Extracellular simian virus 40 induces an ERK/MAP kinase-independent signalling pathway that activates primary response genes and promotes virus entry

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Simian virus 40 (SV40) binding to growth-arrested cells activated an intracellular signalling pathway that induced the up-regulation of the primary response genes c-myc, c-jun and c-sis within 30 min and of JE within 90 min. The up-regulation of the primary response genes occurred in the presence of cycloheximide and when UV-inactivated SV40 was adsorbed to cells. SV40 binding did not activate Raf or mitogen-activated protein kinase (MAP/ERK1), or mobilize intracellular Ca2+. The SV40-induced up-regulation of c-myc and c-jun was blocked by the tyrosine kinase inhibitor, genistein, and by the protein kinase C (PKC) inhibitor, calphostin C, but not by expression of the MAP kinase-specific phosphatase, MKP-1. These results suggest that the SV40-induced signalling pathway includes the activities of a tyrosine kinase and a Ca2+-independent isoform of PKC, but not of Raf or MAP kinase. Finally, SV40 infectious entry into cells was specifically and reversibly blocked by genistein.

Introduction

Mouse polyomavirus was previously shown to induce the up-regulation of the primary or early response genes c-myc, c-fos, c-jun and JE, apparently via a signal transmitted by the polyomavirus receptor at the cell surface (Zullo et al., 1987; Glenn & Eckhart, 1990). Simian virus 40 (SV40) is a papovavirus related to polyomavirus. We showed earlier that class I proteins encoded by the major histocompatibility complex (MHC) [human lymphocyte antigens (HLA) in humans] are an essential component of the SV40 receptor (Atwood & Norkin, 1989; Breau et al., 1992). Although the polyomavirus receptor has not been identified, it is clearly different from the SV40 receptor (Clayson & Compans, 1989).

Here, we investigate whether SV40, which binds to cells via a different receptor, might also transmit a signal. We show that SV40 indeed transmits a signal that results in the up-regulation of primary response genes. Furthermore, the SV40-induced signalling pathway appears to depend on a tyrosine kinase activity and protein kinase C (PKC), but is independent of Raf and mitogen-activated protein kinase (MAP/ERK). Finally, SV40 infectious entry into cells was specifically and reversibly blocked by genistein, an inhibitor of tyrosine-specific protein kinases. A preliminary account of some of these findings was described previously (Norkin & Anderson, 1996).

Methods

Chemicals. Genistein and calphostin C were purchased from Sigma. The calcium probe fura-2 acetoxy-methyl ester (fura-2/AM) and the surfactant Fluronic F-127 were purchased from Molecular Probes. All other chemicals were reagent grade.

Virus and cells. CV-1 African green monkey kidney fibroblast cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Media Tech) plus 10% fetal bovine serum (FBS; Gibco/BRL) supplemented with 0.1 mg/ml gentamicin at 37 °C in a 5% CO2 environment. SV40 wild-type strain 776 was used in all experiments and had been triple plaque-purified on CV-1 cells. Purified SV40 virions were prepared by CsCl density centrifugation essentially as described by Khoury & Lai (1979). In addition, purified virions were dialyzed against PBS pH 7.2 using dialysis tubing with a Mw 5000 cut-off (Spectra/Port 6; Spectrum Medical Industries).

Probes. For mRNA detection, DNA probes against the human c-myc third exon (1.4 kb fragment; Oncor), murine c-jun (JAC.1, 2.6 kb;
ATP and a fragment of myelin basic protein (MBP, Santa Cruz) analysed by 10% SDS-PAGE and autoradiography. The immunoprecipitates were prepared as described above. The immunoprecipitates were incubated for 4 h on ice. The MAP kinase reaction mixtures were terminated by spotting 25 μl of the reaction mixtures onto phosphocellulose discs and rinsing with 0.85% phosphoric acid. Radioactivity was quantified by Cerenkov counting.

mRNA isolation and Northern blot hybridization. CV-1 cells at confluence were cultured for 24 h in media with 0.5% FBS. SV40 was used at an input multiplicity of 1 p.f.u./cell unless otherwise indicated, with all incubations at 37 °C. As a positive control for proto-oncogene activation, either 20% FBS (diluted in PBS) or human PDGF (Sigma) were used. Cells were exposed to SV40, then at 0, 15, 30, 60, 90, 120 min and 24 h after virus addition, cells were scraped, centrifuged and quick-frozen in dry ice-ethanol. At 60 min, cells were washed with PBS and fresh medium was added for any additional incubation times. Poly(A)+ RNA was isolated using the Micro-FastTrack kit (Invitrogen). RNA was run on a formaldehyde gel, transferred to a nitrocellulose membrane (Stratagene) by overnight capillary action with 10× SSC. Hybridization conditions were 50% formamide overnight at 42 °C. Blots were washed for 15 min each with 2× SSC and 1× SSC at room temperature and for 5 min with 0.1× SSC at 60 °C. DNA probes were labelled with [32P]dCTP (6000 Ci/mmol; Amersham) by primer extension (Prime-it II, Stratagene). Blots were stripped between probed by two 10 min washes in 95 °C water. Autoradiograms were obtained using an X-Omat film (Kodak). Densitometry was performed on the autoradiograms using a Hewlett Packard scanner and Pdi Quantity One software (Pdi Software).

Assay of MAP kinase phosphorylation and activity. Confluent CV-1 cells were incubated in serum-free medium for 48 h, rinsed in phosphate-free MEM and then incubated for 5 h at 37 °C with 1 mCi [32P]orthophosphate in 1 ml phosphate-free MEM buffered with HEPS. Cells were then rinsed with phosphate-free MEM and exposed to SV40 at an input multiplicity of 100 p.f.u./cell or to epidermal growth factor (EGF; 100 ng/ml) to establish a positive control, at 4 °C for 30 min. The cells were then incubated at 37 °C for 15 and 30 min for the EGF- and SV40-treated samples, respectively. Cells were rinsed in cold phosphate-free MEM and then incubated for 5 h at 37 °C with 1 mCi [32P]ATP in 40 μl of kinase reaction buffer containing 25 mM-HEPES pH 7.4, 25 mM-β-glycerol phosphate, 1 mM-DTT, 10 mM-MgCl2, 0.1 μM-ATP and 10 μg Syntide II (Santa Cruz Biotechnology) as a peptide substrate. The reactions were terminated by spotting 25 μl of the reaction mixtures onto phosphocellulose discs, followed by rinsing with 0.85% phosphoric acid. Radioactivity was quantified by Cerenkov counting.

Intracellular calcium levels. CV-1 cells were plated onto fibronectin-treated (50 μg/ml) sterile #1 coverslips and cultured in DMEM plus 10% FBS until confluent. Cells were loaded with 2 μM-fura-2/AM plus 0.02% Pluronic F-127 in HEPES-buffered saline (HBS; 20 mM-HEPES pH 7.4, 125 mM-NaCl, 5.4 mM-KCl, 1.8 mM-CaCl2, 0.8 mM-MgSO4, 1 g/l D-glucose) at 37 °C for 30 min. The cells were then rinsed three times with HBS and mounted in the flow chamber of a video-imaging fluorescent microscope. (See Linderman et al. (1990) for a complete description of this quantitative digital fluorescent imaging system, based on a charge-coupled device (CCD) camera that can resolve rapid Ca2+ transients, image collection and processing.) Throughout the experiments the cells were kept in HBS containing 10 mM-Ca2+. The cells were imaged for 140 s prior to treatment. At 150 s SV40 (300 p.f.u./cell) was added. At 750 s the cells were challenged with 1% FBS. Each curve in Fig. 7 (see below) shows the response of an individual cell over time plotted as fluorescence ratio (R = F340/F380), which is proportional to free cytosolic Ca2+ ([Ca2+]i). Note that whereas only a few individual cells can be tracked by the system, the entire field can be viewed visually.

Assay for PKC activation. Confluent CV-1 cells were serum-deprived for 48 h, exposed to SV40 or W6/32 and suspended in lysis buffer (20 mM-Tris–HCl pH 7.5, 0.5 mM-EDTA, 0.5 mM-EGTA, 10 mM-2-mercaptoethanol, 25 μg/ml leupeptin and 25 μg/ml aprotinin). They were then disrupted by passage through a 26-gauge needle and centrifuged. The pellet (membrane) fractions were resuspended in lysis buffer containing 0.5% Triton X-100 and sonicated. PKC was partially purified from the resuspended fractions and the supernatant (cytosolic) fractions on DEAE anion exchange columns. PKC activity in each fraction was determined using the Gibco/BRL Protein Kinase C Assay System (Yasuda et al., 1990), in which the specific substrate for PKC is a synthetic peptide from myelin basic protein (MBP). Ac-MBP(4–14), that is acetylated at its N terminus to maintain its stability.

Results

Extracellular SV40 up-regulates primary response genes

To determine if SV40 initiates a signal from the cell surface that induces the up-regulation of primary response genes, purified SV40 was adsorbed to serum-deprived quiescent CV-1 cells and mRNA was isolated at various times for Northern
Fig. 1. SV40 induces the early up-regulation of primary response gene mRNA. Northern blot analysis of mRNA from serum-deprived CV-1 monkey kidney cells was as described in Methods. (a) mRNA was isolated at 0, 15, 30, 60, 90 and 120 min and 24 h following treatment with SV40 (1 p.f.u./cell) or at 30 min following treatment with 20% FBS. (b) UV-inactivated SV40 induced the up-regulation of c-myc and standard SV40 induced up-regulation in the presence of cycloheximide. Cells only, lanes 1 and 4; cells exposed to non-inactivated SV40, lanes 2 and 5; cells exposed to UV-inactivated SV40, lane 3; cells exposed to non-inactivated SV40 in presence of cycloheximide (10 µg/ml) from -1 to 1 h, lane 6. The mRNA was isolated at 1 h.

The up-regulation of c-myc was induced by UV-inactivated SV40 (Fig. 1b, lanes 1–3), which did not express T antigen. Furthermore, non-inactivated SV40 induced the up-regulation of c-myc in the presence of cycloheximide (Fig. 1b, lanes 4–6). The SV40-induced early up-regulation of these genes, in the absence of virus gene expression or intervening protein synthesis, fulfils the criteria for ligand-activated induction of primary response genes (see Discussion).

The SV40-activated signal pathway is sensitive to the tyrosine protein kinase inhibitor, genistein

A number of extracellular ligands transmit signals via the activation of a tyrosine kinase activity at the cell membrane (reviewed in Fantl et al., 1993). The signal is then passed through the cytoplasm via the sequential activation of serine/threonine kinases, eventually activating nuclear transcription factors (reviewed in Peloch & Sanghera, 1992). The possible involvement of a tyrosine kinase in the SV40-activated signalling pathway was evaluated by examining the effect on the up-regulation of c-myc and c-jun of genistein, a specific inhibitor of tyrosine-specific protein kinases (Akiyama et al., 1987). The SV40-induced up-regulation of both c-myc and c-jun was sensitive to genistein (Fig. 2).
Effects of extracellular SV40 on the activities of Raf and MAP kinase

The MAP kinase cascade (Ras–Raf–MEK–MAP kinase) is activated by a variety of growth factors (Roberts, 1992; Thomas, 1992; Blenis, 1993). This signalling pathway in turn activates several transcription factors including c-myc (Seth et al., 1991) and perhaps c-jun (Baker et al., 1992).

Activation of MAP kinases is correlated with their phosphorylation on threonine and tyrosine residues (Ray & Sturgill, 1988; Sanghera et al., 1991). Thus, we examined the ability of SV40 to induce elevated levels of phosphorylated MAP kinase. Cells were serum-starved and metabolically labelled with [32P] and then exposed to either SV40 or EGF (positive control). Since MHC class I proteins are a component of the SV40 receptor (Atwood & Norkin, 1989; Breau et al., 1992) and MAbs against class I molecules also induce the up-

regulation of c-myc and c-jun (W. C. Breau & L. C. Norkin, unpublished results), the effect of exposing cells to anti-class I MAb W6/32 was also determined (see Discussion). Immunoprecipitates of MAP kinase (ERK1) from lysates of EGF- and W6/32-treated cells showed enhanced levels of phosphorylated MAP kinase (Fig. 3 a). In contrast, SV40-treated samples showed the same levels of phosphorylated MAP kinase as unstimulated controls.

We next directly measured MAP kinase activity by immune complex kinase assays of anti-ERK1 immunoprecipitates, using a fragment of MBP containing the MAP kinase-specific consensus sequence as the substrate. Whereas EGF was readily observed to induce elevated levels of MAP kinase activity, samples from cells exposed to SV40 showed the same levels of MAP kinase activity as unstimulated control samples. (Fig. 3 b). Note that immune complex kinase assays, in which the ligand was W6/32, were done in parallel with the above experiment. The MAb induced an increase in MAP kinase activity similar to that induced by EGF (N. S. Dangoria & L. C. Norkin, unpublished results).

We next considered the possibility that SV40 actually does activate MAP kinase, but this activation is not seen here because at some point SV40 also activates a protein phosphatase activity that modulates MAP kinase. To test this possibility, we measured the effect of SV40 on MAP kinase
activity when cells were pretreated with okadaic acid and sodium orthovanadate (inhibitors of protein phosphatase 2A and tyrosine phosphatases, respectively; Brown & Gordon, 1984). Treatment of cells with the two inhibitors alone resulted in a slight increase in MAP kinase activity (data not shown), consistent with the premise that protein phosphatases normally function as suppressors of growth factor signalling pathways (Mumby & Walter, 1993). However, pretreatment of cells with the inhibitors, followed by exposure to SV40, did not result in a significant activation of MAP kinase beyond that induced by treatment with the inhibitors alone. Thus, blocking protein phosphatase activity did not lead to potentiation of an effect by SV40 on MAP kinase activity.

The above experiments do not exclude the possibility that SV40 might be inducing the up-regulation of MAP kinase below the level of detection of our assays. To obtain direct evidence that the SV40-induced up-regulation of c-myc and c-jun is not dependent on the activation of MAP kinase, cells were transfected with the gene 3CH134, which encodes the MAP kinase-specific phosphatase, MKP-1. This phosphatase inactivates MAP kinase activity both in vitro and in vivo (Sun et al., 1993). Expression of 3CH134 in CV-1 cells had no observable effect on the SV40-induced up-regulation of c-myc and c-jun (Fig. 4). In contrast, expression of 3CH134 under the same conditions blocked the up-regulation of c-myc and c-jun by W6/32 (N.S. Dangoria & L.C. Norkin, unpublished results). Taken together, the above results show that MAP kinase is not required for the SV40-induced up-regulation of c-myc and c-jun, nor does SV40 binding appear even to activate MAP kinase.

We next asked whether extracellular SV40 might induce the activation of Raf, an upstream effector of the MAP kinase-dependent signalling cascade. Raf activity was measured directly in immune complexes from cells exposed to SV40. Consistent with the absence of SV40-induced activation of MAP kinase, the virus did not activate Raf under conditions in which Raf was activated by exposing cells to EGF (Fig. 5).

**PKC may be a mediator of the SV40 signalling pathway**

PKC mediates a number of cellular responses including mitogenesis (reviewed in Hug & Sarre, 1993). The translocation of PKC to the plasma membrane is a well-established indicator of PKC activation (Woodgett & Hunter, 1987). By this criterion, exposing serum-deprived CV-1 cells to SV40 did not appear to activate PKC (Fig. 6). In the experiment shown, cells were exposed to SV40 at an input multiplicity of 10 p.f.u./cell. Similar results were obtained at inputs of 1 and 100 p.f.u./cell (data not shown). For the purpose of comparison and to establish a positive control, cells were also treated with W6/32, which did induce the translocation of PKC activity.

A straightforward way to evaluate the role of PKC in a cellular response is to inhibit the activity of the enzyme in intact cells. For this purpose, we measured the SV40-induced up-regulation of c-myc and c-jun in cells treated with calphostin C, a highly specific and potent inhibitor of PKC (Kobayashi et al., 1989; see Discussion). The SV40-induced up-regulation of both c-myc and c-jun was sensitive to calphostin C (Fig. 4). In contrast, calphostin C did not block the up-regulation of c-myc in cells...
by EGF. Thus, the effect of calphostin C on the up-regulation of the primary response genes was specific for the SV40-induced response.

**Extracellular SV40 does not mobilize Ca^{2+}**

Ca^{2+} is a downstream mediator in a number of neural, endocrine and peptide growth factor-induced responses (Rasmussen et al., 1986). Extracellular signals may cause a rise in cytosolic Ca^{2+} from either the extracellular fluid or from intracellular stores. This leads to the activation of Ca^{2+}-sensitive signalling pathways in the cell.

The effect of extracellular SV40 on cytosolic Ca^{2+} levels was followed in individual cells over time using a quantitative digital fluorescent imaging system (Fig. 7). No cells were seen to respond when challenged with SV40 (300 p.f.u./cell), whereas essentially all cells responded within seconds when challenged with serum. The cells, which were attached to coverslips in these experiments, were subsequently rinsed several times and incubated at 37 °C for 48 h, at which time virtually all were seen to be infected as indicated by indirect immunofluorescent staining for SV40 T antigen (data not shown). The above experiment was initially done at 30 p.f.u./cell and then at 150 p.f.u./cell, with the same results as shown above (data not shown).

**SV40 infectious entry is dependent on signal transmission**

We developed an assay to measure the timing of SV40 infectious entry based on our finding that SV40 at the cell surface can be neutralized with anti-SV40 antiserum (H. A. Anderson & L. C. Norkin, unpublished results). SV40 was preadsorbed to cells at 4 °C. Cultures were then shifted to 37 °C. Adding neutralizing antiserum at various times after the shift enabled us to time infectious entry. Infected cells were identified by immunofluorescent staining for SV40 T antigen at 48 h. Using our post-adsorption neutralization assay we found that SV40 infectious entry is notably slow, with most preadsorbed virus remaining susceptible to neutralization for more than 1-5 h after the shift to 37 °C (H. A. Anderson & L. C. Norkin, unpublished results). However, entry was complete by 4 h. Note that bulk SV40 entry, as measured by flow cytometry, followed a similar time course (H. A. Anderson & L. C. Norkin, unpublished results). Thus, in the experiment shown in Fig. 8, if the cells were not treated with genistein to block the SV40-induced signal, then they were not affected by adding antiserum at 4 h. However, when cells were treated with genistein from 0.5 h before to 4 h after infection and then exposed to SV40 antiserum, there was an 85% decrease in the number of infected cells. The effect of the genistein was partly reversible if the cells were not treated with antiserum at 4 h. Note that the genistein treatment did not impair SV40 binding to cells, as shown by FACScan analysis (data not shown).

Receptor-mediated endocytosis is usually constitutive. To control for any non-specific effects of genistein that might impair endocytosis in general, we measured the effect of the drug on the endocytosis of transferrin. ^{125}I-labelled transferrin was preadsorbed to cells at 4 °C. Cultures were then shifted to 37 °C. The amount of preadsorbed ^{125}I-labelled transferrin
internalized in 5 min was measured after removing surface 125I-labelled transferrin with pronase. Pretreatment of the cells with genistein for 4 h followed by incubation in genistein during transferrin adsorption at 4 °C and the subsequent incubation at 37 °C had no effect on transferrin internalization (data not shown). Note that in contrast to the time-course of SV40 entry, most transferrin internalized within minutes of transfer to 37 °C (data not shown).

Discussion

We show here that SV40, like polyomavirus (Zullo et al., 1987; Glenn & Eckhart, 1990), also transmits a receptor-mediated signal that results in the transcriptional up-regulation of primary response genes.

The primary response genes have also been referred to as 'immediate early' and 'early growth response' genes (Herschman, 1991). However, the term 'primary response' may be more useful since it is based on the operational criterion that induction does not require intervening protein synthesis (Yamamoto & Alberts, 1976; Herschman, 1991). Instead, induction of primary response genes usually results from the activation of signalling pathways initiated from the cell surface, which activate pre-existing transcriptional modifiers. In this regard, SV40 was able to induce the transcriptional up-regulation of c-myc in the presence of cycloheximide. Furthermore, UV-inactivated SV40 was able to induce the up-regulation of c-myc. Thus, the SV40-induced up-regulation of primary response genes fulfils the criteria for a receptor-mediated primary response.

SV40 was previously reported to induce the up-regulation of primary response genes (Morike et al., 1988). However, in that study up-regulation of primary response genes was not seen in cells exposed to UV-irradiated virus. In contrast, we observed up-regulation of these genes in cells exposed to UV-irradiated virus as well as in cells infected in the presence of cycloheximide. Thus, unlike the present study, the earlier report does not describe a receptor-mediated process. It is noteworthy that the induction of the signalling pathway described here occurred at virus inputs of 1 p.f.u./cell. Thus, these events occur under physiologically relevant conditions and are not merely phenomena that might be associated with unusually high virus multiplicities.

The serine/threonine kinases Raf and MAP kinase are components of a major ubiquitous intracellular signalling pathway that is induced by a number of mitogenic stimuli (Peloch & Sanghera, 1992; Roberts, 1992), in particular those that activate protein tyrosine kinases. Thus, it was somewhat surprising that SV40 did not appear to activate Raf and MAP kinase, since the SV40-induced signalling pathway appeared to include a tyrosine kinase activity. We considered the possibility that SV40 might indeed be activating Raf and MAP kinase, but that this activation might be concealed if the virus was also activating a phosphatase that modulated the MAP kinase pathway. However, SV40 did not appear to activate MAP kinase when cells were pretreated with okadaic acid and sodium vanadate. We also considered the possibility that SV40 might induce MAP kinase activation below our level of detection. However, SV40 induced the up-regulation of c-myc and c-jun under conditions in which MAP kinase activity was blocked by expression of the MAP kinase-specific phosphatase, MKP-1. Thus, neither Raf nor MAP kinase appeared to be effectors of the SV40-induced signalling pathway.

As noted above, Ca²⁺ is a second messenger in a variety of signal responses. Using a highly sensitive CCD-based video-imaging fluorescent microscope (Linderman et al., 1990) we found no indication that SV40 binding induced an increase in free intracellular Ca²⁺. The SV40-induced signal pathways might possibly involve spatially highly localized calcium transients that are not resolved by this system or they might require longer incubation times before being expressed. However these possibilities appear unlikely since we readily detected Ca²⁺ mobilization in every cell within seconds of exposure to 1% serum. EG, insulin and CSF-1 provide examples of other ligands that can transmit a signal without mobilizing Ca²⁺ (Downing et al., 1989; Nishibe et al., 1990; Franklin & Kraft, 1992).

SV40 did not appear to activate PKC, based on the criterion of translocation of PKC activity from a cytosolic to a membrane fraction. Nevertheless, activation of PKC may have occurred, as suggested by our finding that the SV40-induced up-regulation of c-myc and c-jun was blocked by calphostin C. There is always concern over the specificity of pharmacological kinase inhibitors since most do not show absolute specificity. However, calphostin C is highly specific for PKC. For example, although calphostin C also inhibits protein kinase A and src kinase (IC₅₀ > 50 μM) and protein kinase G (IC₅₀ > 25 μM), it does so at much higher concentrations than are required to block PKC (IC₅₀ = 50 nM) (Kobayashi et al., 1989). A likely reason for the high specificity of calphostin C for PKC is that this drug interacts with the common regulatory, rather than the catalytic region, of the PKC family of enzymes (Kobayashi et al., 1989). Regardless, the high specificity of calphostin C makes it a valuable reagent. Our finding that calphostin C at 0.5 μM nearly completely blocked the up-regulation of c-myc and c-jun strongly suggests that PKC is an effector of the SV40-induced signalling pathway leading to the up-regulation of primary response genes. Furthermore, calphostin C was specific for the SV40-induced response since it did not block the up-regulation of c-myc by EGF.

Since SV40 did not appear to mobilize Ca²⁺, it is interesting to note that several Ca²⁺-independent isoforms of PKC have been described (reviewed in Hug & Sarre, 1993). The mechanism for the activation of the atypical isoforms of PKC is not known. Unlike the classical isoforms, the atypical ones require neither Ca²⁺ nor association with membrane diacylglycerol for their activation (McGlynn et al., 1992; Nakanishi & Exton, 1992), perhaps accounting for our results.
Taken together, the above results imply that the SV40-induced signal that up-regulates c-myc and c-jun is dependent on a tyrosine kinase and PKC, but is independent of Raf and MAP kinase. We do not yet know how the signal might be transmitted from the tyrosine kinase to PKC, nor do we know what effectors might be downstream of PKC. Other MAP kinase-independent mitogenic signalling pathways have recently been described (for a review see Eisenman & Cooper, 1995).

The SV40-induced signal appeared to promote SV40 infectious entry. This was implied by our finding that preadsorbed SV40 remains susceptible to neutralization by anti-SV40 antiserum while genistein is present, but that infectious entry can occur upon genistein reversal. Furthermore, the lack of an effect of genistein on the internalization of transferrin, which occurs constitutively through clathrin-coated vesicles, shows that genistein is not inhibiting endocytosis in general.

The likelihood that SV40 infectious entry is promoted by a signal transmitted from the cell surface is interesting with respect to the following: firstly, whereas most viruses that enter cells by endocytosis enter in clathrin-coated vesicles (Marsh & Helenius, 1989), SV40 enters cells in non-coated vesicles (Maul et al., 1978; Kartenbeck et al., 1989). These non-coated vesicles might at this time be referred to as caveolae (e.g. compare Fig. 1 of Maul et al., 1978, to Fig. 1 of Rothberg et al., 1992). Indeed, we found that SV40 infectious entry was blocked by caveolae-disrupting drugs such as nystatin (H. A. Anderson & L. C. Norkin, unpublished results). In control experiments, these drugs had no effect on internalization of transferrin. In contrast, SV40 infectious entry was not sensitive to acidification of the cytosol (H. A. Anderson & L. C. Norkin, unpublished results), which impairs endocytosis through clathrin-coated vesicles (Sandvig et al., 1987). In control experiments, cytosol acidification blocked internalization of transferrin (H. A. Anderson & L. C. Norkin, unpublished results). Furthermore, it was suggested that caveolae have a role in trans-membrane signalling (Anderson, 1993; Lisanti et al., 1994) and that the internalization of caveolae might be regulated by phosphorylation (Parton et al., 1994). Studies to clarify the mechanism by which the trans-membrane signal might promote SV40 entry are in progress.

The primary or early response genes are generally activated by PDGF and are associated with the passage of quiescent cells into the G₁ phase of the cell-cycle (reviewed in Pardee, 1989). It was suggested that the activation of these genes by polyomavirus might prime cells to support virus replication (Zullo et al., 1987; Glenn & Eckhart, 1990). In an experiment not described here, we found that treatment with genistein from 0:5 to 3 h caused a delay of several hours in the onset of SV40 DNA synthesis. An important control in the experiment was that genistein had no effect on virus DNA synthesis when present between 5 and 7 h. Since these results could be explained at least in part by the effect of genistein on entry, we do not know whether the signal might also promote replication. Regardless, to the best of our knowledge, our results are the first to show that a virus-induced trans-membrane signal actually promotes infection, at the point of penetration or otherwise. Note that human immunodeficiency virus (HIV) was reported to induce a signal mediated by its receptor, CD4, that resulted in the phosphorylation of CD4 by a PKC-dependent pathway (Fields et al., 1988). However, other studies did not find evidence for an HIV-induced signal transduction pathway (Horak et al., 1990; Hoxie et al., 1988) or for the inhibition of HIV entry by agents that block signal transduction (Orloff et al., 1991).

We previously reported that MHC class I molecules are a necessary component of the SV40 receptor (Atwood & Norkin, 1989; Breau et al., 1992). Although class I proteins are best known for their important role in immunity (reviewed in Yewdell & Bennink, 1992), they have also been found in association with several peptide hormone and growth factor receptors (reviewed in Eddin, 1988). Also, cross-linking class I proteins with antibodies triggers partial activation of T cells (Geppert et al., 1988; Dissing et al., 1990).

As a first step in asking whether MHC class I proteins might play a role in transducing the SV40 signal beyond merely facilitating virus binding, we found that cross-linking class I proteins with anti-MHC-I MAb also induced the transcriptional up-regulation of c-jun and c-myc (W. C. Breau & L. C. Norkin, unpublished results). However, unlike SV40, anti-MHC-I MAb clearly activated Raf and MAP kinase, as well as PKC (N. S. Dangoria & L. C. Norkin, unpublished results).

Despite the above, it remains possible that MHC class I molecules might mediate the SV40-activated signals. The differences between the signals activated by SV40 and anti-MHC-I might be explained by differences in the extent of receptor cross-linking or conformational changes induced by each ligand, or perhaps in their selective recruitment of distinct accessory signalling molecules. Studies to clarify the role of MHC class I proteins in the SV40-activated signals are in progress.

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