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Dominant Inhibitory Ras Delays Sindbis Virus-Induced Apoptosis in Neuronal Cells

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Mature neurons are more resistant than dividing cells or differentiating neurons to Sindbis virus-induced apoptotic death. Therefore, we hypothesized that mitogenic signal transduction pathways may influence susceptibility to Sindbis virus-induced apoptosis. Since Ras, a 21-kDa GTP-binding protein, plays an important role in cellular proliferation and neuronal differentiation, we investigated the effect of an inducible dominant inhibitory Ras on Sindbis virus-induced death of a rat pheochromocytoma cell line, PC12 cells. Dexamethasone induction of dominant inhibitory Ras (Ha Ras^{Asn17}) expression in transfected PC12 cell lines (MMTV-M17-21 and GSrasDN6 cells) resulted in a marked delay in Sindbis virus-induced apoptosis, compared with infected, uninduced cells. The delay in death after Sindbis virus infection in induced versus uninduced PC12 cells was not associated with differences in viral titers or viral infectivity. No delay in Sindbis virus-induced apoptosis was observed in Ha Ras^{Asn17}-transfected PC12 cells if dexamethasone induction was initiated less than 12 h before Sindbis virus infection or in wild-type PC12 cells infected with a chimeric Sindbis virus construct that expresses Ha Ras^{Asn17}. The delay in Sindbis virus-induced apoptosis in induced Ha Ras^{Asn17}-transfected PC12 cells was associated with a decrease in cellular DNA synthesis as measured by 5′-bromo-2′-deoxyuridine incorporation. Thus, in PC12 cells, inducible dominant inhibitory Ras inhibits cellular proliferation and delays Sindbis virus-induced apoptosis. These findings suggest that a Ras-dependent signaling pathway is a determinant of neuronal susceptibility to Sindbis virus-induced apoptosis.

Sindbis virus (SIN), the prototype alphavirus, is a single-stranded positive RNA virus that replicates lytically in most mammalian cell lines and produces an age-dependent fatal encephalitis in mice. We have shown that lytic replication in several cell lines is due to the induction of apoptosis (16), a genetically encoded cell suicide program that is important for the removal of harmful or superfluous cells from multicellular organisms. SIN-infected cells display light microscopic changes (e.g., membrane blebbing, cytoplasmic condensation, and nuclear condensation), electron microscopic changes (e.g., chromatin condensation, cytoplasmic vacuolization, and loss of surface microvilli), and endonucleosomal DNA cleavage patterns that are characteristic of apoptosis (16). In addition, apoptosis is observed in the neurons of SIN-infected neonatal mice (which develop fatal encephalitis) (14), but not in the brains of older mice that develop asymptomatic disease (18). A number of antiapoptotic genes have been shown to inhibit SIN-induced apoptosis both in vitro (2, 16, 24) and in vivo (14), suggesting that differential expression of endogenous inhibitors of apoptosis (or proteins with which they interact) may be a major determinant of resistance to SIN-induced apoptosis in certain cell types such as mature neurons. However, other factors may also be important in the regulation of apoptosis in SIN-infected cells.

The resistance of fully differentiated neurons to lytic SIN replication may provide an additional clue to cellular factors regulating apoptosis induced by SIN infection. Nearly all mammalian cell lines that are susceptible to SIN infection demonstrate cytopathic effects within 12 to 48 h after infection. Similarly, rat embryonic dorsal root ganglion neurons also die within 24 to 48 h after SIN infection if they are infected within the first several days after explantation (16). In contrast, in the presence of nerve growth factor, these cells develop a mature neuron phenotype and develop resistance to SIN-induced death within 3 to 4 weeks after explantation (15, 16). This phenotypic dichotomy, susceptibility to SIN-induced death in proliferating cells and actively differentiating neurons versus resistance to SIN-induced death in fully differentiated neurons, suggests that a mitogenic signal transduction pathway may influence susceptibility to SIN-induced apoptosis.

Ras is a 21-kDa, GTP-binding protein that plays an important role in signal transduction pathways mediating both cellular proliferation and nerve growth factor-induced neuronal differentiation (reviewed in references 20 and 37). A mutation in Ha Ras (Asn-17) confers a dominant negative phenotype by preferentially binding GDP rather than GTP and thereby competing with endogenous cellular p21 Ras for upstream activators (5). Previous studies have shown that Ha Ras^{Asn17} inhibits proliferation of NIH 3T3 cells (6), neuronal differentiation of rat pheochromocytoma PC12 cells (34), apoptosis in naive and neuronally differentiated PC12 cells deprived of trophic factor support (7), and Fas-induced apoptosis in Jurkat cells (10). In addition, Ha Ras^{Asn17} inhibits activation of the c-Jun NH_2-terminal protein kinase (JNK) kinase signal transduction cascade (28, 29) and inhibits activation of c-Jun by polyomavirus middle-sized tumor antigen (33). The JNK signaling cascade plays a critical role in the induction of apoptosis in PC12 cells (38), and c-Jun activation plays a critical role in the induction of apoptosis in sympathetic neurons (12) deprived of nerve growth factor. Therefore, Ras may be a common upstream element of independent downstream signal transduction pathways involved in both cellular proliferation and differentiation and in cellular apoptosis.

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The role of p21 Ras in signal transduction pathways regulating cell proliferation, differentiation, and, potentially, cellular apoptosis in neuronal cells deprived of trophic factor support led us to investigate whether a Ras-dependent pathway influences susceptibility of neuronal cells to SIN-induced apoptosis. To address this question, we examined the susceptibility to SIN-induced apoptosis of previously described PC12 cell lines that express high levels of either inducible or constitutive dominant inhibitory Ras. In addition, we studied the effects of Ha Ras^{Asn17} expression in PC12 cells by using a double subgenomic SIN vector. Our results indicate that a Ras-dependent signal transduction pathway may be a determinant of cellular susceptibility to SIN-induced apoptosis.

MATERIALS AND METHODS

Plasmid constructions. A double subgenomic SIN vector, dsTE12Q, was constructed by ligating the *Bss*HII-*Xho*I fragment from SIN strain dsTE12 into the *Bss*HII-*Xho*I sites of the previously described neurovirulent double subgenomic SIN vector, ds633 (14). A 570-bp fragment spanning the coding region of dominant inhibitory Ha Ras^{Asn17} was amplified by PCR from the template pXCR17^{N} (provided by Larry A. Feig, Tufts University School of Medicine, Boston, Mass.), incorporating *Bst*EII sites into the upstream and downstream primers, and ligated into the *Bst*EII site downstream of the double subgenomic promoter of dsTE12Q to generate the plasmid dsTE12Q/*ras*^{Asn17}. The correct sequence of the dominant inhibitory *ras*^{Asn17} insert was confirmed by the dideoxy chain termination sequencing method of Sanger et al. (31). As a control for the presence of the foreign insert, plasmid dsTE12Q/CAT was constructed by ligating the *Bst*EII fragment from the previously described clone ds633/CAT (14) into dsTE12Q.

Production of recombinant viruses. The viruses SIN/CAT and SIN/*ras*^{Asn17} were generated from the clones dsTE12Q/CAT and dsTE12Q/*ras*^{Asn17} by previously described methods (14). Briefly, 5′-capped RNA transcripts were synthesized from cDNA clones linearized with *Xho*I and transcribed in vitro with SP6 DNA-dependent RNA polymerase at 38°C for 60 min. BHK-21 cells in 35-mm dishes (~5×10^5 cells) were transfected with RNA transcripts (~200 ng) mixed with 6 μg of Lipofectin (GIBCO/BRL) according to the manufacturer's instructions. At 24 h after transfection, virus particles were harvested from the transfected cell monolayers and frozen in aliquots at −70°C. Virus harvested from transfected cell monolayers were used in all experiments. Viral stock titers were determined by plaque assay titration on BHK-21 cells. Because of the possibility that p21 Ras^{Asn17} might protect against SIN-induced cell death and interfere with plaque formation, the titers of infectious virus particles were also determined by an indirect immunofluorescence assay as previously described (14).

Cell culture. Wild-type PC12 cells and PC12 subclones transfected with Ha *ras*^{Asn17}, mouse mammary tumor virus (MMTV)-M17-21, M-M17-26 (34) (provided by Geoffrey M. Cooper, Dana-Farber Cancer Institute, Boston, Mass.), and GSrasDN6 cells (35) (provided by Simon Halegoua, State University of New York at Stony Brook, Stony Brook) were cultured as previously described on collagen-coated dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (9). In MMTV-M17-21 cells and GSrasDN6 cells, Ha *ras*^{Asn17} expression is driven by the MMTV long terminal repeat and inducible by dexamethasone. In M-M17-26 cells, Ha *ras*^{Asn17} is expressed from the metallothionein promoter at high basal levels and cannot be further induced by exposure to zinc.

Virus infections and titrations. Dexamethasone (Sigma, St. Louis, Mo.) was added to cell cultures 24 h prior to virus infection at a final concentration of 10 μm. Virus infections were performed with either SIN/CAT or SIN/*ras*^{Asn17} at a multiplicity of infection of 5 PFU per cell in RPMI 1640 medium supplemented with 2% fetal bovine serum. Viral supernatant titers in triplicate wells were measured at serial time points after infection by plaque assay titration on BHK-21 cells.

DNA fragmentation assays. PC12 cells were harvested 18 h after mock infection or SIN infection, and cellular DNA was extracted in situ and resolved by agarose gel electrophoresis as described elsewhere (32).

Cell counts. At serial time points after infection, PC12 cells, MMTV-M17-21 cells, M-M17-26 cells, and GSrasDN6 cells were processed as described by Batistou and Greene (1), and the remaining viable nuclei were counted in a hemocytometer. Counts were performed on triplicate wells and are presented as means ± standard errors of the mean. The results are presented relative to the cell number initially plated per well (designated as 100).

Infectious center assays. At 6, 12, and 24 h after infection, dexamethasone-treated and untreated MMTV-M17-21 cells were washed three times with Hanks balanced salt solution, detached by scraping, washed 10 times by centrifugation in Dulbecco modified Eagle medium–2% fetal bovine serum, and resuspended evenly in Dulbecco modified Eagle medium–2% fetal bovine serum. To neutralize extracellular virus, cell suspensions were incubated with 1 mg of a neutralizing anti-E2 monoclonal antibody, R6 (25), per ml for 30 min at 37°C, and then serial dilutions of cells were plated in triplicate on BHK cells for plaque assay. Infectivity was calculated as the percentage of MMTV-M17-21 cells releasing virus based on the number of viable cells plated.

Western blots (immunoblots). A total of 5×10^5 wild-type PC12 cells or MMTV-M17-21 cells were infected with SIN/CAT or SIN/*ras*^{Asn17} or mock infected with Hanks balanced salt solution. Twenty-four hours after infection, cell lysates were prepared and 20 μg of total protein was subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and transferred to nitrocellulose by the protocol described in reference 19. The nitrocellulose membrane was incubated for 1 h with Pan-Ras Ab-3 (1:80 dilution) (Oncogene Science, Cambridge, Mass.) and then for 1 h with horseradish peroxidase-conjugated horse anti-mouse immunoglobulin G (1:3,000) (Amersham, Arlington Heights, Ill.). The positive reaction was visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Cell proliferation assays. Cellular proliferation of dexamethasone-treated and untreated wild-type PC12 cells, MMTV-M17-21 cells, and M-M17-26 cells was assayed by measuring incorporation of 5′-bromo-2′-deoxyuridine (BrdU) into newly synthesized DNA, by using the BrdU labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Briefly, PC12, MMTV-M17-21, and M-M17-26 cells were seeded in 96-well microtiter plates in the medium described above; seeding densities were adjusted for differences in basal rates of proliferation so that all wells contained 10^5 cells at the initiation of BrdU labeling. After 24 h in culture, 10 μM dexamethasone was added to half of the wells. After 48 h in culture, 10 μl of BrdU labeling solution was added per well, and wells were incubated for 12 h at 37°C. Cells were then fixed with 70% ethanol in HCl and subjected to nuclease partial digestion of cellular DNA. BrdU incorporation was measured with a peroxidase-conjugated anti-BrdU antibody and ABTS substrate. Results are presented as mean optical densities of triplicate wells.

RESULTS

SIN kills PC12 cells by inducing apoptosis. The PC12 line of rat pheochromocytoma cells is a well-characterized model for studying the mechanisms by which neuronal cells undergo apoptosis when deprived of trophic factor support (1, 7, 8, 22, 23, 27, 30, 38). To determine whether the PC12 cell line is also a useful model for studying the mechanisms by which SIN induces apoptosis of neuronal cells, we observed light microscopic changes and investigated whether internucleosomal DNA cleavage occurs after SIN infection. Beginning 18 h after infection, SIN-infected cells displayed morphologic changes, including nuclear condensation, membrane blebbing, and cytoplasmic condensation, that are characteristic of apoptotic death (Fig. 1C). In addition, gel electrophoresis of PC12 cellular DNA revealed fragmentation of chromatin into 180- to 200-bp oligonucleosomal bands (Fig. 1A). These morphologic and biochemical features demonstrate that SIN induces apoptosis in PC12 cells.

Expression of inducible dominant inhibitory Ras delays SIN-induced apoptosis in MMTV-M17-21 cells. To investigate the effects of an inducible dominant inhibitory Ras on SIN-induced apoptosis in PC12 cells, we used a previously characterized PC12 cell line, MMTV-M17-21 cells (34). MMTV-M17-21 cells express a dexamethasone-inducible dominant inhibitory mutant of Ras, Ha Ras^{Asn17}, under the control of the MMTV promoter. Ha Ras^{Asn17} does not exchange bound GDP and therefore functions as a dominant negative mutant by competing with endogenous cellular p21 Ras for upstream activators. Expression of Ha Ras^{Asn17} is increased in MMTV-M17-21 cells by treatment with dexamethasone, and under these conditions, MMTV-M17-21 cells display several phenotypic changes, including the absence of neurite outgrowth in response to nerve growth factor (34) and resistance to serum deprivation-induced apoptotic death (7).

We compared cell viability after SIN infection in dexamethasone-induced versus uninduced MMTV-M17-21 cells. To control for potential effects of dexamethasone on SIN-induced death, we also studied cell viability of SIN-infected nontransfected PC12 cells in the presence or absence of dexamethasone. Figure 2 shows that MMTV-M17-21 cells treated with dexamethasone survive longer than cells grown in the absence

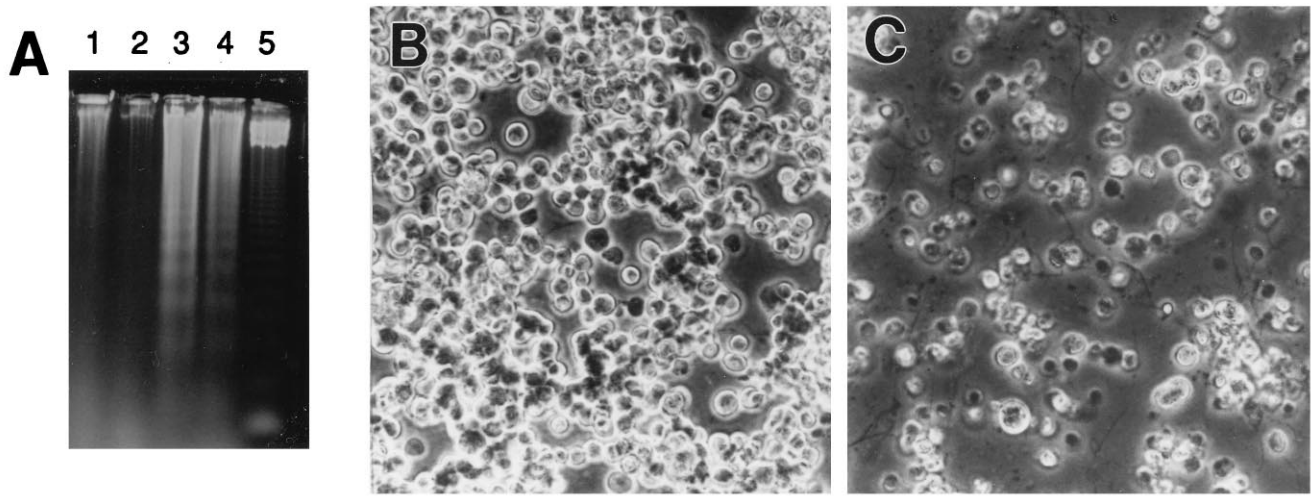

FIG. 1. Induction of apoptosis in SIN/CAT-infected PC12 cells. (A) Nucleosomal laddering detected by in situ extraction-agarose gel electrophoresis 18 h after mock infection (lanes 1 and 2) or infection with SIN/CAT (lanes 3 and 4). Lane 5 shows a 123-bp standard. (B and C) Photomicrographs of PC12 cells 18 h after mock infection (B) or SIN/CAT infection (C).

of dexamethasone. At 24 h after infection, the number of surviving dexamethasone-treated cells was higher than the baseline number, whereas only 25% (compared with baseline) of untreated MMTV-M17-21 cells were alive. These differences are illustrated in representative photomicrographs in Fig. 3. Similarly, at 48 h after SIN infection, 61% of dexamethasone-induced MMTV-M17-21 cells were viable compared with 4% of uninduced cells. In contrast, dexamethasone treatment of PC12 cells had no effect on the time course of cell death after SIN infection, suggesting that the delay in SIN-induced death of dexamethasone-treated MMTV-M17-21 cells was due to the induction of dominant inhibitory Ras expression. The lower rate of cell death in nontransfected PC12 cells versus untreated MMTV-M17-21 cells may be due to the tendency of the nontransfected PC12 cell line to grow in clumps and be more resistant to synchronous virus infection.

Inducible dominant inhibitory Ras expression does not affect SIN replication or infectivity. To address the possibility that inducible dominant inhibitory Ras expression might delay SIN-induced apoptosis by suppressing viral replication, we compared viral titers in the supernatants of SIN-infected MMTV-M17-21 cells grown in the presence and absence of dexamethasone. The mean viral titers in SIN-infected dexamethasone-treated cells were identical to those in untreated MMTV-M17-21 cells at 4, 8, 12, and 24 h after infection (Fig. 4A), suggesting that induction of dominant inhibitory Ras does not significantly affect rates of SIN replication. However, because of differences in rates of cell death in dexamethasone-treated MMTV-M17-21 cells versus untreated cells and antiproliferative effects of Ha Ras^{Asn17} expression (see below), the number of cells in dexamethasone-treated versus untreated wells may not be identical at later time points. Thus, we cannot exclude the possibility that subtle differences may exist in the peak titers of treated versus untreated MMTV-M17-21 cells. However, the observation of identical titers at early time points after infection (when no cell death or cell proliferation has occurred) suggests that an inhibitory effect of Ha Ras^{Asn17} on SIN replication is not likely to be an important factor accounting for the delay in SIN-induced death.

To further confirm that the presence of dexamethasone and/or the expression of dominant inhibitory Ras does not affect SIN replication, we performed infectious center assays to determine the percentage of MMTV-M17-21 cells that were infected at various time points after SIN infection (Fig. 4B). At 6 h after infection, 48% of dexamethasone-treated cells versus 51.2% of untreated cells were infected, and at 12 h after infection, 69% of dexamethasone-treated cells versus 58.7% of untreated cells were infected ($P = 0.276$ and 0.381, respectively; t test). By 24 h, 92% of both dexamethasone-treated and untreated cells were infected. These data indicate that SIN infectivity is comparable in induced versus uninduced MMTV-M17-21 cells and that the delay in SIN-induced death in induced cells cannot be attributed to effects on viral infectivity.

Expression of dominant inhibitory Ras in a SIN vector does not delay SIN-induced apoptosis. To construct a recombinant SIN that expresses dominant inhibitory Ras in virally infected cells, we cloned Ha *ras*^{Asn17} downstream of a duplicated subgenomic promoter in the SIN vector, dsTE12Q, to generate a recombinant chimeric virus called SIN/*ras*^{Asn17}. Previously, this approach has been used successfully to demonstrate an antiapoptotic effect of Bcl-2, $Bclx_L$, p35, and CrmA in SIN-infected cells (2, 14, 24). To evaluate whether expression of Ha

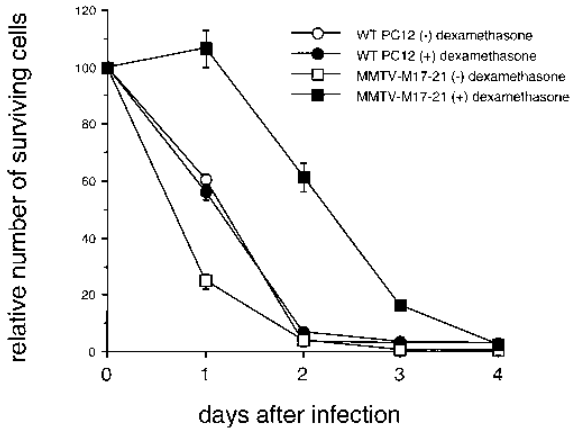

FIG. 2. Cell viability of wild-type PC12 cells and MMTV-M17-21 cells after SIN/CAT infection. Similar results were obtained in more than five independent experiments.

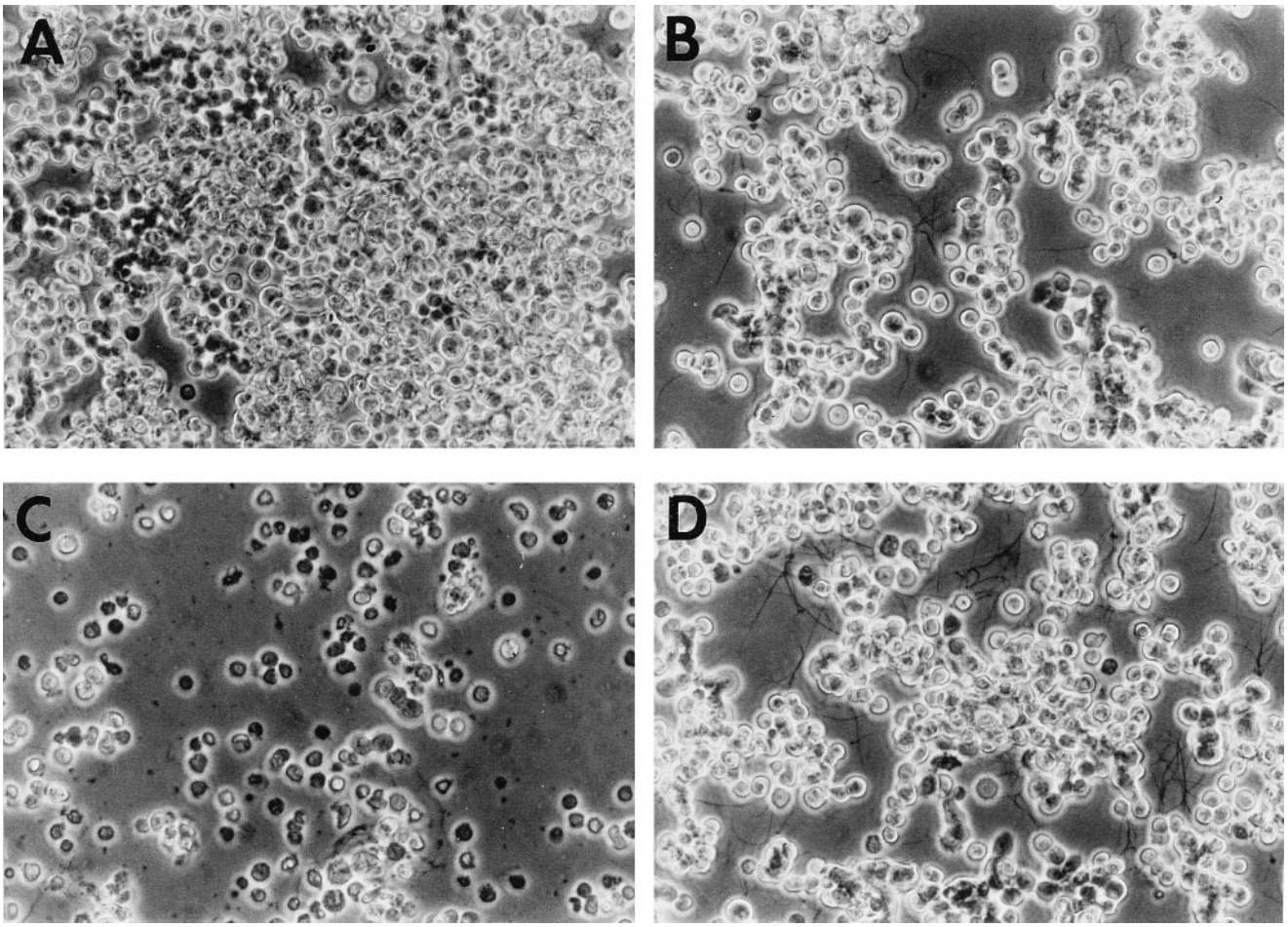

FIG. 3. Photomicrographs of MMTV-M17-21 cells grown in the absence (A and C) or presence (B and D) of dexamethasone 24 h after mock infection (A and B) or infection with SIN/CAT (C and D).

Ras^{Asn17} by SIN delays apoptosis, cell viability of PC12 cells was determined 24 and 48 h after infection with SIN/*ras*^{Asn17} and a control chimeric virus encoding the chloramphenicol acetyltransferase reporter gene, SIN/CAT. No difference was observed in cell viability after infection with SIN/*ras*^{Asn17} compared with SIN/CAT (Fig. 5B). However, Western blot analysis of PC12 cells infected with SIN/*ras*^{Asn17} demonstrated an increase in Ras immunoreactivity of a similar magnitude as the increase in Ras immunoreactivity observed in MMTV-M17-21 cells after dexamethasone treatment (Fig. 5A). Therefore, the

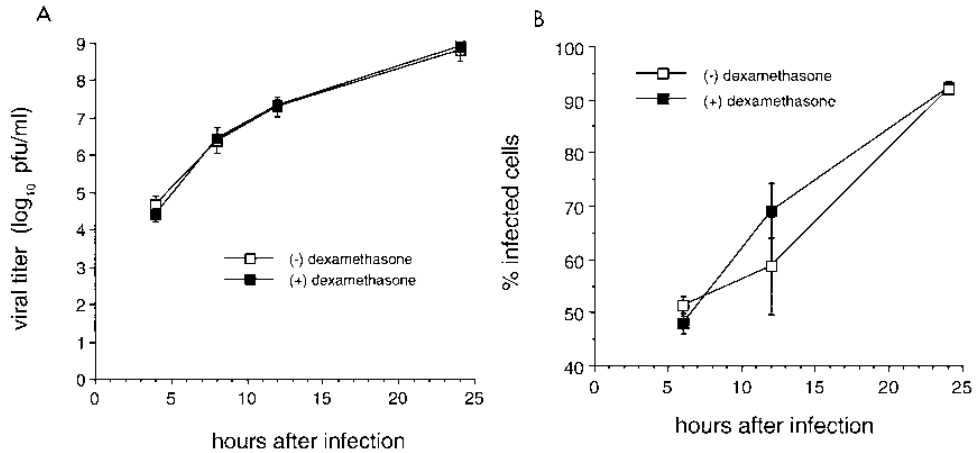

FIG. 4. Effect of dominant inhibitory Ras^{Asn17} expression on SIN replication (A) and SIN infectivity (B) in MMTV-M17-21 cells.

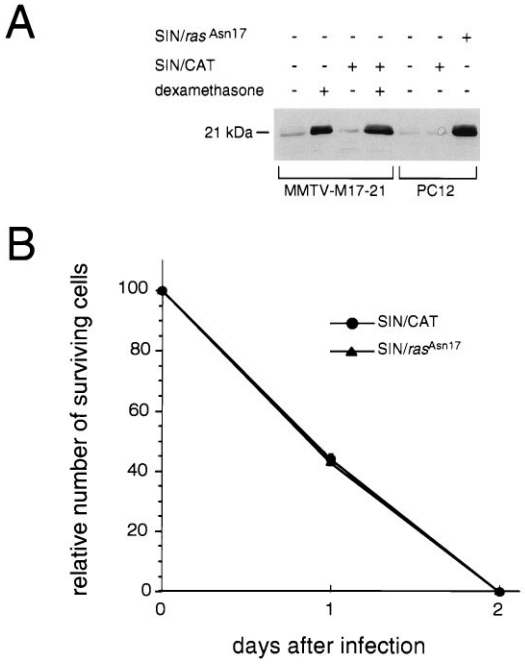

FIG. 5. (A) Western blot analysis of Ras expression in MMTV-M17-21 and PC12 cells infected with SIN/CAT and SIN/*ras*^{Asn17}. (B) Cell viability of PC12 cells infected with SIN/CAT and SIN/*ras*^{Asn17}.

absence of a delay in death in SIN/*ras*^{Asn17}-infected cells could not be attributed to lower levels of Ha Ras^{Asn17} expression than are seen in dexamethasone-treated MMTV-M17-21 cells.

To determine whether the absence of a delay in death in SIN/*ras*^{Asn17}-infected PC12 cells reflects a requirement for inhibition of Ras prior to viral gene expression, we examined the effects of inducing Ras^{Asn17} in MMTV-M17-21 cells at various times prior to and after SIN/CAT infection (Fig. 6). Maximal protection against SIN-induced death was observed when dexamethasone treatment was initiated 24 h prior to infection, and significant protection was still observed when dexamethasone treatment was initiated 12 h prior to infection. However, if dexamethasone treatment was begun 6 h before SIN/CAT infection, at the time of infection, or after infection, no protective effects were observed. These data suggest that dominant inhibitory Ras^{Asn17} needs to be expressed prior to SIN infection in order to delay SIN-induced death.

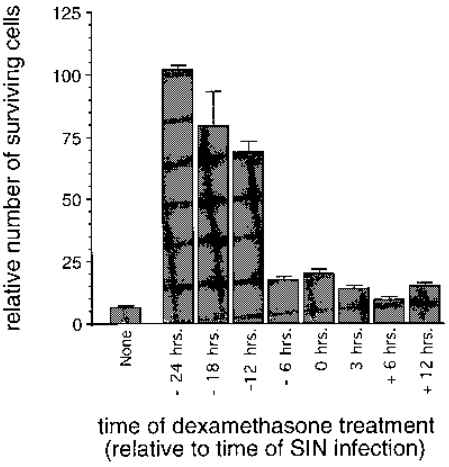

FIG. 6. Effect of timing of dexamethasone treatment on survival of MMTV-M17-21 cells 24 h after infection with SIN/CAT.

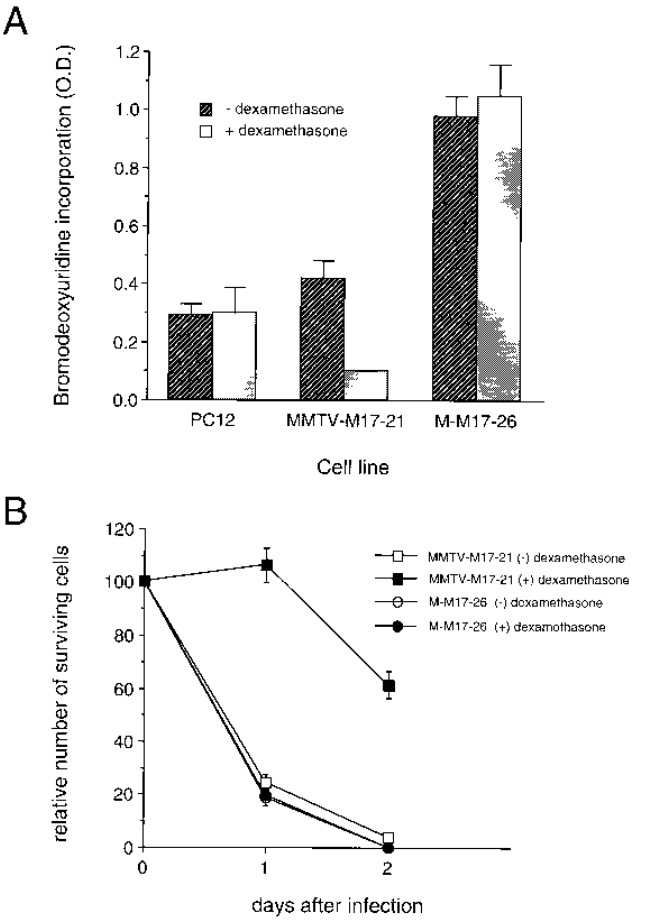

FIG. 7. (A) Cellular DNA synthesis in PC12, MMTV-M17-21, and M-M17-26 cells in the absence and presence of dexamethasone. (B) Cell viability of MMTV-M17-21 and M-M17-26 cells infected with SIN/CAT.

Association between delay in SIN-induced death and proliferative arrest in PC12 cells. Previous studies have suggested that dominant inhibitory Ras protects naive PC12 cells from apoptotic death induced by serum deprivation by causing a proliferative arrest (7). Therefore, we examined whether dominant inhibitory Ras protection of PC12 cells against SIN-induced apoptosis is also associated with proliferative inhibition. First, we confirmed that induction of dominant inhibitory Ras expression in MMTV-M17-21 cells results in proliferative inhibition. We measured DNA synthesis in PC12 cells, MMTV-M17-21 cells, and M-M17-26 cells (a PC12 cell line that constitutively expresses Ha Ras^{Asn17} [34]) in the presence and absence of dexamethasone by detecting BrdU incorporation into cellular DNA (Fig. 7A). Marked inhibition of cellular DNA synthesis was observed in dexamethasone-treated MMTV-M17-21 cells compared with untreated MMTV-M17-21 cells. Dexamethasone treatment had no effect on cellular DNA synthesis in nontransfected PC12 cells or M-M17-26 cells, suggesting that the inhibition of DNA synthesis in dexamethasone-treated MMTV-M17-21 cells was due to the induction of dominant inhibitory Ras.

Next, we investigated whether SIN-induced apoptosis is delayed in the M-M17-26 cells. Previous studies have already

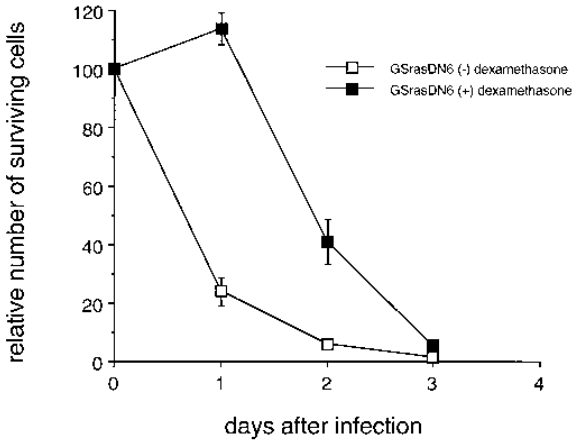

FIG. 8. Cell viability of GSrasDN6 cells after SIN/CAT infection. Similar results were obtained in more than five independent experiments.

established that activation of mitogen-activated protein kinase, a downstream target in Ras-dependent mitogenic signaling, is blocked in M-M17-26 cells (39). However, despite constitutive expression of dominant inhibitory Ras and a block in mitogen-activated protein kinase activity, M-M17-26 cells divide normally (and proliferate more rapidly than nontransfected PC12 cells [Fig. 7A]), suggesting the presence of compensatory cellular changes that permit proliferation despite inhibition of endogenous Ras function. No delay in SIN-induced apoptosis was observed in M-M17-26 cells compared with SIN-infected, untreated MMTV-M17-21 cells or SIN-infected PC12 cells. Thus, dominant inhibitory Ha Ras^{Asn17} expression, without concurrent inhibition of cellular proliferation, is not sufficient to result in a delay in SIN-induced apoptosis.

Expression of inducible dominant inhibitory Ras delays SIN-induced apoptosis in GSrasDN6 cells. The absence of a delay in SIN-induced death in M-M17-26 cells and in PC12 cells infected with SIN/*ras*^{Asn17} raised the possibility that the observed delay in SIN-induced apoptosis in MMTV-M17-21 cells was due to a clonal artifact rather than a true protective effect of Ras^{Asn17}. To rule out this possibility, we investigated the effects of inducible Ras^{Asn17} expression on SIN-induced apoptosis by using a different stably transfected PC12 cell line, GSrasDN6 cells (Fig. 8). At 24 h after infection, the number of surviving dexamethasone-induced GSrasDN6 cells was higher than the baseline number, whereas only 24% of uninduced GSrasDN6 cells were alive compared with baseline. At 48 h after infection, 40% of induced cells were alive versus 6% of uninduced cells. Thus, these results in GSrasDN6 cells are consistent with the results described above in MMTV-M17-21 cells; inducible dominant negative Ras expression delays SIN-induced death in PC12 cells.

DISCUSSION

In the present study, we examined the effects of dominant inhibitory Ras expression on SIN-induced apoptosis of rat pheochromocytoma cells, by using transfected cell lines that express high levels of either inducible or constitutive Ha Ras^{Asn17} as well as wild-type PC12 cells infected with chimeric SIN/*ras*^{Asn17}. Our results demonstrate that induction of dominant inhibitory Ras in a rat pheochromocytoma cell line results in proliferative inhibition and a delay in SIN-induced apoptosis. In contrast, rapidly proliferating PC12 cells that constitutively express high levels of dominant inhibitory Ras are not protected against SIN-induced apoptosis. In addition, expression of dominant inhibitory Ras by a SIN vector does not delay SIN-induced apoptosis. Thus, dominant inhibitory Ras exerts a protective effect only if it is expressed prior to viral gene expression and only if its expression is associated with inhibition of cellular proliferation. Taken together, these observations suggest that a Ras-dependent mitogenic signal transduction pathway is a determinant of neuronal cell susceptibility to SIN-induced apoptosis. However, these data do not rule out a more direct role for Ras in a SIN-induced cell death signaling pathway in PC12 cells.

The association between inhibition of cellular proliferation and delay in SIN-induced apoptosis suggests that Ras^{Asn17} may protect PC12 cells by inducing a proliferative arrest. This interpretation is consistent with accumulating evidence (reviewed in references 3 and 21) in both neuronal and nonneuronal cells that miscoordination between cell cycle components leads to apoptosis and, as a corollary, that manipulations that remove cells from the cycle can protect them against apoptosis (4). In previous studies, Ferrari and Greene postulated that interference with p21 Ras function by overexpression of Ras^{Asn17} protects PC12 cells against apoptosis caused by withdrawal of trophic factor support by preventing them from undergoing a fatal attempt to traverse the cell cycle (7). We have found that SIN-infected cells continue to synthesize new cellular DNA but then undergo death rather than cell division (17). Thus, SIN infection, like withdrawal of trophic factor support, may prevent successful completion of the cell cycle, and Ha Ras^{Asn17} may thereby delay SIN-induced death by inducing a proliferative arrest in PC12 cells.

However, our data do not permit us to distinguish between this interpretation and the alternative interpretation that p21 Ras functions as a required element in a specific signal transduction pathway that leads to SIN-induced apoptosis. Gulbins et al. have shown that Fas-induced apoptosis in Jurkat cells is mediated via a ceramide-initiated Ras signaling pathway that is blocked by Ras^{Asn17} (10). Although a direct relationship between Ras function and a specific cell death signaling pathway has not yet been firmly established in PC12 cells, Ha Ras^{Asn17} has previously been shown to inhibit activation of the JNK signaling cascade in COS (28) and human embryonic kidney (29) cells. In turn, the JNK signaling cascade has been shown to play a central role in apoptosis in PC12 cells deprived of trophic factor support (38). Therefore, it is possible that Ras^{Asn17} blocks activation of downstream effectors of cell death in a JNK signaling pathway that is activated during SIN infection. We are presently evaluating this hypothesis by studying whether activation of downstream effectors in this pathway occurs in SIN infection, whether such activation is blocked by Ha Ras^{Asn17}, and whether dominant inhibitors of downstream molecules also inhibit SIN-induced apoptosis.

Although the lack of protection against SIN-induced death in M-M17-26 cells suggests that dominant inhibitory Ras protects by inducing a proliferative inhibition, it does not rule out a more direct role of Ras in apoptosis. To develop a stably transfected cell line that constitutively expresses Ras^{Asn17}, compensatory changes that permit cellular proliferation despite inhibition of Ras function must occur. If Ras functions as a common upstream element in divergent, yet integrated, signaling pathways that regulate cell death and cell proliferation and differentiation, it is possible that such compensatory changes may also bypass a specific requirement for Ras in a cell death signaling pathway. The ability of Ha Ras^{Asn17} to delay SIN-induced death in MMTV-M17-21 but not in M-M17-26 cells is unlikely to be related to a clonal artifact present in MMTV-M17-21 cells since SIN-induced death is also delayed in an

independently derived PC12 cell line, GSrasDN6 cells (35), that expresses inducible dominant inhibitory Ha Ras^{Asn17}.

The absence of a delay in death in PC12 cells infected with SIN/ras^{Asn17} (compared with SIN/CAT) most likely reflects a requirement for inhibition of Ras prior to viral gene expression. This conclusion is supported by experiments demonstrating that dexamethasone induction of Ha Ras^{Asn17} in transfected PC12 cells must be initiated at least 12 h before infection in order for a protective effect to be observed. The lack of delay in death in PC12 cells infected with SIN/ras^{Asn17} cannot be attributed to lower levels of expression by the SIN vector compared with expression in dexamethasone-induced transfected PC12 cells. Although we demonstrated comparable levels of Ras expression in PC12 cells infected with SIN/ras^{Asn17} and dexamethasone-induced MMTV-M17-21 cells, the rapid death of SIN/ras^{Asn17}-infected PC12 cells makes it difficult to perform experiments to demonstrate that the expressed dominant inhibitory Ras is functional. Thus, we cannot exclude the possibility that Ha Ras^{Asn17} expressed by a SIN vector does not function as a dominant inhibitory mutant, either as a result of altered protein trafficking or posttranslational modifications or because of some other artifact relating to the SIN expression system. As the SIN vector system has already been used successfully to express a large number of functional heterologous proteins (2, 11, 13, 14, 26), this, however, seems like an unlikely possibility.

Our results with SIN/ras^{Asn17} differ from those of other studies in which the SIN vector system has been used successfully to demonstrate a delay in SIN-induced apoptosis by the antiapoptotic genes *bcl-2* and *bclxL* (2, 14). The observed effects of different genes expressed by SIN may be determined, in part, by whether they act as early or late regulators of apoptosis. *bcl-2* and *bclxL* both act as distal inhibitors of apoptosis induced by a wide variety of stimuli, and therefore, expression concurrently with SIN proteins may be sufficient to delay SIN-induced apoptosis. In contrast, if *ras* functions as an early component in either a cellular proliferation or a cellular apoptotic pathway involved in regulating SIN-induced apoptosis, inhibition of this pathway prior to infection may be required to observe a protective effect. Therefore, the basal levels or function of Ras-dependent downstream effectors or the ability of such downstream effectors to be activated by SIN at the time of viral infection may be a critical determinant of the cellular apoptotic response.

The mechanism by which Ras^{Asn17} delays SIN-induced apoptosis in PC12 cells does not appear to relate to difference in rates of viral replication or in viral infectivity. However, we cannot exclude the possibility that Ras^{Asn17} exerts subtle effects on viral replication that were not detected by virus titration and infectious center assays. The lack of apparent antiviral effect of Ras^{Asn17} contrasts with previous reports in which the protective effects of the antiapoptotic gene *bcl-2* are associated with suppression of SIN replication (14, 36). The relationship between levels of viral replication and the ability of SIN to induce apoptosis remains an important area for further investigation.

In summary, our findings support a role for a Ras-dependent signal transduction pathway in determining neuronal susceptibility to SIN-induced apoptosis. Future studies are required to determine whether Ras-dependent cellular proliferation indirectly renders PC12 cells more susceptible to apoptosis or whether a Ras-dependent signaling pathway directly mediates apoptosis induced by SIN. Dual regulation of cellular proliferation and cellular apoptosis by proteins such as Ras may provide insight into mechanisms underlying the propensity of SIN and other viruses to kill actively proliferating cells.

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