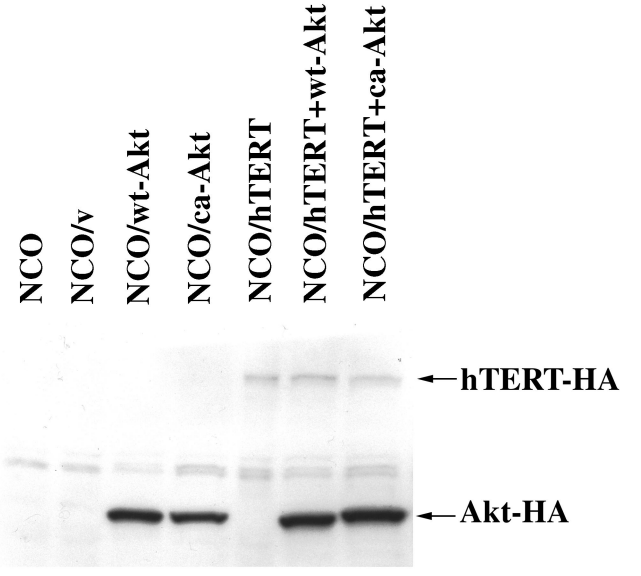

FIG. 4. Expression of Akt and hTERT in NCO cells. NCO cells were infected with a retrovirus encoding wild-type Akt (NCO/wt-Akt), constitutively active Akt (NCO/ca-Akt), hTERT (NCO/hTERT), or both (NCO/hTERT+wt-Akt and NCO/hERT+ca-Akt). Cells were selected for puromycin resistance. Cell lysates were prepared and separated on a 4 to 20% Tris-glycine gradient gel. The separated proteins were transferred to a PVDF membrane and used for Western blotting with anti-HA monoclonal antibody.

role in the inhibition of apoptosis (19, 20, 27). For example, activated Akt can promote cell survival by phosphorylating several of the components of the apoptotic pathway, including Bad (16) and caspase 9 (11). We have shown that st significantly increases Akt phosphorylation (Fig. 2A and 3A), which is critical for Akt kinase activation (2). The higher level of phosphorylated Akt in st-expressing cells is probably the result of PP2A inhibition by st, since studies have shown that phosphorylated Akt can be dephosphorylated and inactivated by PP2A (3). In support of this possibility, transfection of primary human fibroblasts with constructs that express WT st, but not mutants that block PP2A interaction, leads to Akt phosphorylation (K. Fahrbach and K. Rundell, unpublished observations). This is consistent with findings published while our manuscript was in review, showing that the transformation of human cells requires the inhibition of PP2A by st (24).

A key activity in cellular immortalization is the maintenance of telomeres, either by activation of the catalytic subunit of telomerase (which is normally not expressed in primary cells) or by alternative mechanisms that preserve chromosome ends (17, 23). Exogenous introduction of hTERT is sufficient to immortalize fibroblasts (9) but not many other human cell types, including keratinocytes (18, 29). Given the central role of telomerase in immortalization, we examined telomerase activity in keratinocytes that expressed SV40 proteins. There was no detectable telomerase in HFK/T cells. However, introduction of st into HFK/T cells induced high telomerase activity (Fig. 2B), suggesting that st may contribute to HFK immortalization by activating telomerase. This may result from the activation of Akt and inactivation of PP2A by st, since in vitro studies have shown that telomerase is positively regulated by Akt (32) and negatively regulated by PP2A (26). In this case, subthreshold levels of preexisting telomerase protein may be activated by st, allowing the enzyme activity to be measured. Clear evidence that st can cause this activation is apparent from experiments with the NCO cells, where levels of telomerase detectable in E6/E7-expressing cells are clearly elevated when st is introduced. In human mesothelial cells, st also plays an important role in the induction of telomerase activity (21). Although transcriptional activation of telomerase by st is also a formal possibility, it should be noted that neither LT nor st activates the transcription of telomerase in human fibroblasts and that fibroblasts transformed by SV40 early region proteins are not immortal (30, 38).

While contributions of st to immortalization may reflect its activation of Akt and telomerase, mechanisms that drive the anchorage-independent growth of SV40-transformed cells are not defined. Telomerase itself is not required for anchorage-independent growth, as evidenced by the presence SV40-transformed fibroblasts that are fully transformed yet nonimmortal, as described above. Telomerase is more likely to allow the prolonged growth of transformed cells but not contribute to the actual transformed phenotypes.

Our results also demonstrate that st induces anchorage-independent growth in HFKs expressing HPV E6/E7 (Table 2). Since growth in agar is closely associated with acquisition of the tumorigenic phenotype in human keratinocytes, it should be possible to use st to define the cellular pathways involved in the conversion from the nontumorigenic to the tumorigenic state. This would be particularly appropriate for the analysis of progression from cervical dysplasia to cervical carcinoma in women. While the HPV E6/E7 genes play a critical role in the genesis of cervical cancer (most probably achieving the "immortalized state" and inducing altered cellular differentiation), they are clearly insufficient for inducing malignant neoplasia. Our present data showing that st converts HPV-immortalized cells to the anchorage-independent state, coupled with our preliminary unpublished data that Akt is hyperphosphorylated in several cervical cancer cell lines, suggest that this signaling pathway may be a critical target for activation during malignant progression. However, rather than being involved in the direct conversion of cells to anchorage independence (Table 2), it is possible that Akt plays a separate role in the transformation of primary keratinocytes, possibly by generating resistance to anoikis.

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