Infection of Glioma Cells with Sindbis Virus Induces Selective Activation and Tyrosine Phosphorylation of Protein Kinase C δ

IMPLICATIONS FOR SINDBIS VIRUS-INDUCED APOPTOSIS*

Sindbis virus (SV) is an alpha virus used as a model for studying the role of apoptosis in virus infection. In this study, we examined the role of protein kinase C (PKC) in the apoptosis induced by SVNI, a virulent strain of SV. Infection of C6 cells with SVNI induced a selective translocation of PKC δ to the endoplasmic reticulum and its tyrosine phosphorylation. The specific PKC δ inhibitor rottlerin and a PKC δ kinase-dead mutant increased the apoptosis induced by SVNI. To examine the role of the tyrosine phosphorylation of PKC δ in the apoptosis induced by SVNI we used a PKC δ mutant in which five tyrosine residues were mutated to phenylalanine (PKC δ5). PKC δ5-overexpressing cells exhibited increased apoptosis in response to SVNI as compared with control cells and to cells overexpressing PKC δ. SVNI also increased the cleavage of caspase 3 in cells overexpressing PKC δ but did not induce cleavage of PKC δ or PKC δ δ. Using single tyrosine mutants, we identified tyrosines 52, 64, and 155 as the phosphorylation sites associated with the apoptosis induced by SVNI. We conclude that PKC δ exerts an inhibitory effect on the apoptosis induced by SV and that phosphorylation of PKC δ on specific tyrosines is required for this function.

Sindbis virus (SV) is a single-stranded positive-strand RNA alphavirus that has been used as a model for studying the molecular mechanisms underlying virus-induced apoptosis (1). Infection of cells with SV results in either persistent or lytic infection depending on the cell type and the viral strain (1, 2). Lytic infection induces apoptosis, which can be inhibited by overexpression of anti-apoptotic proteins such as Bcl-2 (3). Similarly, overexpression of anti-apoptotic genes such as bcl-2, beclin, and crmA inhibits mortality of mice following SV infection (4) and converts lytic to persistent infection (3). Because the neurovirulence of the different viral strains correlates with mortality in mice and with the apoptosis of cultured cells, it has been suggested that the apoptosis plays a central role in the pathogenesis of the virus (5). Little is known about the signal transduction pathways involved in the apoptotic effect of SV. In a recent study, SV has been reported to increase the phosphorylation of p38 and Hsp27 (6). In addition, SV induces ceramide formation (7) and activation of double-strand RNA-dependent protein kinase (8) and nuclear factor κB (9).

The protein kinase C (PKC) family of serine-threonine kinases plays important roles in signal transduction and in various cellular functions (10–12). At least 12 isoforms have been described so far showing diversity in their structures and biological functions (13, 14). The PKC isoforms are grouped on the basis of their structures and cofactor requirements into three main subclasses, the classical PKCs (α, β1, β2, and γ), the novel PKCs (δ, ε, η, and θ), and the atypical PKCs (PKCζ and PKC ν). The two other members, PKC μ and PKC ν, represent a fourth subclass with unique characteristics (15, 16). The regulation of PKC activity involves phosphorylation on serine and threonine residues (17, 18). In addition, recent studies suggest that phosphorylation on tyrosine residues can modulate the activity and substrate recognition of PKC (19).

The PKCs have been implicated as important regulators of cell apoptosis (20, 21). PKCa, PKCe, and PKCd have been associated with inhibition of cell apoptosis (22, 23), whereas PKCδ, θ, and μ have been described as proapoptotic kinases, and their cleavage by caspase 3 has been shown to be important for their action (24, 25). Indeed, apoptotic stimuli such as etoposide and ionizing radiation induce the cleavage of these isoforms and the accumulation of active catalytic fragments (25, 26). PKCδ has been reported to play a role in the apoptosis induced by etoposide (27) and in the apoptosis of keratinocytes and LNCaP cells in response to PMA (28, 29). Although most studies suggest that PKCδ is associated with the induction of apoptosis, an anti-apoptotic effect for PKCδ has been reported for some systems (30).

In a recent study, we reported that etoposide induced apoptosis of C6 cells via activation of PKCδ and its tyrosine phosphorylation on specific tyrosine residues (31). In the present study, we report that infection of C6 cells with SVNI, a neurovirulent strain of Sindbis virus, also induced selective activation and tyrosine phosphorylation of PKCδ. Furthermore, we found that this phosphorylation was essential for the ability of PKCδ to protect C6 cells from SVNI-induced apoptosis. Our
results emphasize that the action of PKC depends critically on its biological context.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-PKC/H9254 antibody was obtained from Transduction Laboratories (Lexington, KY), and polyclonal anti-PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor antibodies and the endoplasmic reticulum (ER) marker DiOC5 (3) were from Molecular Probes (Eugene, OR). An anti-active caspase 3 antibody was obtained from New England Biolabs (Beverly, MA). Cell fragmentation (anti-histone) ELISA and lactate dehydrogenase kits were from Roche Molecular Biochemicals. Leupeptin, aprotinin, phenylmethylsulfonyl fluoride, sodium vanadate, and anti-actin antibody were obtained from Sigma.

Viruses—The variant of Sindbis virus used in this study was described previously (32). Briefly, the neurovirulent strain SVNI was isolated by serial passages of an SV strain in brains of suckling and weanling mice.

Virus Plaque Assay in Vero Cells—Growth of SVNI was quantified on Vero cells by plaque formation as described previously (33). Briefly, a dilution of virus was added to Vero cell monolayers in Petri dishes and incubated at 37°C for 1 h to permit viral absorption. The monolayer was overlaid with 2× minimum Eagle’s medium and 2% tragacanth (gum tragacanth grade III; Sigma) containing 2% fetal bovine serum and 2.4% NaHCO3. Cultures were incubated for 48 h, and plaques were counted after staining with 0.05% neutral red.

Site-directed Mutagenesis of PKC/H9254—Site-directed mutagenesis of PKC/H9254 was performed using the Transformer site-directed mutagenesis kit from CLONTECH (Palo Alto, CA). Conversion of tyrosine residues at sites 52, 64, 155, 187, and 565 into phenylalanine was performed as described previously (34). PKC/H9254 and the PKC/H9254 mutants were subcloned into the metallothionein promoter-driven eukaryotic expression vector (MTH).

C6 Glial Cultures and Cell Transfection—C6 cells were grown in medium consisting of Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (0.05 mg/ml). The cells were transfected with either the empty vectors or with the PKC8 and PKC8 mutant expression vectors using LipofectAMINE PLUS (Invitrogen) as described (34). For the translocation studies, cells were transiently transfected with the PKC8-GFP fusion protein as described (34). Experiments were carried out routinely on two clones of the transfected cells.

Measurements of Cell Apoptosis—Cell apoptosis was measured by flow cytometry after propidium iodide staining, by Hoechst staining, and by ELISA using anti-histone antibodies. Cells were infected with SVNI for 24 or 36 h. Detached cells and trypsinized adherent cells were
collected, fixed in 70% ethanol for 1 h on ice, washed with PBS, and treated for 15 min with RNase (200 μg/ml) at room temperature. Cells were stained with propidium iodide (5 μg/ml) and analyzed on a BD PharMingen cell sorter. For Hoechst staining, cells were fixed with methanol and incubated for 10 min with 1 μg/ml Hoechst 33258. Cells were then viewed and counted under UV illumination for the visualization of the Hoechst-stained nuclei.

For anti-histone ELISA, fragmented DNA was extracted from the control and infected cells and was incubated in 96-well plates coated with anti-histone antibodies for 2 h. Plates were incubated with anti-DNA antibodies conjugated to peroxidase for an additional 2 h. Substrate solution was added, and absorbance was measured at 405 nm.

**Immunoblot Analysis**—Cells were washed twice in cold PBS, scraped with a rubber policeman, and sonicated in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotonin, 50 μg/ml leupeptin, 0.5 mM Na3VO4) for 15 s. Sample buffer (2×) was added, and the samples were boiled for 5 min.

Lysates (30 μg of protein) were resolved by SDS-PAGE and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in phosphate-buffered saline and subsequently stained with the primary antibody. Specific reactive bands were detected using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad), and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham Biosciences).

**Immunoprecipitation**—Immunoprecipitation was performed as described previously (34). Briefly, C6 cells were infected with SVNI for different periods of time. The samples were pre-absorbed with 25 μl of protein A/G-Sepharose for 10 min, and immunoprecipitation was performed using 4 μg/ml of anti-PKCδ or anti-PKCε antibody for 1 h at 4 °C followed by incubation with 30 μl of A/G-Sepharose for an additional hour. Following washes, the pellets were resuspended in 25 μl of SDS sample buffer and boiled for 5 min. The samples were subjected to Western blotting.

**Immunofluorescence Staining**—Cells were infected with SVNI for 1–6 h. Cells were then washed with PBS and fixed in 4% paraformaldehyde for 15 min. Subsequently, cells were washed in PBS and, after blocking the staining buffer (2% bovine serum albumin and 0.1% Triton X-100 in PBS) for 30 min at room temperature, were incubated with the specific anti-PKC antibodies followed by incubation with an anti-rabbit Alexa Fluor 488 or with anti-mouse Alexa Fluor 546 antibodies for an additional hour. Cells were mounted using FluoroGuard antifade reagent and were viewed and photographed using confocal microscopy with ×63 magnification at excitation wavelengths of 543 and 488 nm. For ER staining, fixed and permeabilized cells were incubated for 30 min with the ER marker, DiOC5 (3).

**Statistical Analysis**—The results are presented as the mean values ± S.E. Data were analyzed using analysis of variance and a Student’s t test.

**RESULTS**

**Sindbis Virus Induces Apoptosis of C6 Cells**—SVNI infection induced apoptosis of the C6 cells, which was detected 24 h post-infection and reached maximal levels after 36 h. Using FACS analysis of propidium iodide-stained cells, we found that ~25–35% of the cells were apoptotic in response to SVNI after 24 h of treatment, and ~60% of the cells underwent apoptosis after 36 h (Fig. 1A). Infection of the cells with SVNI reduced the number of the cells and resulted in the appearance of rounded and detached cells, which are characteristic of apoptotic cells (Fig. 1B). Visualization of Hoechst-positive cells (Fig. 1C) and anti-histone ELISA (Fig. 1D) provided further measures of the induction of apoptosis.

**Sindbis Virus Induces a Selective Translocation of PKCδ**—PKCδ has been implicated as an important regulator of cell apoptosis (20, 21). To study the possible involvement of PKC in the apoptosis induced by SVNI, we first examined the effect of SVNI on the translocation of the different PKC isoforms expressed in the C6 cells. For these experiments we infected C6 cells with SVNI for 1, 3, and 6 h and followed the translocation of the different PKC isoforms using immunofluorescence and confocal microscopy. SVNI induced translocation of PKCδ, as is evident both in its depletion from the nucleus and in its accumulation in the perinuclear region (Fig. 2A). Translocation was already observed after 1 h, reached maximal levels after 3 h, and returned to control levels after 6 h. PKCδ, -β, -ζ, -μ, and -ι did not show detectable translocation after infection of the cells with SVNI (data not shown).

To further characterize the translocation of PKCδ in response to SVNI infection we visualized the ER using the ER marker DiOC5 (3). As shown in Fig. 2B, the ER marker DiOC5 (3) accumulated in the perinuclear region in a similar pattern to that of PKCδ in SVNI-treated cells. Merged images showed
clearly colocalization of the fluorescence of the DiOC₅(3) and of the Alexa Fluor 546 used to visualize anti-PKC/H₉₂₅₄, suggesting that SVNI induced translocation of PKC/H₉₂₅₄ to the ER.

The pattern of translocation of PKC/H₉₂₅₄ obtained using GFP-tagged PKC/H₉₂₅₄ was similar to that determined by immunofluorescence. Thus, SVNI infection of cells transiently transfected with GFP-PKC/H₉₂₅₄ induced translocation of PKC/H₉₂₅₄ to the perinuclear region (Fig. 2C). It should be noted that this pattern of translocation upon treatment of the cells with PMA. As has been described elsewhere (39), PMA induced translocation of PKCδ to the plasma and nuclear membranes (Fig. 2C).

PKCδ Protects C6 Cells from SVNI-induced Apoptosis—Because SVNI induced a selective translocation of PKCδ, we examined the role of this PKC isoform in the apoptotic effect of SVNI. As an initial approach, we used the PKCδ selective inhibitor rottlerin. Treatment of C6 cells with rottlerin (5 μM) or with the vehicle control Me₂SO (data not shown) did not affect the basal level of cell apoptosis. However, rottlerin increased the apoptotic effect of SVNI by ~30–40%, suggesting that PKCδ may exert a protective effect on SVNI-infected C6 cells (Fig. 3A). Because the specificity of rottlerin for PKCδ has been questioned (36–38), we examined further the role of PKCδ in the apoptotic effect of SVNI by using a PKCδ kinase-dead mutant (PKCδ KD; K376R). Cells stably expressing the PKCδ KD mutant were infected with SVNI for 24 h. Infection of the PKCδ KD-expressing cells induced increased cell apoptosis compared with CV cells as observed by cell morphology (Fig. 3B) and by anti-histone ELISA (Fig. 3C). The results reflect the means ± S.E. of triplicate measurements in each of three experiments (C and D). * p < 0.001 as compared with cells overexpressing PKCδ KD.

FIG. 3. Role of PKCδ in the apoptosis of C6 cells infected with SVNI. C6 cells were infected with SVNI (m.o.i. 5) in the absence and presence of rottlerin (5 μM) for 24 h (A). Alternatively, cells overexpressing CV, PKCδ KD mutant (B and C), or PKCδ (D) were infected with SVNI. Cell apoptosis was determined using PI staining and FACS analysis (A and D) or anti-histone ELISA (C). The channels corresponding to a sub-G₁ DNA content, reflecting apoptosis, are indicated by the arrows. The morphology of the cells (24 h) was monitored under a phase contrast light microscope (B). The optical density values of SVNI-infected PKCδ KD-overexpressing cells were designated as 100% (total apoptosis), and the other values were expressed relative to this (C). The results represent the means ± S.E. of triplicate measurements in each of three experiments (C and D). * p < 0.001 as compared with cells overexpressing PKCδ KD.
Infection of C6 Cells with SVNI Induces Tyrosine Phosphorylation of PKCδ—PKCδ undergoes tyrosine phosphorylation in response to various apoptotic stimuli (31, 35, 40). To examine whether infection with SVNI induced tyrosine phosphorylation of PKCδ, we infected C6 cells with SVNI for 1–4 h. PKCδ was immunoprecipitated, and the membrane was blotted with anti-phosphotyrosine antibody. As shown in Fig. 4A, SVNI induced tyrosine phosphorylation of PKCδ in a time-dependent manner, and maximal phosphorylation was obtained after 2 h. We did not detect tyrosine phosphorylation of PKCε (Fig. 4B) or of the other PKC isoforms (data not shown).

Tyrosine Phosphorylation of PKCδ Is Necessary for Its Protective Effect on SVNI-induced Apoptosis—To explore the importance of the tyrosine phosphorylation of PKCδ in the apoptosis induced by SVNI, we employed a PKCδ mutant in which five putative tyrosine phosphorylation sites, 52, 64, 155, and 187 in the regulatory domain and tyrosine 565 in the catalytic domain, were mutated to phenylalanine (34). The expression of this mutant in C6 cells and its effects on cell proliferation, glutamine synthetase expression, and cell apoptosis in response to etoposide have been described (31, 34, 39). We found that SVNI did not induce tyrosine phosphorylation of the PKCδ5 mutant (Fig. 5A). In contrast to the results observed in response to etoposide, PKCδ5-overexpressing cells exhibited a significantly increased cell apoptosis, compared with control vector cells and with cells overexpressing PKCδ, both as determined by PI staining (Fig. 5B) and by the morphological appearance of the cells (Fig. 5C).

Replication of SVNI in Cells Overexpressing PKCδ and PKCδ5—To determine whether the differential effect of SVNI on the apoptosis of cells overexpressing PKCδ and PKCδ5 is because of changes in virus replication, we measured viral replication by plaque assay. Cells were infected with SVNI (m.o.i. 5) for 9, 24, and 48 h. Using plaque assay, we found that the replication of SVNI was similar in control cells and in cells overexpressing PKCδ and PKCδ5 (Table 1).

Cleavage of Caspase-3, PKCδ, and PKCδ5 in Response to SVNI Infection—The apoptosis induced by SVNI is mediated by caspases (41). In a recent study we demonstrated that tyrosine-phosphorylated PKCδ regulated the cleavage of caspase 3, which in turn cleaved PKCδ to release the active catalytic domain (31). To further explore the role of tyrosine phosphorylation of PKCδ in the apoptosis induced by infection of SVNI, we compared the cleavage of caspase 3 in cells overexpressing control vector, PKCδ, and the PKCδ5 mutant. Using a specific antibody that recognizes the cleaved product (17 kDa) of caspase 3, we detected a cleaved fragment of caspase 3 in SVNI-infected control vector cells. Cells overexpressing PKCδ exhibited very low levels of cleaved caspase 3, whereas large amounts of the cleaved product were obtained in cells overexpressing PKCδ5 (Fig. 6A).

To examine whether SVNI infection induced cleavage of PKCδ or PKCδ5, we infected C6 cells overexpressing PKCδ and PKCδ5 with SVNI for 12 and 24 h and analyzed cell lysates by Western blotting. Using the ε tag, which could detect the cleaved catalytic domain of the exogenous PKCδ and PKCδ5, we found no detectable cleaved product in either of the infected cells (Fig. 6B), suggesting that although SVNI infection induced activation of caspase 3, caspase 3 did not cleave PKCδ or PKCδ5.

Role of Specific Tyrosine Mutants in the Apoptosis Induced by SVNI—To examine the role of the specific tyrosine residues in the effect of PKCδ on cell apoptosis induced by SVNI, we used C6 cells stably transfected with different PKCδ mutants in which each one of the tyrosines (52, 64, 155, 187, 565) was mutated individually to phenylalanine. The expression of these mutants in C6 cells was described previously (39).

We found that cells overexpressing PKCδY52F, PKCδY64F, and PKCδY155F exhibited an enhanced apoptotic response to SVNI albeit to a lesser extent than cells overexpressing PKCδ5. In contrast, infection with SVNI of cells overexpressing PKCδY187F or PKCδY565F resulted in a lower apoptotic response, similar to the response observed in cells overexpressing PKCδ5 (Fig. 7A).

We also found that infection with SVNI induced a smaller increase in the tyrosine phosphorylation of PKCδY52F, PKCδY64F, and PKCδY155F as compared with PKCδ (Fig. 7B). The lowest degree of the tyrosine phosphorylation was obtained in cells overexpressing the PKCδY155F mutant, as compared with the other single tyrosine mutants. Correspondingly, this mutant exhibited the highest degree of cell apoptosis in response to SVNI (Fig. 7B).

DISCUSSION

In this study we explored the role of PKCδ in the apoptosis induced by Sindbis virus infection. We found that infection of the cells with SVNI induced a selective translocation and tyrosine phosphorylation of PKCδ and that the phosphorylated PKCδ exerted a protective effect against SVNI-induced apoptosis.

SVNI, a neurovirulent strain of Sindbis virus, has been reported to induce apoptosis in a variety of cell types (1, 2). The mechanisms involved in the apoptotic effect of SVNI have been described mainly with regard to the roles of apoptotic-related proteins such as beclin (4), Bcl2, Bax (42, 43), and caspases (41). Thus, SVNI induces high expression of Bax (42), and its apoptotic effects are blocked by high expression of Bcl2 (3). In addition, various studies indicate that the effects of SVNI are mediated by caspase activation, because CrmA (41), caspase 3, and caspase 8 inhibitors (41, 44) blocked the apoptotic effect of the virus. In contrast, only a few reports exist with regard to the signal transduction pathways activated by SV infection. SV was reported to induce activation of sphingomyelinase and ceramide release in N18 cells (7) and to activate p38 and the small heat shock protein HSP27 in Vero cells (6). Similarly, SV infection was reported to activate the double-strand RNA-dependent protein kinase (9) and nuclear factor κB (8).

We found that SVNI induced a selective translocation of PKCδ to the ER within 3 h of infection, whereas it did not affect
FIG. 5. Tyrosine phosphorylation and cell apoptosis in SVNI-infected cells overexpressing PKCε and PKCδ5. C6 cells overexpressing CV, PKCδ, or PKCδ5 were infected with SVNI (m.o.i. 5) for 120 min (A) or for 24 h (B and C). Immunoprecipitation (IP) of PKCδ and PKCδ5 was performed using anti-PKCε antibody, and membranes were blotted with anti-phosphotyrosine antibody (anti-PY) or with an anti-PKCδ antibody (A). The results represent one of three separate experiments, which gave similar results. For measurement of apoptosis, cells were harvested after 24 h of treatment and analyzed using PI staining and FACS analysis (B). The channels corresponding to a sub-G, DNA content, reflecting apoptosis, are indicated by the arrows. The morphology of the cells (36 h) was monitored under a phase contrast light microscope (C). IB, immunoblot.
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Replication of SVNI in cells overexpressing CV, PKCδ, and PKCδ5

C6 cells overexpressing control vector (CV), PKCδ, or PKCδ5 were infected with SVNI (m.o.i. 5) for various periods of time. The replication of SVNI was determined using plaque assay as described under “Experimental Procedures.” The results represent one of three separate experiments, which gave similar results.

| Time (h) | CV | PKCδ | PKCδ5 |
|---------|----|------|-------|
| 9       | 5.5 × 10^7 | 5.0 × 10^7 | 9.5 × 10^7 |
| 24      | 1.5 × 10^10 | 8.9 × 10^7 | 1.4 × 10^9 |
| 48      | 1.4 × 10^9 | 9.5 × 10^7 | 1.1 × 10^9 |

The translocation of the other PKC isoforms. PKCδ has been reported to undergo differential translocation to distinct cellular compartments depending on the specific stimulus and on the specific cell type. Thus, in C6 cells PKCδ translocates to the plasma and nuclear membranes in response to PMA, in contrast to its translocation to the ER in response to SVNI. In keratinocytes, PKCδ undergoes translocation to the mitochondria in response to PMA (29) and in HeLa cells PKCδ translocates to the Golgi in response to ceramide (45). Various PKC isoforms are also found in the nucleus and in subnuclear compartments (46). It is currently not clear what is the function of PKCδ in the ER and which proteins can be phosphorylated by PKCδ in the ER membrane.

SVNI induced tyrosine phosphorylation of PKCδ. This phosphorylation appeared to be essential for the protective effect of PKCδ against SVNI-induced apoptosis, because cell apoptosis was decreased in cells overexpressing PKCδ and was increased significantly in cells overexpressing the PKCδ5 mutant. Indeed, the PKCδ5 mutant acts in an opposite way to PKCδ in its effect on cell apoptosis in response to SVNI. Likewise, PKCδ and PKCδ5 induced opposite effects on the expression of the astrocytic marker GS (34), on cell proliferation (39), and on cell apoptosis in response to etoposide (31). In contrast to the effect on apoptosis, production of progeny virus was similar in cells expressing PKCδ, PKCδ5, and CV, suggesting that the differential apoptotic response observed in cells overexpressing PKCδ and PKCδ5 cannot be attributed to differences in virus replication.

Of the five sites of phosphorylation mutated in PKCδ5, we found that phosphorylation on tyrosines 52, 64, and 155 in the regulatory domain was essential for the protective effect of PKCδ. Phosphorylation of PKCδ on tyrosine residues occurs in response to a large number of stimuli including PMA (34), platelet-derived growth factor (39, 47), epidermal growth factor (48), activation of the IgE receptor (19, 49), and apoptotic stimuli such as γ-irradiation (40), H2O2 (35), and etoposide (31). Tyrosine phosphorylation of PKCδ occurs on different tyrosine residues. Thus, platelet-derived growth factor and PMA induced tyrosine phosphorylation on tyrosines 187 and 155, respectively (39), and activation of the IgE receptor induced phosphorylation on tyrosine 52 (19). In addition, apoptotic stimuli such as etoposide (31) and H2O2 (35) induce tyrosine phosphorylation in either the regulatory or the catalytic domain, respectively, of PKCδ. It is not known currently why phosphorylation in the regulatory domain of PKCδ is essential for its protective effect against apoptosis induced by SVNI. Changes in the catalytic activity and cellular localization via binding to specific scaffold proteins could provide the basis for this effect.

A striking finding was that PKCδ played opposite roles in apoptosis induced by SVNI and by etoposide, although tyrosine phosphorylation of PKCδ was essential for both effects. The opposite effects of PKCδ on cell apoptosis in response to SVNI and etoposide and the differential role of the tyrosine phosphorylation in PKCδ effects may be attributed to a number of factors. First, PKCδ underwent differential translocation by etoposide and SVNI. Thus, distinct translocation of PKCδ to the nucleus by etoposide (31) or to the ER by SVNI could lead to different effects because of the phosphorylation of different substrates and to the association of PKCδ with specific proteins present in these locations. Second, etoposide and SVNI induced phosphorylation of different tyrosine residues on PKCδ. The effects of tyrosine phosphorylation on the activity of PKCδ or on its function are dependent on the specific system and stimulus; however, it is believed currently that tyrosine phosphorylation can alter the affinity of PKCδ toward its different substrates (19). Thus, the phosphorylation of PKCδ on specific residues might confer differential affinity of PKCδ for distinct apoptotic-related proteins. Consequently, the activation of different down-stream pathways including caspases or other apoptosis-related proteins by these two stimuli could provide the basis for the divergent effects of PKCδ on cell apoptosis.

Apoptosis by SV infection has been reported to involve caspase activation (41, 44). Indeed, we found that SVNI induced activation of caspase 3. This activation was increased further in cells overexpressing PKCδ5 and was decreased in cells overexpressing PKCδ. These results are in contrast to our recent studies, which demonstrated that etoposide induced an increased activation of caspase 3 in cells overexpressing PKCδ and a decreased response in cells overexpressing PKCδ5 (31). One explanation for these differences could be a differential activation of upstream caspases by PKCδ and PKCδ5 in response to etoposide and SVNI, which will eventually converge in the activation of caspase 3.

Interestingly, and in contrast to the results obtained with etoposide (31), SVNI infection did not induce caspase-dependent cleavage of PKCδ or PKCδ5. In various systems, the apoptotic effect of PKCδ has been associated with a cleavage of the catalytic domain from the regulatory domain (27). Cleavage of the catalytic domain of PKCδ by caspases has been reported in cells treated with ionizing radiation, tumor necrosis factor-α,
and etoposide (25, 27, 50), whereas no cleavage of PKCδ was observed in LNCaP cells undergoing apoptosis in response to PMA (28). Indeed, our recent studies (31) and others (27) have suggested the presence of a positive loop between PKCδ and caspase 3 in etoposide-treated cells, which was not observed in SVNI-treated cells. Because caspase 3 has been reported recently to undergo nuclear translocation in response to apoptotic stimuli (51), it is possible that the lack of PKCδ cleavage by caspase 3 is because of a different cellular localization of the two proteins.

One of the interesting issues remaining to be investigated is the identity of the tyrosine kinases and phosphatases that phosphorylate and dephosphorylate PKCδ in response to SVNI. PKCδ has been reported to associate and undergo tyrosine phosphorylation by different tyrosine kinases such as Src, Lyn (19, 49), Fyn (39, 47), and c-Abl (40). We found differential phosphorylation of tyrosines 52, 64, and 155 in response to SVNI and differential sensitivity of these tyrosine mutants to SVNI-induced apoptosis. The ability of PKCδ to be phosphorylated on multiple tyrosine residues suggests that PKCδ can associate with different tyrosine kinases via distinct tyrosine residues, which can then lead to diverse cellular outcomes. Moreover, it appears that phosphorylation at one tyrosine residue may influence phosphorylation at the others. The identity of the tyrosine kinases involved in the phosphorylation of PKCδ in SVNI-treated cells is currently being studied. Our results so far suggest that Fyn, which is involved in the tyrosine phosphorylation of PKCδ in response to platelet-derived growth factor, is probably not involved in the apoptosis induced by either etoposide (31) or SVNI.

The mechanisms by which SVNI induces translocation and tyrosine phosphorylation of PKCδ are not yet understood, but several options can be considered. The translocation and phosphorylation of PKCδ induced by SVNI were observed following 1–3 h of infection, suggesting that early events such as binding of the virus to its cellular receptor or its entrance via the plasma membrane activated this signaling pathway. Indeed, infection with SVNI induces a rapid activation of ceramide (7) and p38 (6). Moreover, it was reported recently that the entrance of the virus to the cells is sufficient for the induction of apoptosis by the virus (52).

Apoptosis induced by viral infection is a defense mechanism of the host cells, because it limits virus production and prevents the infection of neighboring cells (53). Most viruses inhibit or delay early apoptosis by inducing the expression of endogenous anti-apoptotic cellular proteins or by using their own anti-apoptotic genes (53). Thus, the tyrosine phosphorylation of PKCδ by SVNI may represent a mechanism by which the virus inhibits cell apoptosis, whereas activation of tyrosine phosphatases that dephosphorylate PKCδ may reflect a cell response that aims at inducing cell apoptosis.

In summary, we demonstrated that the apoptosis induced by SVNI infection involves tyrosine phosphorylation and translocation of PKCδ to the ER. The tyrosine-phosphorylated PKCδ protected the cells from the apoptosis induced by SVNI.

Unpublished data.

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**FIG. 7.** Apoptosis and tyrosine phosphorylation in SVNI-infected C6 cells overexpressing single PKCδ tyrosine mutants. C6 cells overexpressing PKCδ or the different PKCδ mutants were infected with SVNI (m.o.i. 5) for 24 h, and cell apoptosis was measured by PI staining and FACS analysis. The results represent the means ± S.E. of triplicate measurements in each of five experiments. Tyrosine phosphorylation of PKCδ and the PKCδ tyrosine mutants was assessed in cells infected with SVNI for 2 h. Immunoprecipitation (IP) of the PKCδ and the PKCδ mutants was performed using anti-PKCδ antibody as described under “Experimental Procedures” (B). Membranes were then stained (IB) with anti-phosphotyrosine antibody (anti-PY) or with an anti-PKCD antibody. The results represent one of three similar experiments. *, p < 0.001 as compared with cells overexpressing PKCδ52 and PKCδ64 mutants.
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whereas the dephosphorylated PKCδ rendered the cells more sensitive to the apoptosis induced by SVNI. The effect of the tyrosine phosphorylation of PKCδ on the apoptosis in C6 cells in response to SVNI may be mediated by altering the affinity of PKCδ toward downstream apoptosis-related substrates or by altering the activity of a tyrosine kinase that is associated with PKCδ. Thus, the differential phosphorylation of specific tyrosine residues and the distinct localization of PKCδ may provide the basis for the anti- and pro-apoptotic functions of this kinase.

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REFERENCES

1. Griffin, D. E., and Hardwick, J. M. (1997) Annu. Rev. Microbiol. 51, 565–592
2. Griffin, D. E., and Hardwick, J. M. (1999) Trends Microbiol. 7, 155–160
3. Levine, B., Huang, Q., Isaacs, J. T., Reed, J. C., Griffin, D. E., and Hardwick, J. M. (1993) Nature 361, 739–742
4. Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., and Levine, B. (1989) J. Virol. 72, 8586–8596
5. Griffin, D. E. (1998) Neurosci. Biobehav. Rev. 22, 721–723
6. Nakatsume, T., Kato, I., Nakamura, S., Takahashi, Y., Ikawa, Y., and Yoshinaka, Y. (1998) Biochem. Biophys. Res. Commun. 253, 59–64
7. Jan, J. T., Chatterjee, S., and Griffin, D. E. (2000) J. Virol. 74, 6425–6432
8. Lin, K. I., DiDonato, J. A., Hoffmann, A., Hardwick, J. M., and Ratan, R. R. (1994) J. Biol. Chem. 269, 6140–6148
9. Hayashi, A., Seki, N., Hattori, A., Kuzuma, S., and Saito, T. (1999) Biochim. Biophys. Acta 1450, 99–106
10. Newton, A. C. (2002) Methods Enzymol. 345, 499–506
11. Newton, A. C. (1995) Curr. Biol. 5, 973–976
12. Salem-Smith, H., Chang, E. Y., Szallasi, Z., Blumberg, P. M., and Rivera, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9112–9116
13. Dempsey, R. C., Newton, A. C., Mochly-Rosen, D., Fields, A. P., Reiland, M. E., Insel, P. A., and Messing, R. O. (2000) Am. J. Physiol. Lung Cell Mol. Physiol. 278, L429–L438
14. Coon, S. S., Leitch, D., Hinz, N., Deacon, E., Salmon, M., and Lord, J. M. (2000) Exp. Cell Res. 256, 34–41
15. Gubina, E., Rinaudo, M. S., Szallasi, Z., Blumberg, P. M., and Mufson, R. A. (1996) Biochim. Biophys. Acta 129, 429–439
16. Ruvolo, P. P., Deng, X., Carr, B. K., and May, W. S. (1998) J. Biol. Chem. 273, 25436–25442
17. Endo, K., Oki, E., Biedermann, V., Kojima, H., Yoshida, K., Johannes, F. J., Kufe, D., and Datta, R. (2000) J. Biol. Chem. 275, 18476–18481
18. Ron, D., and Kazanietz, M. G. (1999) Front. Biosci. 4, 1415–1479
19. Jaken, S. (1996) Curr. Opin. Cell Biol. 8, 336–342
20. Appel, E., Katzoff, A., Ben Moshe, T., Kazimirsky, G., Kobiler, D., Lustig, S., and Brodie, C. (2000) J. Cell Biol. 149, 8547–8558
21. Weng, Q., Bhattacharyya, D., Garfield, S., Marquez, V. E., and Blumberg, P. M. (1999) J. Biol. Chem. 274, 37233–37239
22. Li, W., Chen, X. H., Kelley, C. A., Alimandi, M., Zhang, J., Chen, Q., Bottaro, D. P., and Pierce, J. H. (1996) J. Biol. Chem. 271, 26404–26409
23. Denning, M. F., Duggan, A. A., Threadgill, D. W., Magnuson, T., and Tsai, S. H. (1996) J. Biol. Chem. 271, 5265–5271
24. Song, J. S., Swann, P. G., Szallasi, Z., Blank, U., Blumberg, P. M., and Rivera, J. (1998) Oncogene 16, 3357–3368
25. Kornya, H., Kouchi, Z., Umeda, T., Saido, T. C., Momoi, T., Ishiura, S., and Nishizuka, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6587–6592
26. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Biochem. J. 351, 95–105
27. Gschwendt, M., Muller, H. J., Kelbassa, K., Rang, P., Kittstein, W., Rincke, M., and Marks, F. (1998) Biochem. Biophys. Res. Commun. 199, 93–98
28. Kornya, H., Umeda, T., Saido, T. C., Momoi, T., Ishiura, S., and Nishizuka, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6587–6592
29. Kornya, H., Umeda, T., Saido, T. C., Momoi, T., Ishiura, S., and Nishizuka, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6587–6592
30. Yuan, Z. M., Utsugisawa, T., Ishiko, T., Nakada, S., Huang, Y., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1998) Oncogene 16, 1643–1648
31. Appel, E., Katzoff, A., Ben Moshe, T., Kazimirsky, G., Kohiler, D., Lustig, S., and Brodie, C. (2000) Virology 276, 238–242
32. Lewis, J., Oyler, G. A., Ueno, K., Fannjiang, J. V., Chau, B. N., Vornov, J., Korsmeyer, S. J., Zou, S., and Hardwick, J. M. (1999) Nat. Med. 5, 832–835
33. Sard, R., Ben Moshe, T., Kazimirsky, G., Kohiler, D., Lustig, S., and Brodie, C. (2001) Cell Death Differ. 8, 1224–1231
34. Kaino, M., Ohmori, S., Shirai, Y., Sakai, N., and Saito, N. (2001) Mol. Cell. Biol. 21, 1769–1783
35. Wang, Q. M., Bhattacharyya, D., Garfield, S., Marquez, V. E., and Blumberg, P. M. (1999) J. Biol. Chem. 274, 37233–37239
36. Li, W., Chen, X. H., Kelley, C. A., Alimandi, M., Zhang, J., Chen, Q., Bottaro, D. P., and Pierce, J. H. (1996) J. Biol. Chem. 271, 26404–26409
37. Denning, M. F., Duggan, A. A., Threadgill, D. W., Magnuson, T., and Tsai, S. H. (1996) J. Biol. Chem. 271, 5265–5271
38. Song, J. S., Swann, P. G., Szallasi, Z., Blank, U., Blumberg, P. M., and Rivera, J. (1998) Oncogene 16, 3357–3368
39. Majumder, P. K., Pandey, P., Sun, X., Cheng, K., Datta, R., Saxena, S., Kharbanda, S., and Kufe, D. (2000) J. Biol. Chem. 275, 21793–21796
40. Koide, I., and Branton, P. E. (1997) J. Virol. 71, 1739–1746
41. Koide, I., and Branton, P. E. (1997) J. Virol. 71, 1739–1746