THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 17, Issue of April 29, pp. 16772–16783, 2005
Printed in U.S.A.

Cell Signaling through the Protein Kinases cAMP-dependent Protein Kinase, Protein Kinase Cε, and RAF-1 Regulates Amphotropic Murine Leukemia Virus Envelope Protein-induced Syncytium Formation*

Received for publication, October 12, 2004, and in revised form, February 18, 2005
Published, JBC Papers in Press, March 1, 2005, DOI 10.1074/jbc.M411537200

Wei Wang‡, Zsolt Jobbagy‡§, Terry H. Bird‡¶, Maribeth V. Eiden‖, and Wayne B. Anderson‡**

From the ‡Laboratory of Cellular Oncology, NCI, National Institutes of Health and the ‖Laboratory of Cellular and Molecular Regulation, NIMH, National Institutes of Health, Bethesda, Maryland 20892

Amphotropic murine leukemia virus (A-MuLV) utilizes the PiT2 sodium-dependent phosphate transporter as its cell surface receptor to infect mammalian cells. The process of A-MuLV infection requires cleavage of the R peptide from the envelope protein. This occurs within virions thereby rendering them competent to fuse with target cells. Envelope proteins lacking the inhibitory R peptide (*e.g.* envelope (R-) proteins) induce viral envelope-mediated cell-cell fusion (syncytium). Here we have performed studies to determine if cell signaling through protein kinases is involved in the regulation of PiT2-mediated A-MuLV envelope (R-)-induced syncytium formation. Truncated A-MuLV retroviral envelope protein lacking the inhibitory R peptide (R-) was used to induce viral envelope-mediated cell-cell fusion. Signaling through cyclic AMP to activate PKA was found to inhibit envelope-induced cell-cell fusion, whereas treatment of cells with PKA inhibitors H89, KT5720, and PKA Catα siRNA all enhanced this cell fusion process. It was noted that activation of PKC, as well as overexpression of PKCε, up-regulated A-MuLV envelope protein-induced cell-cell fusion, whereas exposure to PKC inhibitors and expression of a kinase-inactive dominant-negative mutant of PKCε (K437R) inhibited syncytium formation. *v-ras* transformed NIH3T3 cells were highly susceptible to A-MuLV envelope-induced cell-cell fusion, whereas expression of a dominant-negative mutant of Ras (N17Ras) inhibited this cell fusion process. Importantly, activation of Raf-1 protein kinase also is required for A-MuLV envelope-induced syncytium formation. Expression of constitutively active BXB Raf supported, whereas expression of a dominant-negative mutant of Raf-1 (Raf301) blocked, A-MuLV-induced cell-cell fusion. These results indicate that specific cell signaling components are involved in regulating PiT2-mediated A-MuLV-induced cell-cell fusion. Selective pharmacological modulation of these signaling components may be an effective means of altering cell susceptibility to viral-mediated cytopathic effects.

Retrovirus entry into cells is initiated by the binding of virus envelope glycoproteins to specific cell surface receptors. This is followed by fusion of the viral membrane with the receptor-bearing cellular plasma membrane, which allows the viral core to be released into the cell (1). Retrovirus infection also can result in virus-induced cell-cell fusion to form multinucleated giant cells (syncytia) (2, 3). Syncytium formation is dependent on the interactions between viral envelope protein on the surface of infected cells and virus receptors on the surface of uninfected cells (4). Syncytium formation is an important consequence of virus infection. Yet, the cellular regulatory mechanisms that allow retrovirus-mediated cell-cell fusion, including activation or inhibition of cellular signal transduction pathways, remain to be elucidated.

Several studies have suggested that cellular signal transduction pathways and associated protein kinases could be involved in regulating both retrovirus infection and virus-induced cell-cell fusion. Mohagheghpour *et al.* (5) reported that treatment of $CD4^+$ T cells with 1-oleoyl-2-acetyl glycerol, an analog of the protein kinase C (PKC)[1] activator, diacylglycerol, enhanced syncytium formation with human immunodeficiency virus-1 (HIV-1)-envelope-positive cells. Root *et al.* (6) reported that inhibition of PKC decreased influenza virus entry into cells. Other studies have suggested that fusion regulatory proteins (FRPs) and protein kinases regulate HIV gp160 envelope-mediated cell-cell fusion (7). Cyclic AMP (cAMP), a second messenger that activates cAMP-dependent protein kinase (PKA), also has been implicated in the regulation of HIV infection and virus-induced cell-cell fusion by up-regulating HIV-1 co-receptor CXCR4 expression (8, 9).

Cell surface receptors for amphotropic murine leukemia virus (A-MuLV) have been demonstrated to serve as sodium-dependent phosphate (Na^+/P_i) transporters in a variety of cells. Based on their structural and functional characteristics, these molecules, together with the gibbon ape leukemia virus (GALV) receptor, were classified as type III Na^+/P_i transporters and were designated as PiT2 and PiT1, respectively (for review, see Ref. 10). It has been shown that both the PiT1 and PiT2 phosphate transporter and virus receptor activities are altered in response to changes in extracellular inorganic phosphate

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Dept. of Pathology, Dartmouth Hitchcock Medical Center, One Medical Center Dr., Lebanon, NH 03756.

¶ Present address: Dept. of Biology, Loyola College, 4501 N. Charles St., Baltimore, MD 21210-2699.

** To whom correspondence should be addressed: Laboratory of Cellular Oncology, NCI, National Institutes of Health, Bldg. 37, Rm. 4124, 37 Convent Dr., Bethesda, MD 20892. Tel.: 301-496-9247; Fax: 301-480-0471; E-mail: andersow@mail.nih.gov.

[1] The abbreviations used are: PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PKA Catα, PKAα catalytic subunit; A-MuLV, amphotropic murine leukemia virus; GALV, gibbon ape leukemia virus; PMA, phorbol 12-myristate 13-acetate; EAR, hamster PiT2; N17Ras, dominant-negative mutant of Ras; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; TM. transmembrane; HIV, human immunodeficiency virus; siRNA, short interfering RNA; SU, surface subunit.

This paper is available on line at http://www.jbc.org

concentrations (11–13). In studies carried out in CHO cells, the levels of PiT2 at the cell surface remained unchanged following variations of the phosphate supply, whereas changes in the efficiency of PiT2-mediated phosphate and virus entry occurred within minutes in response to changes in extracellular phosphate concentration (14). Recently, Salaun *et al.* (15) reported that changes in extracellular phosphate concentration appear to switch PiT2 assemblies at the cell surface between compact and relaxed configurations, and that it is the compact arrangement that is required for enhanced PiT2 activities. These results indicated that PiT2 activities may be regulated by unspecified post-translational modifications of the cell surface PiT2 proteins, and that these modifications are required for activation of phosphate transporter and retrovirus receptor functions. In earlier studies we reported that activation of PKC by treatment of cells with phorbol 12-myristate 13-acetate (PMA) enhanced, whereas activation of PKA inhibited, Na^+/P_i uptake (16). In more recent studies, it was established that PMA treatment of cells enhances Na^+/P_i uptake via stimulation of PiT2, and that this effect is specifically mediated through PMA activation of PKCϵ (17). However, it remains to be determined if changes in protein kinase activities also can modulate PiT2 viral receptor function.

GALV and A-MuLV are closely related γ-retroviruses. Their envelope proteins are composed of a surface (SU) subunit and a transmembrane (TM) subunit that are cleaved by a host protease from a common precursor protein (18). Evidence indicates that SU is the subunit responsible for binding to the cell surface receptor whereas the TM subunit contributes to the fusion process. The R peptide, the C-terminal region of the cytoplasmic tail of TM polypeptide, is further cleaved by a viral protease. After binding of SU to the viral receptor, TM mediates fusion of virion and host membranes. However, R peptide acts to inhibit fusion until it is cleaved from the C terminus of TM (19, 20). Truncated retroviral envelope proteins not bearing R peptides (R-) and expressed in appropriate cells induce syncytium formation (19, 20).

Here we have expressed truncated A-MuLV and GALV envelope proteins (R-) in certain cell lines to determine if signal transduction pathways and the modulation of specific protein kinase activities can act to regulate PiT1 and PiT2 receptor function and modulate A-MuLV and GALV envelope-induced cell-cell fusion. It was found that activation of PKCϵ is required for A-MuLV-, but not GALV-, induced syncytium formation, whereas cell signaling through cyclic AMP to activate PKA inhibited the A-MuLV-induced fusion process. Further, activation of Raf protein kinase also is required for A-MuLV-induced syncytium formation.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and the PLATINUM *Taq* DNA polymerase high fidelity PCR kit were purchased from Invitrogen. PMA, rottlerin, Gö6976, GF109203X, PD98059, SB202190, 8-Br-cAMP, KT5720, forskolin, and H89 were from Calbiochem. The PKA Catα siRNA was purchased from Dharmacon (Lafayette, CO). The pCAETR plasmid coding for the A-MuLV envelope protein (R-) (not bearing R peptide) was a gift from Dr. Jack A. Ragheb (National Institutes of Health, Bethesda, MD). Plasmids coding N17Ras, Raf301, and ERK185 were kindly provided by Dr. Toren Finkel (National Institutes of Health) with Dr. Silvio Gutkind's (National Institutes of Health) permission. The pCIGALV plasmid carrying the GALV envelope cDNA, pLNHA.PiT1 encoding an HA tagged PiT1, pcDNAMoMLV gag-pol, and pCI4070A encoding the A-MuLV envelope protein (21) were used in this study. Polyfect transfection reagent is a product of Qiagen (Valencia, CA). pCI-neo vector and pGEM-T easy vector were from Promega (Madison, WI). The BCA protein assay kit was purchased from Pierce.

Antibodies

The PiT2-specific rabbit antiserum was produced in our laboratory as described previously (22). A-MuLV pig antiserum (lot no. 77S000445) was obtained from the NCI/BCB Reagents Repository (Camden, NJ). Mouse monoclonal anti-β-actin (AC-15), horseradish peroxidase-labeled anti-FLAG, and anti-HA tag monoclonal antibodies were purchased from Sigma. Rabbit polyclonal anti-PKAα catalytic subunit, anti-Raf-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-p44/42 MAP kinase antibodies were from Cell Signaling (Beverly, MA). Mouse monoclonal Pan-Ras antibody (Ab-3) was purchased from Calbiochem. Peroxidase-labeled goat anti-rabbit and anti-swine Igs were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

Cell Culture

All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum. The ϵMTH vector contains a zinc-inducible promoter (23). Thus, cells transfected utilizing the ϵMTH vector were incubated in the presence and absence of 75 μM zinc acetate to further induce synthesis of the indicated recombinant proteins. Because of significant constitutive expression of the encoded recombinant proteins in the absence of zinc in NIH3T3 cells, similar results were obtained in studies carried out without zinc induction.

DNA Construct of pCIGALVTR Coding Truncated GALV Envelope Protein (R-) (no R peptide)

(i) Primers—PCR primers used were as follows: 1) AATTCACGCGTGGTACCTCTAGAAG; 2) TTTCTGCGGCCGCTAGATCTACAGAATTTTAACTGCAC; 3) AGACAGGATATCAGTGGTCC; 4) TTTTCCCAGTCACGACGTTG.

Recognition sites for restriction endonucleases used for cloning are underlined. Primer pairs 1–2 were used to produce GALVTR that encodes truncated GALV envelope protein (R-) (not bearing R peptide). Primer pairs 3–4 were used to amplify 3′-untranslated and poly(A) in pCAETR.

(ii) pCIGALVTR Construct—pCIGALV DNA (21) was used as template to produce the high fidelity PCR fragment GALVTR using primer pairs 1–2. The purified product GALVTR was cloned into the pGEM-T (Promega, Madison, WI) easy vector. GALVTR then was cut out with XbaI-NotI and ligated into the pCI-neo vector (Promega) cut with XbaI-NotI to form new plasmid pCIGALVTR-a. To create pCIGALVTR that has the same 3′-UTR and poly(A) as pCAETR, high fidelity PCR product from plasmid pCAETR was produced using primer pairs 3–4, then cut with BglII-NotI and inserted into the BglII-NotI site of pCIGALVTR-a to form pCIGALVTR. The validity of the pCIGALVTR construct was confirmed by DNA sequencing.

DNA Construct of pϵMTH.EAR.FLAG Coding for FLAG Epitope-tagged Hamster PiT2

(i) Primers—PCR primers used were as follows: 1) 5′-SalI primer, GAGGTCGACATGGCCATGGATGAGTATTTGTGG; 2) 3′-FLAG primer, *CTTGTCATCGTCGTCCTTGTA*GTCTACATGGATTTTGTGCAAC; 3) 5′-FLAG primer, *TACAAGGACGACGATGACAAG*CTTAAAGGCCCTGAGGAAAAGCTCT; 4) 3′-MluI primer, GTATACGCGTCACATATGGAAGGATCCCATACATG; Recognition sites for restriction endonucleases used for cloning are underlined; overlapping sequences are italicized.

(ii) pϵMTH.EAR.FLAG Construct—Insertion of the FLAG epitope tag into the cytoplasmic loop of hamster PiT2 was accomplished in two steps by overlapping PCR using primer pairs 1–2 and 3–4 in the first rounds of PCRs. The products of these reactions then were purified, annealed, and used as template for a final PCR using primers 1 and 4. The purified product was cut with SalI-MluI and ligated into the pϵMTH vector cut with XhoI-MluI.

Cell Lines

The NIH3T3 Cl-7 cell line and the *v-Ha-ras* and *v-K$_i$*-ras transformed NIH3T3 cells were kindly provided by Drs. J. DeClue and D. Lowy (National Institutes of Health). NIH3T3/hamster PiT2.FLAG and NMU34m/hamster PiT2.FLAG cells expressing FLAG epitope-tagged hamster PiT2 were constructed using the pϵMTH.EAR.FLAG construct described above, and were generated as described previously (17). The PKCα-, PKCβ-, PKCδ-, and PKCϵ-overexpressing NIH3T3 cell lines were generated as described previously utilizing the ϵMTH vector (17). NIH3T3/DN-PKCϵ cells overexpressing the kinase-inactive mutant of PKCϵ were constructed by converting lysine 437 within the catalytic domain to an arginine and then cloned into the epitope-tagging vector

pεMTH, as described previously (24). NIH3T3/BXB Raf cells overexpressing the N-terminally truncated, activated Raf-1 were produced by cloning into the pεMTH vector (25).

Generation of NIH3T3/HA.PiT1 Cell Line—The pCI4070A plasmid encoding the A-MuLV envelope protein was co-transfected along with the pcDNAMoMLV gagpol plasmid and the pLNHA.PiT-1 plasmid encoding HA-tagged PiT1 into 293T cells using the calcium phosphate precipitation method (Promega) (21). Supernatant containing enveloped pseudoretrovirus was harvested at 48 h post-transfection. NIH3T3 cells at about 70% confluency were exposed to retroviral vector containing supernatant that had been passed through a 0.22-μm Millipore (Bedford, MA) filter and then were adjusted to contain 10 μg of polybrene/ml. Thirty-six hours later, the medium was changed, and cell lines stably expressing HA.PiT1 were selected with G418 (0.8 mg/ml) as previously described (21).

siRNA Transfection

PKAα catalytic subunit (PKA Catα) siRNA oligonucleotides (SMARTpool), and Luciferase siRNA oligonucleotides (target sequence 5′-cgtacgcggaatacttcga-3′) from Dharmacon (Lafayette, CO) were transfected into NIH3T3 cells with *Trans*IT-TKO transfection reagent (Mirus Corporation, Madison, WI) according to the manufacturer's instructions. Cells were transfected with 50 nM PKA Catα siRNA and control Luciferase siRNA (final concentration present in the cell culture media).

Syncytium Formation Assays

The cell types indicated were transiently transfected with 1.8 μg of the envelope-encoding plasmids pCAETR or pCIGALVTR using PolyFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Cells were plated on 35-mm tissue culture dishes and incubated overnight to reach about 50% confluency at the time of transfection. The DNA-PolyFect-DMEM mixture (1.8 μg of DNA, 10 μl of PolyFect, and 100 μl of DMEM) was incubated for 10 min at room temperature. The cells were washed once with 2.5 ml of PBS, and 1.5 ml of cell culture medium was added to the cells. After 10 min of incubation, 600 μl of cell culture medium were added to the DNA-PolyFect-DMEM mixture, and the resulting solution was gently loaded onto the cells. Syncytium formation in 4 random fields was assessed at the indicated time points post-transfection. There were about 1000 cells in the fields. A syncytium with more than 5 nuclei was counted as syncytium formation. The fusion number is expressed as the percentage of N/T (N is the number of nuclei in syncytia, and T is the total number of nuclei in the field).

Western Blot Analysis

Whole cell lysates, cellular membrane, and cytosolic fractions were prepared as described previously (22). Proteins present in cell lysates were separated by precast 4–20% SDS-polyacrylamide gel (Owl Separation Systems, Portsmouth, NH) electrophoresis, and then electrophoretically transferred from the gel onto Protran membranes (Schleicher & Schuell, Keene, NH). Immunoreactive proteins were detected with appropriate antibodies as described elsewhere (23).

RESULTS

Transfection of cells with viral envelope protein expression vectors in the absence of other viral proteins has been used successfully to induce extensive cell-cell membrane fusion (2, 19, 26). In this study we employed pCAETR, an A-MuLV envelope protein (R-) expression vector, and pCIGALTR, a GALV envelope protein (R-) expression vector, to transfect cells and express the indicated envelope protein lacking the R inhibitory peptide as described by Ragheb and Anderson (19). The ability of cell-expressed envelope protein to induce multinuclear syncytium formation is termed fusion-from-within, and was used here as a measure of viral envelope-induced cell-cell fusion. Such syncytium formation also is thought to serve as a model for fusion between viral envelope and the host cell membrane that precedes viral entry. Here, we have used this model system to characterize the cell signaling events that regulate PiT2-dependent A-MuLV envelope-mediated syncytium formation. When the indicated cell lines were transiently transfected with empty vector (no expression of A-MuLV envelope) in the presence and absence of the indicated inhibitors and activators, no syncytium formation was observed under any of the conditions described.

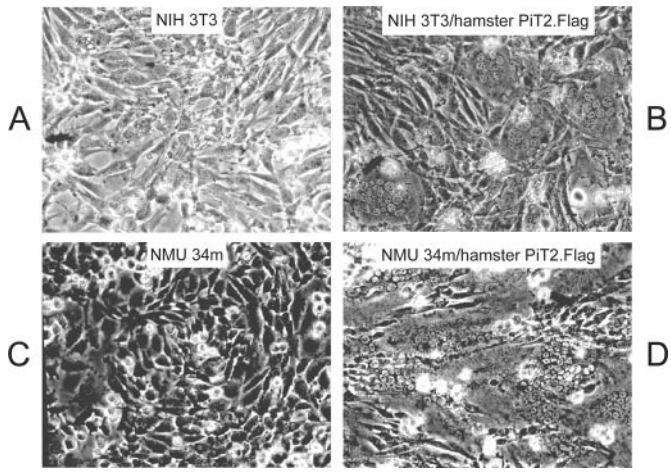

FIG. 1. **Syncytium formation in cells expressing the A-MuLV envelope protein (R-) is dependent on the level of PiT2 transporter/viral receptor.** NIH3T3 (*panel A*), NIH3T3 overexpressing FLAG epitope-tagged hamster PiT2 (*panel B*), NMU34m (*panel C*), and NMU34m overexpressing FLAG epitope-tagged hamster PiT2 (*panel D*) cells were transiently transfected with pCAETR to express the A-MuLV envelope protein (R-). Cells were photomicrographed under phase contrast 48 h post-pCAETR transfection. Numerous syncytia of 5 or more nuclei were observed only in the NIH3T3/hamster PiT2.FLAG and NMU34m/hamster PiT2.FLAG cells overexpressing PiT2 (magnification, ×40).

The Level of PiT2 Expression Affects A-MuLV Envelope Protein-induced Syncytium Formation—In agreement with the results reported by Ragheb and Anderson (19), little detectable syncytium formation (<1%) was observed when NIH3T3 cells were transfected with pCAETR to express A-MuLV envelope protein (R-) (Fig. 1, *panel A*). However, expression of hamster PiT2 in NIH3T3 cells to increase the level of PiT2, the A-MuLV receptor, significantly enhanced the ability of expressed A-MuLV envelope to induce cell fusion (Fig. 1, *panel B*). To support this finding that the efficiency of A-MuLV envelope-induced syncytium formation was dependent on the level of PiT2 receptor, studies also were carried out with NMU34m cells, a nitrosylmethylurea (NMU)-transformed cell line that like BHK cells lack functional A-MuLV receptors are resistant to A-MuLV infection (27). Again it was observed that numerous and large multinucleated syncytia formed on NMU34m cells expressing hamster PiT2 (*panel D*), but not on parental NMU34m cells (*panel C*), with expression of A-MuLV envelope protein (R-).

Inhibition of A-MuLV Envelope Protein-induced Syncytium Formation by PKA—Previously, it was established that preincubation of NIH3T3 cells with 8-Br-cAMP to activate PKA resulted in a decrease in P_i uptake (16). Here studies were carried out to determine the possible involvement of cellular PKA activity in the regulation of A-MuLV-induced cell-cell fusion in these cells. The results presented in Fig. 2, *panel A*, and in Table I, show that treatment of NIH3T3 cells expressing FLAG epitope-tagged hamster PiT2 with the PKA activator 8-Br-cAMP inhibited A-MuLV envelope-induced cell-cell fusion. Likewise, exposure of NIH3T3 cells expressing FLAG epitope-tagged hamster PiT2 to forskolin to increase intracellular levels of cAMP to activate PKA also decreased A-MuLV envelope-induced syncytium formation (Table I). As noted in Fig. 1, expression of the A-MuLV envelope (R-) in mock control NIH3T3 cells was not capable of inducing syncytium formation.

The reagents H89 and KT5720 both are well established inhibitors of PKA activity. As reported by the supplier (Calbiochem), H89 is a specific inhibitor of PKA (K_i, 50 nM) compared

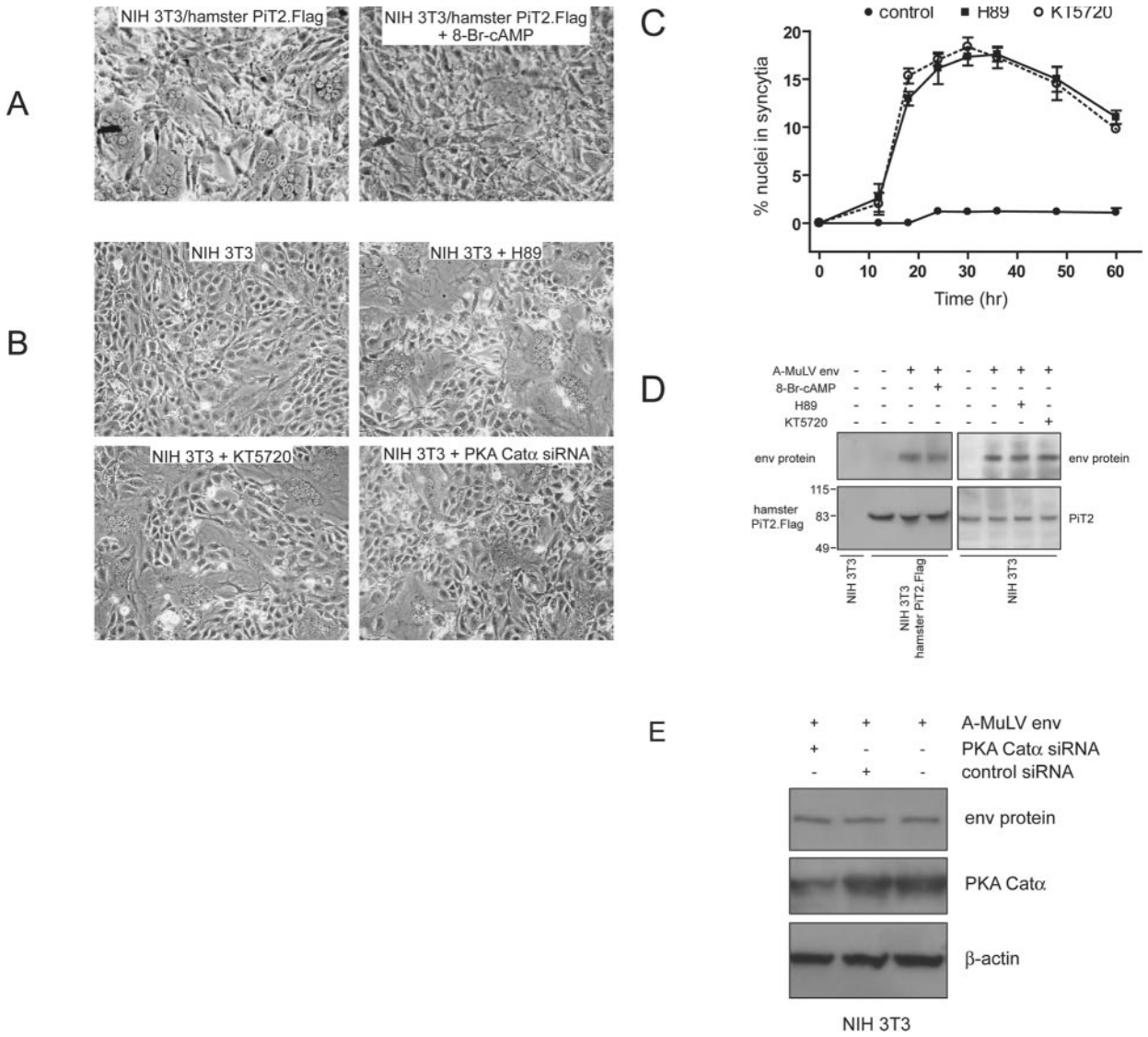

FIG. 2. **Syncytium formation in cells expressing the A-MuLV envelope protein (R-) is regulated by the cAMP-PKA signaling pathway.** *Panel A*, stable transfectants of NIH3T3 cells overexpressing FLAG epitope-tagged hamster PiT2 were treated without (*left panel*) or with (*right panel*) 8-Br-cAMP (200 μM) as indicated 3 h following transient transfection with pCAETR to express the A-MuLV envelope protein (R-) for 48 h. *Panel B*, wild-type NIH3T3 cells were incubated with PKA inhibitors H89 (100 nM) or KT5720 (100 nM) added 3 h following transient transfection with pCAETR to express the A-MuLV envelope protein (R-). NIH3T3 cells also were transfected with PKA Catα siRNA as described under "Experimental Procedures" 4 days prior to transient transfection with pCAETR to express the A-MuLV envelope protein (R-). Cells were photomicrographed under phase contrast 48 h after pCAETR transfection (magnification, ×40). *Panel C*, time course for A-MuLV envelope protein-induced cell-cell fusion in NIH3T3 cells incubated in the presence of PKA inhibitors of H89 (100 nM) (■) or KT5720 (100 nM) (○) and in the absence of inhibitors (●). The percent of nuclei in syncytia (cell fusion) was determined at the indicated time points following H89 or KT5720 addition to cells transiently transfected with pCAETR. The results are expressed as the means of three similar experiments run in duplicate, with S.D. indicated by the *vertical bars*. *Panel D*, Western blot analysis of total cell lysates prepared from NIH3T3 (*right panels*) and NIH3T3/hamster PiT2.FLAG (*left panels*) cells in the presence (+) and absence (−) of A-MuLV envelope protein (R-) expression, along with 8-Br-cAMP (200 μM), H89 inhibitor (100 nM), and KT5720 (100 nM) as indicated for 45 h. Electrophoresis of aliquots of the indicated cell lysates containing 25 μg of total protein were carried out on 4–20% SDS-polyacrylamide gels. Immunoblot analysis to determine possible changes in the levels of A-MuLV envelope protein (R-), FLAG epitope-tagged hamster PiT2, or PiT2 was carried out with anti-A-MuLV pig antiserum (1:750), horseradish peroxidase-conjugated anti-FLAG monoclonal antibody (1:2000), and anti-PiT2 rabbit antiserum (1:500), respectively. *Panel E*, Western blot analysis of total cell lysates prepared from control and PKA Catα siRNA-transfected NIH3T3 cells was performed as described under "Experimental Procedures" in the presence (+) of A-MuLV envelope protein (R-) expression, as indicated, for 45 h. Electrophoresis of aliquots of the indicated cell lysates containing 25 μg of total protein were carried out on 4–20% SDS-polyacrylamide gels. Immunoblot analysis to determine possible changes in the levels of A-MuLV envelope protein (R-), PKAα catalytic subunit, or β-actin as loading control was carried out with anti-A-MuLV pig antiserum (1:750), anti-PKAα catalytic subunit rabbit antibody (1:700), and anti-β-actin monoclonal antibody (1:5000), respectively.

with PKC (K_i, 76 μM). KT5720 also is an effective inhibitor of PKA (K_i, 56 nM), but does not inhibit PKC or PKG. In studies utilizing H89 and KT5720 in the treatment of intact cells in culture, these agents commonly have been used at high concentrations (1–10 μM). However, in a recent report Fu *et al.* (28) showed that treatment of intact cells with only 50 nM H89 for 24 h resulted in significant and specific inhibition of PKA activity. Thus, to significantly enhance the specificity of H89 and KT5720 as selective inhibitors of PKA activity, we used these two PKA inhibitors at a concentration of 100 nM to treat cells for 24–48 h in the present study. Interestingly, treatment of NIH3T3 cells with low concentrations (100 nM) of the PKA inhibitors H89 and KT5720 markedly stimulated cell-cell fusion in response to expression of A-MuLV envelope (R-) (Fig. 2, *panel B* and Table I). In the presence of either H89 or KT5720, syncytium formation was observed within 12–18 h after the

TABLE I

Activation of the cAMP-PKA signaling pathway inhibited A-MuLV envelope protein (R-)-induced cell-cell fusion

Stable transfectants of NIH3T3 cells overexpressing PKCε, FLAG epitope-tagged hamster PiT2, or the control empty vector (M) were treated with 8-Br-cAMP (200 μM), forskolin (4 μM), H89 (100 nM), or KT5720 (100 nM) as indicated 3 h following transient transfection with pCAETR to express the A-MuLV envelope protein (R-). The PKA Catα siRNA was transfected into NIH3T3 as described under "Experimental Procedures" 4 days prior to transient transfection with pCAETR. The percent of nuclei in syncytia (cell fusion number) was determined 48 h after transfection. Results are expressed as the mean ± S.D. of four similar experiments run in triplicate.

Cell line	Nuclei in syncytia					
	Untreated	8-Br-cAMP	Forskolin	H89	KT5720	PKA Catα siRNA
	%					
NIH3T3 (M)	<1	<1	<1	15 ± 2.4[a]	14 ± 1.6[a]	7 ± 1.8[a]
NIH3T3/PKCε	12 ± 1.1[b]	7 ± 2[a]	8 ± 1.5[a]	11 ± 2.2	12 ± 2.3	ND[c]
NIH3T3/hamster PiT2.FLAG	17 ± 0.2[b]	11 ± 1.3[a]	12 ± 2.4[a]	20 ± 1.7[a]	19 ± 1.1[a]	ND

[a] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to similar untreated cells.
[b] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to untreated NIH3T3 cells.
[c] ND, not determined.

transfection of A-MuLV envelope expression pCAETR (Fig. 2, *panel C*). Western blot analysis of lysates prepared from these cells showed that treatment with 8-Br-cAMP and with the H89 and KT5720 PKA inhibitors had no effect on the cellular levels of either expressed A-MuLV envelope protein or PiT2 protein (Fig. 2, *panel D*).

To gain additional evidence to support the involvement of the cAMP-PKA signaling pathway in the regulation of PiT2/A-MuLV envelope protein (R-)-induced cell-cell fusion, NIH3T3 cells were transfected with 50 nM PKA Catα siRNA to decrease the intracellular level of the α catalytic subunit of PKA. Four days after transfection of cells with PKA Catα siRNA, these cells were transiently transfected with pCAETR to express the A-MuLV envelope protein (R-). As shown in Fig. 2, *panel D*, and in Table I, treatment of NIH3T3 cells with PKA Catα siRNA to decrease the level of PKA Catα subunit protein (see Fig. 2, *panel E*) significantly enhanced A-MuLV envelope-induced syncytium formation. We were unable to detect PKA catalytic subunit β by Western blot analysis, and transfection of NIH3T3 cells with PKA Catβ siRNA did not promote A-MuLV envelope-induced cell-cell fusion.[2] Taken together, these results reported in Fig. 2 and Table I clearly indicate that elevated PKA activity inhibited, whereas decreased PKA activity stimulated, syncytium formation induced by A-MuLV envelope.

Stimulation of A-MuLV Envelope Protein-induced Syncytium Formation by PKC—In earlier studies, it was established that activation of PKC by treatment of cells with PMA increased PiT2-mediated P_i uptake, and that this effect was mediated through PMA activation of the PKCε isoform (17). Thus, studies were initiated to determine if PKCε also was involved in mediating A-MuLV envelope-induced cell-cell fusion. NIH3T3 cells have been shown to express the PKCα, -δ, -ε, and -ζ isoforms (29, 30). Since PMA does not activate the atypical PKCζ isoform, and since it was shown not to alter PiT2-mediated P_i uptake (17), PKCζ was not included in the present study. NIH3T3 cells overexpressing classical PKCα and PKCβ, and novel PKCδ and PKCε were used to determine the ability of each of these isotypes to alter A-MuLV envelope-induced cell-cell fusion in the absence of PMA treatment. As shown in Fig. 3, *panel A*, and in Table II, only overexpression of the PKCε isoform was found to appreciably increase A-MuLV envelope (R-)-induced syncytium formation.

In other studies, inhibitors and activators of cellular PKC activity were used to further establish a selective role for PKCε in the regulation of A-MuLV-induced cell-cell fusion. Three different PKC inhibitors were used to define a selective role for PKCε in up-regulating A-MuLV envelope-induced syncytium formation. Addition of the pan-specific bisindolylmaleimide inhibitor GF109203X, which inhibits both the classical and novel PKC isoforms, to NIH3T3 cells overexpressing either PKCε or hamster PiT2 resulted in significant inhibition of envelope-induced syncytium formation (Table II). Conversely, treatment of these cell types with the Gö6976 PKC inhibitor, which selectively inhibits only classical PKC isotypes, including PKCα, did not result in significant inhibition of A-MuLV envelope-induced cell-cell fusion. These results further indicate that the PKCα isoform present in NIH3T3 cells is not involved in mediating up-regulation of this A-MuLV fusion process. A third protein kinase inhibitor, Rottlerin, also was examined. Rottlerin has been reported to be a good inhibitor of PKCδ (31). As indicated in Table II, treatment with Rottlerin did not result in inhibition of syncytium formation induced in cells overexpressing either PKCε or PiT2. However, activation of PKC with PMA did up-regulate A-MuLV envelope-induced cell-cell fusion in cells overexpressing PKCε and PiT2, but not in cells overexpressing PKCα, -β, or -δ (Table II). These results lend additional support for an important role for PKCε in the modulation of this cell fusion process.

Finally, to confirm that PKCε is the specific isoform involved in up-regulating A-MuLV envelope protein-induced syncytium formation, studies were carried out with NIH3T3 cells overexpressing a kinase inactive dominant-negative PKCε K437R mutant (DN-PKCε). This mutant was constructed with an amino acid substitution at Lys-437 in the catalytic domain to prevent ATP binding (24). As presented in Table III, A-MuLV envelope protein-induced cell-cell fusion was observed in NIH3T3 cells treated with H89 to inhibit PKA activity. A-MuLV envelope-induced cell fusion noted in the presence of H89 was decreased by the PKCε and PKCδ inhibitor GF109203X, but not by the PKCα inhibitor Gö6976. Of particular interest are results that showed that the presence of the dominant-negative PKCε K437R mutant blocked envelope-induced cell-cell fusion in cells exposed to H89. Western blot analysis of lysates prepared from cells treated with the various PKC activators and inhibitors indicated in Fig. 3 and Tables II and III, showed that these agents had no effect on the cellular levels of either expressed A-MuLV envelope protein or PiT2 protein (Fig. 3, *panel C*). Together, these data provide strong evidence and support for a crucial role for PKCε in the up-regulation of A-MuLV envelope-induced syncytium formation.

PKA and PKC Regulation of A-MuLV Envelope Protein-induced Syncytium Formation with NMU34m Cells—To determine if PKA and PKC demonstrate similar regulation of PiT2- and A-MuLV envelope-induced syncytium formation in a cell type other than NIH3T3 cells, kinase activator and inhibitor studies also were carried out with NMU34m cells. As shown above in Fig. 1, expression of A-MuLV envelope protein (R-) in mock control NMU34m cells did not result in syncytium for-

[2] W. Wang and W. B. Anderson, unpublished results.

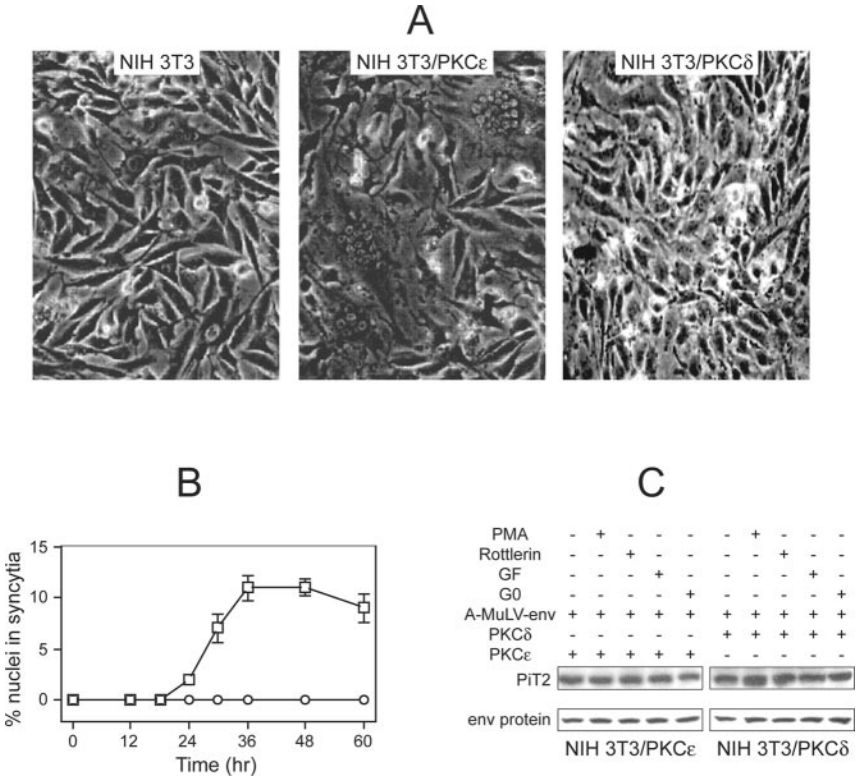

FIG. 3. **Syncytium formation in cells expressing the A-MuLV envelope protein (R-) is up-regulated in cells overexpressing PKCε.** *Panel A*, NIH3T3 cells (*left*), NIH3T3 cells overexpressing PKCε (*middle*), and NIH3T3 cells overexpressing PKCδ (*right*) were transiently transfected with pCAETR to express the A-MuLV envelope protein (R-). Cells were photomicrographed under phase contrast 48-h post-pCAETR transfection. Numerous syncytia of 5 or more nuclei are observed only in the NIH3T3 cells overexpressing PKCε (magnification, ×40). *Panel B*, time course for A-MuLV envelope protein-induced syncytium formation in NIH3T3 cells overexpressing PKCε. The percent of nuclei in syncytia was determined at the indicated time points following transient transfection with empty vector (*open circles*) or pCAETR (*open squares*). The results are expressed as the means of three similar experiments run in triplicate, with S.D. indicated by the *vertical bars*. *Panel C*, Western blot analysis of cell membrane lysates prepared from NIH3T3/PKCε (*left panels*) and NIH3T3/PKCδ (*right panels*) cells expressing the A-MuLV envelope protein (R-) in the presence (+) and absence (−) of GF109203X (1 μM), Gö6976 (1 μM), PMA (10 nM), and Rottlerin (2 μM). Immunoblot analysis was carried out with anti-A-MuLV pig antiserum (1:750) to detect envelope protein, and anti-PiT2 rabbit antiserum (1:500) to detect PiT2.

TABLE II

PKCε up-regulates A-MuLV envelope protein (R-)-induced cell-cell fusion

Stable transfectants of NIH3T3 cells overexpressing different PKC isoforms, FLAG epitope-tagged hamster PiT2, or the control empty vector (M) were treated with GF109203X (1 μM), Gö6976 (1 μM), rottlerin (2 μM), or PMA (10 nM) as indicated 3 h after transient transfection with pCAETR expressing the A-MuLV envelope protein (R-). The percent of nuclei in syncytia (cell fusion number) was determined 48 h after transfection, and is expressed as described in the legend to Table I.

Cell line	Nuclei in syncytia				
	Untreated	GF109203X	Gö6976	Rottlerin	PMA
			%		
NIH3T3 (M)	<1	ND[a]	ND	ND	<1
NIH3T3/PKCα	<1	ND	ND	ND	<1
NIH3T3/PKCβ	<1	ND	ND	ND	<1
NIH3T3/PKCδ	<1	ND	ND	ND	<1
NIH3T3/PKCε	11 ± 0.9[b]	5 ± 0.6[c]	10 ± 1.0	10 ± 0.7	17 ± 1.2[c]
NIH3T3/hamster PiT2.Flag	16 ± 0.1[b]	13 ± 0.3[c]	17 ± 2.2	15 ± 1.4	25 ± 1.2[c]

[a] ND, not determined.
[b] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to untreated NIH3T3 cells.
[c] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to similar untreated cells.

TABLE III

A-MuLV envelope protein (R-)-induced cell-cell fusion is dependent upon PKCε

Stable transfectants of NIH3T3 cells overexpressing PKCε dominant negative mutant or the control empty vector (M) were treated with H89 (100 nM), GF109203X (1 μM), Gö6976 (1 μM), or rottlerin (2 μM) as indicated 3 h after transient transfection with pCAETR expressing the A-MuLV envelope protein (R-). The percent of nuclei in syncytia (cell fusion number) was determined 48 h after transfection, and is expressed as described in the legend to Table I.

Cell line	Nuclei in syncytia				
	Untreated	H89	H89+GF109203X	H89+Gö6976	H89+Rottlerin
			%		
NIH3T3 (M)	<1	14 ± 1.7[a]	9 ± 1.8[b]	15 ± 2.3	15 ± 1.8
NIH3T3/DN-PKCε	<1	<1	ND[c]	ND	ND

[a] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to untreated NIH3T3 cells.
[b] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to similar untreated cells.
[c] ND, not determined.

mation. Moreover, treatment of NMU34m cells with the H89 inhibitor to block PKA did not promote envelope-induced cell-cell fusion as observed with NIH3T3 cells (Table IV). This apparently is due to a lack of functional A-MuLV receptor on NMU34m cells (27). However, as noted in Fig. 1, syncytium formation was induced in NMU34m cells overexpressing FLAG epitope-tagged hamster PiT2. Treatment of NMU34m/hamster PiT2.FLAG cells with 8-Br-cAMP to activate PKA decreased envelope-induced cell-cell fusion, as did inhibition of PKCε with GF109203X (Table IV). The classical PKC isoform inhibitor

TABLE IV

A-MuLV envelope protein (R-)-induced cell-cell fusion in NMU34m/hamster PiT2.FLAG cells is regulated by PKA and PKC

Stable transfectants of NMU34m cells expressing FLAG epitope-tagged hamster PiT2 or the control empty vector (M) were treated with 8-Br-cAMP (200 μM), H89 (100 nM), GF109203X (1 μM), Gö6976 (1 μM), or PMA (10 nM) as indicated 3 h after transient transfection with pCAETR expressing the A-MuLV envelope protein (R-). The percent of nuclei in syncytia (cell fusion number) was determined 48 h after transfection, and is expressed as described in the legend to Table I.

Cell line	Nuclei in syncytia					
	Untreated	8-Br-cAMP	H89	GF109203X	Gö6976	PMA
			%			
NMU34m (M)	<1	ND[a]	<1	ND	ND	<1
NMU34m/hamster PiT2.FLAG	32 ± 0.7	16 ± 0.6[b]	30 ± 2.8	12 ± 1.4[b]	31 ± 3.2	32 ± 3.2

[a] ND, not determined.

[b] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to similar untreated cells.

Gö6976 again was found to have no effect on A-MuLV envelope-induced cell-cell fusion. However, exposure to PMA did not up-regulate envelope-induced cell-cell fusion in NMU34m/hamster PiT2.FLAG cells. This likely is due to the elevated activation state of PKC noted in transformed cells (32, 33). These results indicate that PKA and PKC regulation of A-MuLV envelope-induced cell-cell fusion was similar in both NIH3T3 and NMU34m cells.

Syncytium Formation Induced by GALV Envelope Protein in NIH3T3 Cells Overexpressing PiT1 Is Not Regulated by PKA and PKC—Cell infection with Gibbon ape leukemia virus (GALV) is initiated by binding of its envelope protein to a cell surface receptor called PiT1. The PiT1 receptor is highly homologous to PiT2, with PiT1 sharing 56% amino acid homology with PiT2. PiT1 also has been shown to serve as a sodium-dependent phosphate transporter (10). To determine if GALV envelope induction of syncytium formation is dependent on the level of PiT1, mock control NIH3T3 cells and NIH3T3 cells overexpressing HA epitope-tagged PiT1 were transiently transfected with pCIGALVTR to express GALV envelope protein (R-). No appreciable cell-cell fusion was noted in NIH3T3 cells, whereas cells expressing HA-tagged PiT1 exhibited significant syncytium formation (Fig. 4, *panel A*). Western blot analysis showed HA.PiT1 was efficiently expressed (Fig. 4, *panel B*), and cell-cell fusion was noted 18–24 h following GALV envelope (R-) expression (Fig. 4, *panel C*). To examine if syncytium formation induced via GALV envelope expression was regulated by PKA and PKC, studies were carried out with NIH3T3 cells overexpressing HA-PiT1. Incubation of these cells with 8-Br-cAMP to stimulate cellular PKA, or with H89 to inhibit PKA, had no significant effect on GALV envelope protein-induced cell-cell fusion (Table V). Likewise, treatment with the PKC inhibitors GF109202X and Gö6976, or with the PKC activator PMA, did not affect GALV envelope protein-induced cell-cell fusion. These results are in agreement with our earlier studies, which showed that P_i uptake mediated by PiT2, but not by PiT1, is up-regulated with exposure of cells to PMA (17). Of interest is the finding that the protein kinase inhibitor Rottlerin significantly decreased GALV envelope protein-induced cell-cell fusion (Table V). As shown above in Table II, Rottlerin did not inhibit A-MuLV envelope-induced cell-cell fusion in NIH3T3 cells overexpressing hamster PiT2. This suggests that GALV envelope-induced syncytium formation may be regulated by a Rottlerin-sensitive protein kinase separate from PKA and PKC.

Cell Signaling Regulation of A-MuLV Envelope Protein-induced Syncytium Formation with v-ras Transformed NIH3T3 Cells—Several viruses have been shown to induce cell-cell fusion in oncogenically transformed cells, but not in the parent normal cells (3, 19, 34). Thus, studies were initiated with *v-ras* transformed NIH3T3 cells to determine if signaling through activated Ras influenced the regulatory properties of A-MuLV envelope-mediated syncytium formation. As shown in Fig. 5, *panel A*, *v-Ha-ras* and *v-Ki-ras* transformed cells transiently expressing A-MuLV envelope protein exhibited high levels of syncytium formation. Cell fusion was observed within 12 h of A-MuLV envelope expression in the *v-ras* transformed cells (Fig. 5, *panel B*). Western blot analysis revealed that *v-ras* transformation of NIH3T3 cells did not alter the cellular level of PiT2 protein (*panel C*) or the expression level of A-MuLV envelope protein (*panel D*). These results suggested that *v-ras* transformation of NIH3T3 cells might alter cell signaling to markedly potentiate the A-MuLV envelope-induced cell-cell fusion process.

To further characterize the stimulatory effect of activated *ras*, studies were carried out to determine the influence of various protein kinase activators and inhibitors on A-MuLV envelope protein-mediated syncytium formation with *v-ras* transformed NIH3T3 cells. In agreement with the results found with transformed NMU34m cells overexpressing FLAG epitope-tagged hamster PiT2 (Table IV), treatment of *v-Ha-ras*- and *v-Ki-ras*-transformed NIH3T3 cells transiently expressing the A-MuLV envelope (R-) with 8-Br-cAMP caused significant inhibition of, whereas treatment with H89 inhibitor had no effect on, syncytium formation (Table VI). The inability of H89 to enhance cell fusion in these cells may be due to the low intracellular concentrations of cyclic AMP, and thus low levels of active PKA, found in *v-ras*-transformed cells compared with the levels found in untransformed cells (35, 36). As a consequence, exposure of these transformed cells to the H89 PKA inhibitor would have limited effect on the induced cell-cell fusion process. Treatment of *v-ras*-transformed cells with the PKC inhibitors GF109203X and Gö6976, or the PKC activator PMA, also had no effect on A-MuLV envelope-induced cell-cell fusion. It is not clear why exposure of these cells to the PKCϵ inhibitor GF109203X does not decrease A-MuLV envelope-induced cell-cell fusion. It has been reported that *v-ras*-transformed cells have elevated levels of the PKC activator diacylglycerol (37). This apparently contributes to the increased activation state of PKC noted in transformed cells (32, 33). Thus, the GF 109203X inhibitor may not be able to decrease the presence of active PKCϵ to a level sufficient to result in significant inhibition of this induced cell fusion process. The elevated levels of diacylglycerol, combined with the low levels of cyclic AMP, found in *v-ras*-transformed cells may be responsible, at least in part, for the enhanced ability of these cells to undergo A-MuLV envelope-induced fusion to form multinucleated syncytia.

Ras-Raf-1 Signaling Promotes A-MuLV Envelope Protein-induced Syncytium Formation—To confirm that activated Ras was involved in promoting A-MuLV envelope-induced cell-cell fusion, cells stably expressing different signaling proteins were co-transfected with pCAETR to express A-MuLV envelope protein (R-) along with a plasmid coding for the dominant-negative Ras mutant N17Ras (Table VII). Expression of dominant-negative N17Ras resulted in significant inhibition of A-MuLV en-

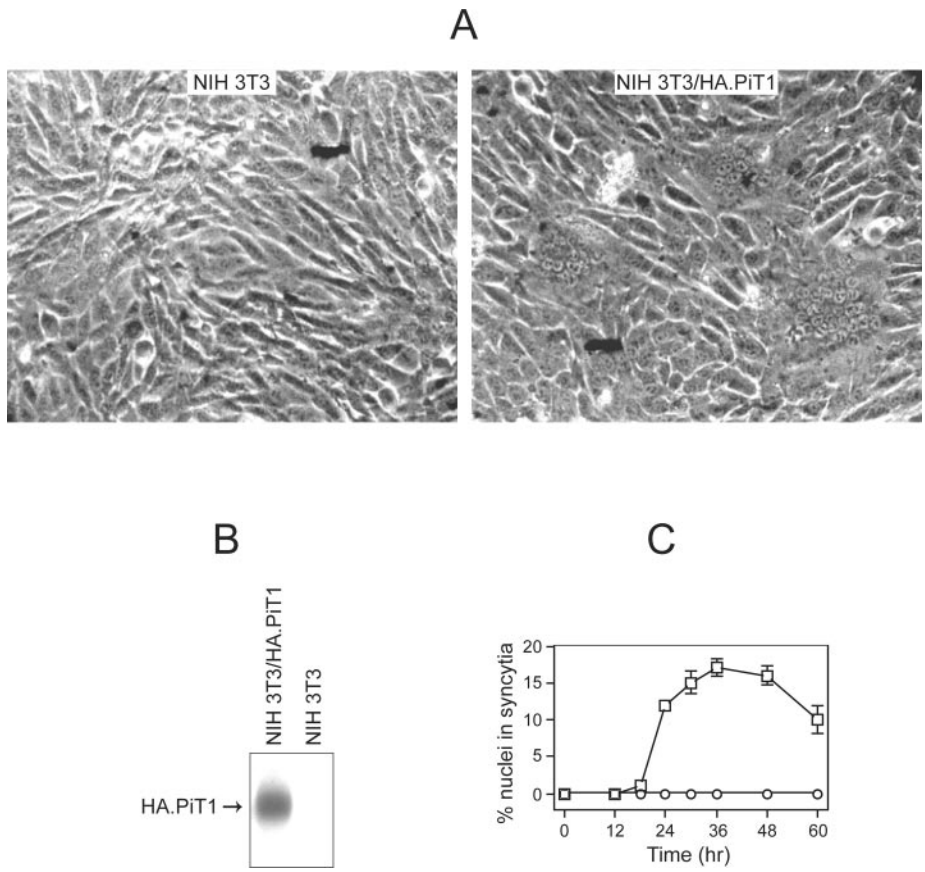

FIG. 4. **Syncytium formation in cells expressing the GALV envelope protein (R-) is dependent on the level of PiT1 transporter/viral receptor.** *Panel A*, NIH3T3 cells (*left*) and NIH3T3 cells overexpressing HA epitope-tagged PiT1 (*right*) were transiently transfected with pCIGALVTR to express the GALV envelope protein (R-). Cells were photomicrographed under phase contrast 48 h post pCIGALVTR transfection. Numerous syncytia of 5 or more nuclei are observed only in the NIH3T3 cells overexpressing PiT1 (magnification, ×40). *Panel B*, Western blot analysis of cell lysates prepared from NIH3T3/HA.PiT1 and NIH3T3 cells. Immunoblot analysis was carried out with HA tag monoclonal antibody to determine the level of HA epitope-tagged PiT1 expression. *Panel C*, time course for GALV envelope protein-induced syncytium formation in NIH3T3 cells overexpressing HA epitope-tagged PiT1. The percent of nuclei in syncytia was determined at the indicated time points following transient transfection with empty vector (*open circles*) or pCIGALVTR (*open squares*). The results are expressed as the means of three similar experiments run in triplicate, with S.D. indicated by the *vertical bars*.

TABLE V

GALV envelope protein (R-)-induced cell-cell fusion is not regulated by PKA and PKC

Stable transfectants of NIH3T3 cells expressing HA epitope-tagged PiT1 or the control empty vector (M) were treated with 8-Br-cAMP (200 μM), H89 (100 nM), GF109203X (1 μM), Gö6976 (1 μM), PMA (10 nM), or Rottlerin (2 μM) as indicated 3 h after transient transfection with pCIGALVTR expressing the GALV envelope protein (R-). The percent of nuclei *in syncytia* (cell fusion number) was determined 48 h after transfection, and is expressed as described in the legend to Table I.

Cell line	Nuclei in syncytia						
	Untreated	8-Br-cAMP	H89	GF109203X	Gö6976	PMA	Rottlerin
				%			
NIH3T3 (M)	<1	<1	<1	ND[a]	ND	<1	ND
NIH3T3/HA.PiT1	17 ± 1.9[b]	15 ± 2.7	16 ± 2.6	16 ± 0.6	16 ± 0.9	18 ± 2.5	5 ± 0.6[c]

[a] ND, not determined.
[b] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to untreated NIH3T3 cells.
[c] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to similar untreated cells.

velope-mediated syncytium formation in NIH3T3 cells expressing FLAG epitope-tagged hamster PiT2. However, co-expression of N17Ras had little effect on A-MuLV envelope-induced cell-cell fusion in cells overexpressing PKCε or BXB Raf, a truncated, constitutively active form of Raf-1 protein kinase. These results indicated that a requirement for activated Ras was negated in cells with elevated levels of PKCε or of activated BXB Raf protein kinase. To further explore a requirement for Raf-1 protein kinase, cells were cotransfected with pCAETR to express A-MuLV envelope protein along with a plasmid coding for the dominant-negative Raf mutant Raf301. Raf301 affects the regulatory domain of Raf-1, but does not inhibit BXB Raf. Expression of dominant-negative Raf301 significantly inhibited A-MuLV envelope-induced syncytium formation in cells overexpressing FLAG epitope-tagged hamster PiT2 and PKCε, as well as with *v-Ha-ras* and *v-Ki-ras*-transformed cells (Table VII). However, dominant-negative Raf301 had no effect on cell-cell fusion noted with NIH3T3 cells expressing constitutively active BXB Raf. To establish that there was efficient expression of the dominant-negative ERK185, N17Ras, and Raf301 proteins used in these studies, Western blot analysis was carried out on cell lysates prepared from the different cell lines transiently transfected with the indicated plasmids coding for these dominant-negative proteins. As shown in Fig. 6, there is a significant increase in the level of ERK, Ras, and Raf proteins expressed in each of the cell lines transfected with plasmids coding for dominant-negative ERK185, N17Ras, and Raf301. These results indicate that activation of Raf-1 protein kinase is required to support optimal A-MuLV envelope-induced cell-cell fusion up-regulated by elevated levels of activated Ras and PKCε.

Cell Signaling through MAPK and p38 Protein Kinase Is Not Required for A-MuLV Envelope Protein-induced Syncytium Formation with v-ras-transformed NIH3T3 Cells—Finally, studies were initiated to determine whether the positive effects of Ras, PKCε, and Raf-1 on the A-MuLV envelope-induced cell-cell fusion process depended on activation of these signaling components themselves or on the subsequent activation of downstream targets of Ras signaling. The Ras-mediated activation of Raf-1 subsequently acts to stimulate MAP kinase

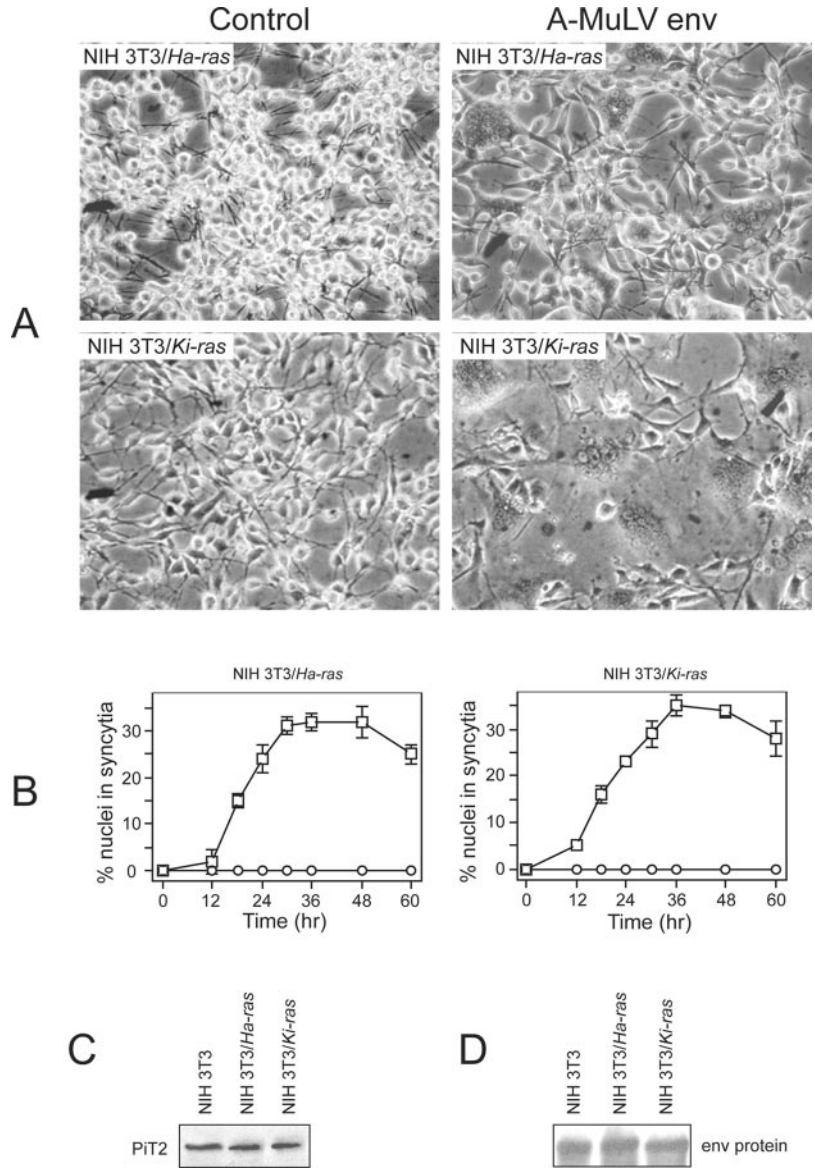

FIG. 5. **Syncytium formation is induced in *v-ras* transformed NIH3T3 cells expressing the A-MuLV envelope protein (R-).** *Panel A*, NIH3T3/*Ha-ras* and NIH3T3/*Ki-ras* were transiently transfected with empty vector (control) or with pCAETR to express the A-MuLV envelope protein (R-) (A-MuLV env). Cells were photomicrographed under phase contrast 48-h post-pCAETR transfection. Numerous syncytia of 5 or more nuclei are observed in the cells expressing the A-MuLV envelope protein (R-) (magnification, ×40). *Panel B*, time course for A-MuLV envelope protein-induced syncytium formation in NIH3T3/*Ha-ras* (*left*) and NIH3T3/*Ki-ras* (*right*) cells. The percent of nuclei in syncytia (cell fusion) was determined at the indicated time points following transient transfection with empty vector (*open circles*) or pCAETR (*open squares*). The results are expressed as the means of three similar experiments run in triplicate, with S.D. indicated by the *vertical bars*. *Panels C* and *D*, Western blot analysis of cell membrane lysates prepared from NIH3T3/*Ha-ras* and NIH3T3/*Ki-ras* cells transiently expressing A-MuLV envelope protein (R-). Immunoblot analysis was carried out with anti-PiT2 rabbit antiserum (1:500) to detect PiT2 (*panel C*) and anti-A-MuLV pig antiserum (1:750) to detect envelope protein (*panel D*).

TABLE VI

A-MuLV envelope protein (R-)-induced cell-cell fusion in v-ras transformed NIH3T3 is regulated by PKA

v-Ha-ras and *v-Ki-ras* transformed NIH3T3 cells were treated with 8-Br-cAMP (200 μM), H89 (100 nM), GF109203X (1 μM), Gö6976 (1 μM), or PMA (10 nM) as indicated 3 h following transient transfection with pCAETR expressing the A-MuLV envelope protein (R-). The percent of nuclei in syncytia (cell fusion number) was determined 48 h after transfection, and is expressed as described in the legend to Table I.

Cell line	Nuclei in syncytia					
	Untreated	8-Br-cAMP	H89	GF109203X	Gö6976	PMA
			%			
NIH3T3/*Ha-ras*	33 ± 1.9	21 ± 2.0[a]	34 ± 2.7	31 ± 2.1	33 ± 1.9	32 ± 2.4
NIH3T3/*Ki*-ras	33 ± 0.9	24 ± 2.2[a]	32 ± 3	33 ± 1.6	32 ± 2.4	32 ± 2.8

[a] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to similar untreated cells.

kinase (MEK), which, in turn, activates MAP kinase (MAPK) (38, 39). Experiments were carried out with pERK185, a dominant-negative inhibitor of extracellular signal-regulated kinase (ERK), as well as with the MAPK kinase (MEK) inhibitor PD98059 and the p38 protein kinase inhibitor SB202190 (Table VII). Expression of dominant-negative ERK185 in the indicated cells had no effect on syncytium formation. Likewise, treatment of the indicated cells with PD98059 to inhibit MEK, or with SB202190 to inhibit p38 protein kinase, did not alter A-MuLV-induced syncytium formation. Exposure of cells to these inhibitory agents did not affect the level of A-MuLV envelope protein expression (data not shown). These results indicate that the up-regulation of A-MuLV envelope-induced syncytium formation through activation of Ras, PKCε, and Raf-1 does not require signaling through MAPK or p38 protein kinase.

DISCUSSION

Protein-mediated membrane fusion plays an important role in the biology of the cell. Membrane fusion is a central process in fertilization, myogenesis, osteogenesis, placenta formation, and virus infection (40). Retrovirus-induced cell-cell fusion and the formation of multinucleated giant cells (syncytia) are induced as a consequence of interactions between the virus en-

TABLE VII

Involvement of the Ras-MAPK cascade in the regulation of A-MuLV envelope protein (R-)-induced cell-cell fusion

Stable transfectants of NIH3T3 cells expressing FLAG epitope-tagged hamster PiT2 or PKCϵ, and *v-Ha-ras, v-Ki-ras*, and BXB Raf-transformed NIH3T3 cells were treated with PD98059 (30 μM) or SB202190 (0.7 μM) as indicated 3 h after transient transfection with pCAETR to express the A-MuLV envelope protein (R-), or these cell lines were co-transfected with 1.8 μg of plasmids coding for N17Ras, Raf301, or ERK185 along with pCAETR, as indicated. The percent of nuclei in syncytia (cell fusion number) was determined 48 h after transfection, and is expressed as described in the legend to Table I.

Cell line	Nuclei in syncytia					
	Untreated	N17Ras	Raf301	ERK185	PD98059	SB202190
			%			
NIH3T3/hamster PiT2.FLAG	18 ± 0.8	8 ± 3[a]	9 ± 2.1[a]	17 ± 1	17 ± 3.2	16 ± 1.7
NIH3T3/PKCϵ	11 ± 1.5	11 ± 0.9	6 ± 1.1[a]	12 ± 2.2	11 ± 2.1	12 ± 0.9
NIH3T3/*Ha-ras*	32 ± 2.0	11 + 1.5[a]	15 ± 2.3[a]	31 ± 3.3	30 ± 1.6	32 ± 1.6
NIH3T3/*Ki-ras*	34 ± 1.7	15 + 2.6[a]	18 ± 1.9[a]	31 ± 1.3	33 ± 3.2	31 ± 1.5
NIH3T3/BXB Raf	15 ± 1.7	13 ± 3.5	13 ± 3.5	14 ± 1.3	14 ± 2.1	14 ± 2.1

[a] Statistical significance was determined by the Student's *t* test. $p < 0.05$, compared to similar untreated cells.

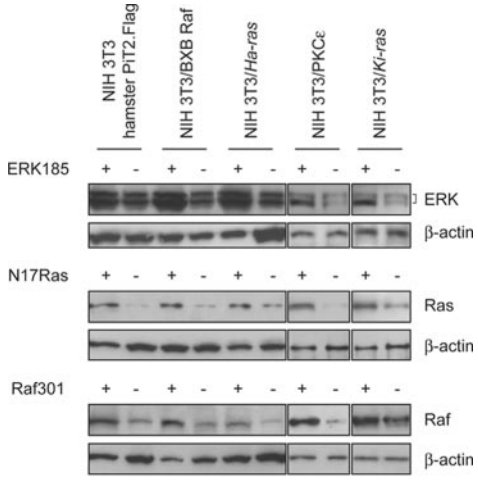

FIG. 6. **Western blot analysis of ERK, Ras, and Raf protein expression levels in NIH3T3 cells expressing FLAG epitope-tagged hamster PiT2 or PKCϵ, and in *v-Ha-ras-*, *v-Ki-ras-*, and BXB Raf-transformed NIH3T3 cells.** These cell lines growing on 6-well plates were co-transfected with (+) or without (−) 1.8 μg of plasmids coding for ERK185, N17Ras, and Raf301 as indicated, along with pCAETR to express the A-MuLV envelope protein (R-). Total cell lysates were prepared from these different cell lines 48 h after transient transfection. Immunoblot analysis then was carried out on these cell lysates with anti-p44/42 MAP kinase rabbit antiserum (1:1000) to detect ERK1 and ERK2, anti-pan Ras monoclonal antibody (1 μg/ml) to detect Ras, anti-Raf-1 rabbit antiserum (1:1000) to detect Raf-1, and anti-β-actin monoclonal antibody (1:5000) to detect β-actin as loading control.

velope protein on infected cells and cell surface virus receptor proteins on uninfected cells (2, 19, 26). In the present study we used viral envelope expression vectors (pCAETR and pCIGALTR) to transiently transfect certain cell lines to express truncated A-MuLV and GALV envelope protein (R-), respectively, and induce cell-cell membrane fusion and multinuclear syncytium formation as described by Ragheb and Anderson (19). This model system of cell-expressed virus envelope-induced cell-cell fusion was used to determine if modulation of signal transduction pathways and specific protein kinase activities might act to alter A-MuLV and GALV envelope protein-induced syncytia formation.

The cell surface receptors for GALV and A-MuLV have been identified, and shown to serve normal cellular functions as sodium-dependent phosphate transporters (PiT1 and PiT2, respectively) (10). Earlier studies established that activation of PKA with 8-bromo cyclic AMP inhibited PiT2-mediated P_i uptake (16). In the present study, it was found that treatment of cells overexpressing hamster PiT2 with 8-Br-cAMP and with forskolin to activate PKA inhibited A-MuLV envelope (R)-induced cell-cell fusion. Interestingly, transient expression of A-MuLV envelope (R-) alone in NIH3T3 cells was found to be insufficient to induce syncytium formation (Fig. 2 and Table I). However, when cells were treated with low concentrations (100 nM) of the PKA inhibitors H89 and KT5760 to inhibit PKA activity, as well as with PKA Catα siRNA to decrease the intracellular level of the PKA catalytic subunit, the A-MuLV envelope (R-) protein now was capable of inducing cell-cell fusion in NIH3T3 cells. This suggests that the endogenous level of active PKA present in NIH3T3 cells is sufficient to block envelope-induced cell-cell fusion under conditions of low cellular levels of PiT2 and PKCϵ, and indicate that cell signaling through the cyclic AMP-PKA pathway can significantly alter the susceptibility of NIH3T3 cells to A-MuLV envelope (R-) protein-induced syncytium formation.

Cyclic AMP-PKA signaling also appears to modulate virus envelope-induced fusion of mononuclear cytotrophoblasts. Syncytin is a protein encoded by the envelope gene of a human endogenous defective retrovirus HERV-W (41), and is found to be expressed at high levels in the placental syncytiotrophoblast (42). Moreover, it was established that this viral envelope protein syncytin is involved in mediating placental cytotrophoblast fusion to form multinucleated syncytiotrophoblast (42). Importantly, in an earlier study Keryer *et al.* (43) showed that the addition of cyclic AMP analogs to activate PKA promoted syncytiotrophoblast formation, whereas treatment with the PKA inhibitor H89 decreased cell fusion. More recently, Frendo *et al.* (44) demonstrated that stimulation of cytotrophoblast cell fusion by cAMP activation of PKA is caused by an induced increase in the level of the HERV-W envelope protein syncytin. This differs from results present here, where levels of the A-MuLV envelope protein are found not be altered in response to changes in cAMP-PKA signaling. Nonetheless, the involvement of PKA in mediating syncytin-induced fusion of cytotrophoblasts does lend support to an important role for cAMP-PKA signaling in the regulation of syncytium formation in response to certain retroviral envelope proteins.

Previously, we demonstrated that stimulation of PKC increased PiT2-mediated P_i uptake, and established that this up-regulation of the Pit2 phosphate transporter was mediated through PMA activation of the PKCϵ isoform (17). Here we have presented results that demonstrate that overexpression, and activation, of PKCϵ enhanced, whereas exposure to the PKCϵ inhibitor GF109203X or expression of a kinase-inactive dominant-negative mutant of PKCϵ (K437R) decreased, A-MuLV envelope-induced syncytium formation (Fig. 3, Tables II and III). These results strongly suggest that cell signaling to activate PKCϵ plays an important regulatory role in mediating A-MuLV envelope-induced cell-cell fusion.

Conversely, in studies carried out with NIH3T3 cells overexpressing PiT1, modulation of PKCϵ and PKA activities had no effect on GALV envelope-induced syncytium formation.

Importantly, however, the protein kinase inhibitor Rottlerin was found to decrease GALV envelope (R-) induced cell-cell fusion whereas it had little effect on A-MuLV envelope-induce syncytium formation (Tables II, III, and V). As noted earlier, Rottlerin was thought to be a selective inhibitor of PKCδ (31). In more recent studies, however, Davies *et al.* (45) showed that Rottlerin was not an effective inhibitor of PKCδ. Rather, they reported that Rottlerin inhibited a number of other protein kinases, including p38-regulated/activated kinase and MAPK-activated protein kinase 2. Additional studies are required to identify a Rottlerin-sensitive protein kinase that appears to be involved in regulating GALV envelope protein-induced cell-cell fusion.

Transformation of NIH3T3 cells with *v-Ha-ras* and *v-Ki-ras* also was found to promote high levels of A-MuLV envelope-induced cell-cell fusion (Fig. 5). These results suggested that *v-ras* transformation of cells might modulate cell signaling pathways to, in turn, enhance A-MuLV envelope-induced syncytium formation. Expression of dominant-negative N17Ras to block Ras function was found to inhibit A-MuLV-induced syncytium formation. However, co-expression of N17 Ras had little effect on A-MuLV-induced cell-cell fusion in cells overexpressing PKCϵ or constitutively active BXB Raf protein kinase (Table VII). Of importance is that these results established that a requirement for activated Ras was negated in cells with elevated levels of activated PKCϵ or Raf-1 protein kinase. Taken together, the results presented in Table VII indicate that activation of Raf-1 protein kinase is required to support optimal A-MuLV envelope-induced cell-cell fusion up-regulated by elevated levels of PKCϵ and by activated Ras. Further, activation of Raf-1 to support this cell fusion process is downstream from signaling through activated Ras and PKCϵ. A critical event in the regulation of Raf-1 protein kinase activity is its binding to activated GTP-Ras and re-localization to the plasma membrane, where Raf-1 is converted to a catalytically active form (39). Importantly, studies have shown that PKCϵ is involved in mediating Raf-1 activation (46–48), whereas PKA has been found to inhibit Raf-1 activity (49–51) Thus, alterations in PKCϵ and PKA activities also might act by directly modulating Raf-1 protein kinase to influence A-MuLV envelope-induced cell fusion. These results support a critical role for Raf-1 activation in A-MuLV envelope-induced cell-cell fusion, and raise the interesting possibility that the use of agents that modulate Raf-1 may be an effective means of altering A-MuLV virus-cell membrane fusion.

Treatment of cells with the different cell signaling activators and inhibitors described was found not to alter the cellular levels of either the PiT2 receptor protein or the expressed levels of the A-MuLV envelope protein (R-). Likewise, exposure of cells to the various agents described to alter cell signaling pathways and modulate A-MuLV envelope-induced cell-cell fusion, had no effect on GALV envelope-induced fusion mediated through the PiT1 phosphate transporter/viral receptor. The PiT1 and PiT2 phosphate transporters/viral receptors are similar, with 56% amino acid identity (52). Thus, if the effectors used are regulating A-MuLV envelope-induced cell fusion via an indirect means (*i.e.* changes in glucose transport, cell migration, cell contact, growth arrest), it is likely they also would influence GALV envelope-induced cell fusion mediated through PiT1. Therefore, the point of cell signaling regulation of induced syncytium formation described here likely takes place at the plasma membrane at the level of A-MuLV envelope association with PiT2 and the initiation of cell fusion events. One possible target for cell signaling modulation of envelope-induced syncytium formation may be through direct post-translational modification(s) of the cell surface PiT2 receptor. The

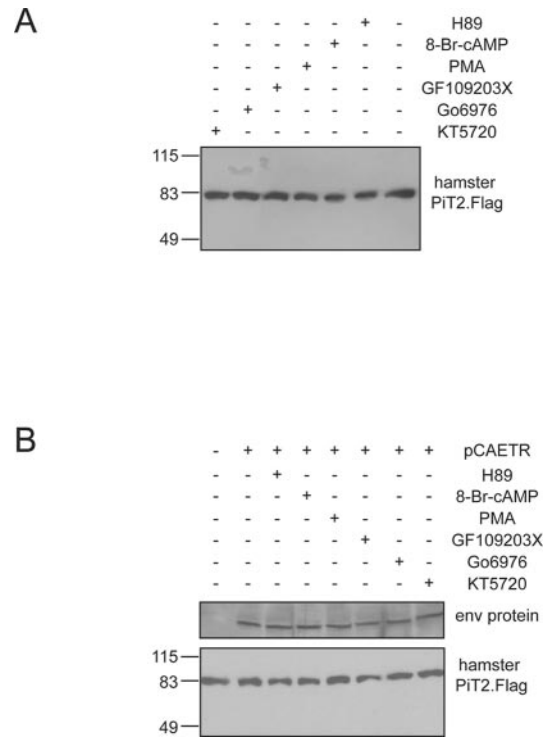

FIG. 7. **Treatment of NIH3T3 cells stably expressing FLAG epitope-tagged hamster PiT2 with PKA and PKC activators and inhibitors does not alter the level of membrane-localized FLAG-tagged hamster PiT2.** NIH3T3/hamster PiT2.FLAG cells were treated with (+) or without (−) PKA inhibitors H89 (100 nM) and KT5720 (100 nM), PKA activator 8-Br-cAMP (200 μM), PKC activator PMA (10 nM), and PKC inhibitors GF109203X (1 μM) and Gö6976 (1 μM) as indicated 3 h following in the absence (*panel A*) or the presence (*panel B*) of transient transfection with pCAETR to express A-MuLV envelope protein (R-). Enriched plasma membrane fractions were prepared from these cells 45 h after treatment with effectors as described previously (22). Immunoblot analysis then was carried out on these membrane fractions with horseradish peroxidase-conjugated anti-FLAG monoclonal antibody (1:2000) to detect FLAG epitope-tagged hamster PiT2 and with anti-A-MuLV pig antiserum (1:750) to detect A-MuLV envelope protein (R-).

PiT2 receptor does contain a number of consensus phosphorylation sites, particularly within the hydrophilic loop domain. Thus, it is conceivable that PKCϵ, PKA, and/or Raf-1 may phosphorylate PiT2, or protein cofactors associated with PiT2, to modulate A-MuLV-induced cell-cell fusion.

In previous studies, Kavanaugh *et al.* (13) showed that P_i uptake was decreased by 50% in cells stably expressing A-MuLV envelope protein, whereas Jobbagy *et al.* (22) found that transiently expressed epitope-tagged PiT2 protein was no longer detected at the plasma membrane in cells productively infected with A-MuLV. In these productively infected cells there is a much longer period of virus, and virus envelope protein, presence within the cell. It has been proposed that under these conditions of prolonged expression of high intracellular levels of A-MuLV envelope protein, complexes of newly synthesized PiT2 receptor and the A-MuLV envelope glycoprotein may form shortly after protein synthesis (22). This complexing of newly synthesized PiT2 likely prevents covalent modifications required for normal PiT2 processing and trafficking to the plasma membrane. In the present study, stably expressed FLAG-tagged PiT2 already is present at high levels at the plasma membrane. When these cells are transiently transfected to produce A-MuLV envelope protein, some newly synthesized FLAG-tagged PiT2 may form a complex with expressed envelope, but this likely would result in only low, undetectable levels of the nascent, unmodified PiT2 (low mo-

lecular weight form), and the pool of elevated PiT2 at the membrane remains relatively stable and sufficient to mediate cell-cell fusion. To determine if the membrane levels of PiT2 are altered by transient (24–48 h) expression of A-MuLV envelope under the conditions used here, Western blot analysis studies were carried with cell membrane lysates prepared from NIH3T3/hamster PiT2.FLAG cells in the presence and absence of transient A-MuLV envelope protein (R-) expression along with exposure to 8-Br-cAMP, the PKA inhibitors H89 and HT5760, PMA, and the PKC inhibitors GF109203X and Gö6976 for 45 h. We were unable to detect any change in the plasma membrane levels of FLAG-tagged PiT2 under any of these conditions (Fig. 7).

Another possible mechanism of cell signaling regulation of A-MuLV envelope-induced syncytium formation may be a result of altered localization of Pit2 at the membrane. Lipid rafts are specific domains on plasma membranes that are enriched in cholesterol and sphingolipids (53, 54). Rafts are known to play an important role in several biological processes, including signal transduction and virus assembly and budding, and a number of signaling proteins, transporters, and cell surface receptors are found associated with lipid rafts at the plasma membrane (54, 55). Lu and Silver (56) reported that the ecotropic murine leukemia virus receptor CAT-1 is found associated within the raft fraction, and that association of mCAT-1 with rafts is an important step in virus infection. Changes in cell signaling could act to re-distribute Pit2, or Pit2-associated proteins, in and out of rafts, or other membrane domains, to alter protein-protein interactions and thus modulate PiT2-mediated A-MuLV envelope-induced syncytium formation.

In summary, we have shown that signaling through cyclic AMP to activate PKA inhibited A-MuLV envelope-induced syncytium formation, whereas activation of PKCϵ is required to promote this fusion process. Importantly, activation of Raf-1 protein kinase also is required for A-MuLV-induced cell-cell fusion. However, signaling through MAPK or p38 protein kinase is not required for A-MuLV envelope-induced cell-cell fusion. It remains to be determined if the cell signaling components (protein kinases) described here act to directly modulate PiT2 itself, to regulate other proteins or lipids associated with a PiT2 complex, or to alter other cellular activities involved in the cell-cell fusion process itself. These observations could lead to the development and use of effective agents to alter the fusogenic potential of A-MuLV, and conceivably modulate the susceptibility of cells to A-MuLV infection.

Acknowledgments—We thank Dr. L. D. Wolff for critical review of the manuscript and Dr. J. A. Ragheb for providing the pCAETR plasmid coding for the A-MuLV envelope protein (R-).

REFERENCES

1. Marsh, M., and Helenius, A. (1989) *Adv. Virus Res.* **36,** 107–151
2. Jones, J. S., and Risser, R. (1993) *J. Virol.* **67,** 67–74
3. Wilson, C. A., Marsh, J. W., and Eiden, M. V. (1992) *J. Virol.* **66,** 7262–7269
4. Coffin, J. (1996) in *Field's Virology* (Fields, B., Knipe, D. M., and Howley, P. M., ed) Vol. 2, Third Ed., pp. 1767–1848, 2 vols., Lippincott, Philadelphia, PA
5. Mohagheghpour, N., Chakrabarti, R., Stein, B. S., Gowda, S. D., and Engleman, E. G. (1991) *J. Biol. Chem.* **266,** 7233–7238
6. Root, C. N., Wills, E. G., McNair, L. L., and Whittaker, G. R. (2000) *J. Gen. Virol.* **81,** 2697–2705
7. Tsurudome, M., and Ito, Y. (2000) *Crit. Rev. Immunol.* **20,** 167–196
8. Cole, S. W., Jamieson, B. D., and Zack, J. A. (1999) *J. Immunol.* **162,** 1392–1400
9. Cristillo, A. D., Highbarger, H. C., Dewar, R. L., Dimitrov, D. S., Golding, H., and Bierer, B. E. (2002) *FASEB J.* **16,** 354–364
10. Overbaugh, J., Miller, A. D., and Eiden, M. V. (2001) *Microbiol. Mol. Biol. Rev.* **65,** 371–389
11. Chien, M. L., Foster, J. L., Douglas, J. L., and Garcia, J. V. (1997) *J. Virol.* **71,** 4564–4570
12. Chien, M. L., O'Neill, E., and Garcia, J. V. (1998) *Virology* **240,** 109–117
13. Kavanaugh, M. P., Miller, D. G., Zhang, W., Law, W., Kozak, S. L., Kabat, D., and Miller, A. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7071–7075
14. Rodrigues, P., and Heard, J. M. (1999) *J. Virol.* **73,** 3789–3799
15. Salaun, C., Gyan, E., Rodrigues, P., and Heard, J. M. (2002) *J. Virol.* **76,** 4304–4311
16. Olah, Z., Lehel, C., and Anderson, W. B. (1993) *Biochim. Biophys. Acta* **1176,** 333–338
17. Jobbagy, Z., Olah, Z., Petrovics, G., Eiden, M. V., Leverett, B. D., Dean, N. M., and Anderson, W. B. (1999) *J. Biol. Chem.* **274,** 7067–7071
18. Pinter, A., and Fleissner, E. (1979) *J. Virol.* **30,** 157–165
19. Ragheb, J. A., and Anderson, W. F. (1994) *J. Virol.* **68,** 3220–3231
20. Rein, A., Mirro, J., Haynes, J. G., Ernst, S. M., and Nagashima, K. (1994) *J. Virol.* **68,** 1773–1781
21. Farrell, K. B., Ting, Y. T., and Eiden, M. V. (2002) *J. Virol.* **76,** 4267–4274
22. Jobbagy, Z., Garfield, S., Baptiste, L., Eiden, M. V., and Anderson, W. B. (2000) *J. Virol.* **74,** 2847–2854
23. Olah, Z., Lehel, C., Jakab, G., and Anderson, W. B. (1994) *Anal. Biochem.* **221,** 94–102
24. Hong, D. H., Petrovics, G., Anderson, W. B., Forstner, J., and Forstner, G. (1999) *Am. J. Physiol.* **277,** G1041–1047
25. Olah, Z., Ferrier, A., Lehel, C., and Anderson, W. B. (1995) *Biochem. Biophys. Res. Commun.* **214,** 340–347
26. Forestell, S. P., Dando, J. S., Chen, J., de Vries, P., Bohnlein, E., and Rigg, R. J. (1997) *Gene. Ther.* **4,** 600–610
27. Miller, D. G., and Miller, A. D. (1993) *J. Virol.* **67,** 5346–5352
28. Fu, M., Rao, M., Wu, K., Wang, C., Zhang, X., Hessien, M., Yeung, Y., Gioeli, D., Weber, M., and Pestell, R. G. (2004) *J. Biol. Chem.* **279,** 29436–29449
29. Lehel, C., Olah, Z., Mischak, H., Mushinski, J. F., and Anderson, W. B. (1994) *J. Biol. Chem.* **269,** 4761–4766
30. Mischak, H., Goodnight, J. A., Kolch, W., Martiny-Baron, G., Schaechtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H., and Mushinski, J. F. (1993) *J. Biol. Chem.* **268,** 6090–6096
31. Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) *J. Biol. Chem.* **273,** 18623–18632
32. Anderson, W. B., Estival, A., Tapiovaara, H., and Gopalakrishna, R. (1985) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19,** 287–306
33. Bell, R. M., Hannun, Y. A., and Loomis, C. R. (1986) *Symp. Fundam. Cancer Res.* **39,** 145–156
34. Ocho, M., Ogura, H., Tanaka, T., and Oda, T. (1980) *Exp. Cell Biol.* **48,** 421–428
35. Pastan, I., and Johnson, G. S. (1974) *Adv. Cancer Res.* **19,** 303–329
36. Pastan, I. H., Johnson, G. S., and Anderson, W. B. (1975) *Annu. Rev. Biochem.* **44,** 491–522
37. Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., and Bell, R. M. (1986) *J. Biol. Chem.* **261,** 8597–8600
38. Ahn, N. G., Seger, R., and Krebs, E. G. (1992) *Curr. Opin. Cell Biol.* **4,** 992–999
39. Newton, A. C. (1995) *J. Biol. Chem.* **270,** 28495–28498
40. Burger, K. N., and Verkleij, A. J. (1990) *Experientia* **46,** 631–644
41. Blond, J. L., Beseme, F., Duret, L., Bouton, O., Bedin, F., Perron, H., Mandrand, B., and Mallet, F. (1999) *J. Virol.* **73,** 1175–1185
42. Mi, S., Lee, X., Li, X., Veldman, G. M., Finnerty, H., Racie, L., LaVallie, E., Tang, X. Y., Edouard, P., Howes, S., Keith, J. C., Jr., and McCoy, J. M. (2000) *Nature* **403,** 785–789
43. Keryer, G., Alsat, E., Tasken, K., and Evain-Brion, D. (1998) *J. Cell Sci.* **111,** 995–1004
44. Frendo, J.-L., Olivier, D., Cheynet, V., Blond, J.-L., Bouton, O., Vidaud, M., Rabreau, M., Evain-Brion, D., and Mallet, F. (2003) *Mol. Cell. Biol.* **23,** 3566–3574
45. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* **351,** 95–105
46. Cacace, A. M., Ueffing, M., Philipp, A., Han, E. K., Kolch, W., and Weinstein, I. B. (1996) *Oncogene* **13,** 2517–2526
47. Cai, H., Smola, U., Wixler, V., Eisenmann-Tappe, I., Diaz-Meco, M. T., Moscat, J., Rapp, U., and Cooper, G. M. (1997) *Mol. Cell. Biol.* **17,** 732–741
48. Lee, M., Petrovics, G., and Anderson, W. B. (2003) *Biochem. Biophys. Res. Commun.* **311,** 1026–1033
49. Cook, S. J., and McCormick, F. (1993) *Science* **262,** 1069–1072
50. Dhillon, A. S., and Kolch, W. (2002) *Arch. Biochem. Biophys.* **404,** 3–9
51. Hafner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994) *Mol. Cell. Biol.* **14,** 6696–6703
52. van Zeijl, M., Johann, S. V., Closs, E., Cunningham, J., Eddy, R., Shows, T. B., and O'Hara, B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1168–1172
53. Simons, K., and Ikonen, E. (2000) *Science* **290,** 1721–1726
54. Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell. Biol.* **1,** 31–39
55. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 111–136
56. Lu, X., and Silver, J. (2000) *Virology* **276,** 251–258