

# Influenza Virus Neuraminidase Activates Latent Transforming Growth Factor $\beta$

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**Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a family of proteins secreted by virtually all cells in a biologically inactive form. TGF- $\beta$  levels increase during many pathophysiological situations, including viral infection. The mechanism for increased TGF- $\beta$  activity during viral infection is not understood. We observed an increase in active TGF- $\beta$  levels within 1 day in mice infected with influenza virus. Further studies showed that the neuraminidase glycoprotein of influenza A and B viruses directly activates latent TGF- $\beta$  in vitro. There are sufficient levels of TGF- $\beta$  activated by virus to induce apoptosis in cells. In addition, influenza virus-induced apoptosis is partially inhibited by TGF- $\beta$ -specific antibodies. These novel findings suggest a potential role for activation of TGF- $\beta$  during the host response to influenza virus infection, specifically apoptosis. This is the first report showing direct activation of latent TGF- $\beta$  by a viral protein.**

Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a family of small polypeptide growth and differentiation factors proteins whose diverse functions influence cellular, physiological, and immunological processes (9, 10, 26). In the immune response, TGF- $\beta$  acts as both an immunosuppressive agent and as a potent proinflammatory molecule through its ability to attract and regulate inflammatory molecules, to induce cytokine secretion, and to stimulate the T-helper-cell type 1 phenotype (10, 26). Thus, TGF- $\beta$  regulates events vital to the initiation, progression, and resolution of immune responses.

Unlike most cytokines, TGF- $\beta$  is secreted by virtually all cells as a biologically inactive protein termed latent TGF- $\beta$  (9). Latent TGF- $\beta$  is composed of an amino-terminal latency-associated peptide (LAP) that remains noncovalently associated with the carboxy-terminal mature TGF- $\beta$  molecule (9). The release of mature TGF- $\beta$  from the LAP is thought to be necessary in order to bind to cellular receptors and induce a biological response. Since expression of the known TGF- $\beta$  receptors is constitutive in most cell types (9), regulation of TGF- $\beta$  resides primarily in the activation of the latent TGF- $\beta$  molecule. Very few proteins activate latent TGF- $\beta$ . The described physiologic activators of latent TGF- $\beta$  are the serine protease plasmin and the extracellular matrix protein thrombospondin (17, 20–23). However, there may be other methods of activation not yet described.

Interestingly, numerous viral infections, including DNA tumor viruses and human immunodeficiency virus, increase TGF- $\beta$  at both the mRNA and protein levels (3, 11), suggesting a potential role for TGF- $\beta$  in viral pathogenesis. However, these observations may not be limited to persistent viral infections. Influenza virus infection results in acute inflammation and increased cytokine secretion at areas of viral replication. The levels of numerous cytokines including interleukin-1, interferons, and tumor necrosis factor increase both in vitro (14, 18) and in vivo (1) after infection with influenza virus. In a mouse model, increased cytokine levels are not observed until

3 days postinfection. These cytokines are thought to be involved in the host response and in clinical effects in the initial phase of the disease (14). The mechanism for the virus-induced increase in cytokine levels is unknown at this time, but it is proposed that a secreted transcription factor may be involved. The ability of TGF- $\beta$  to induce secretion of additional cytokines and its localization at sites of inflammation suggest a potential role in influenza virus pathogenesis. However, in order to induce cytokine secretion, biologically inactive TGF- $\beta$  must be converted to the activated form. Therefore, we hypothesized that TGF- $\beta$  activity is increased during influenza virus infection.

In these studies, we observed an increase in serum TGF- $\beta$  activity within 1 day after infecting mice with influenza virus. The kinetics of activation suggested that the virus directly interacts with latent TGF- $\beta$ , resulting in activation. In vitro studies showed that viral neuraminidase (NA) directly activates latent TGF- $\beta$ , possibly through cleavage of the sialic acid residues on the LAP. This observation is not limited to influenza viruses, since a paramyxovirus also activates latent TGF- $\beta$ . Virally increased levels of TGF- $\beta$  induce apoptosis in cells, suggesting a potential role in pathogenesis. These studies are novel in that they show direct activation of latent TGF- $\beta$  by a viral protein. They also suggest an expanded role for NA in influencing the host response.

## MATERIALS AND METHODS

**Virus growth and cell culture.** The following viruses were obtained from the influenza virus repository at the University of Wisconsin-Madison: avian strains A/Turkey/Ontario/7732/66 (Ty/Ont) (H5N9) and A/Duck/Alberta/60/76 (H12N5); equine strains A/Equine/Kentucky/211/87 (H3N8), A/Equine/Santiago/77 (H7N7), and A/Equine/Kentucky/181/87 (H3N8); swine strain A/Swine/Wisconsin/1915/88 (H1N1); and human strains A/PR/8/34 (H1N1), A/Singapore/1/57 (H2N2), and B/David Breeze/44. Additionally, paramyxovirus 1 (Goose/TN/1/76), i.e., Newcastle disease virus, was used. Viruses were propagated in the allantoic cavities of 11-day-old embryonated chicken eggs for 48 to 72 h at 35°C; the allantoic fluid was harvested, centrifuged for clarification, and stored at –70°C.

Madin-Darby canine kidney (MDCK) cells were grown in modified Eagle's medium (MEM; Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Harlan Bioproducts, Indianapolis, Ind.) and 2 mM glutamine. Normal rat kidney (NRK) cells (NRK-49F; cell line CRL-1570) were purchased from the American Type Culture Collection (Rockville, Md.) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g of glucose per liter, 2 mM glutamine, and 10% calf serum (Hyclone, Ogden, Utah).

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**Purified virus.** Ty/Ont was purified by being pelleted through 30% sucrose as previously described (15).

**Conditioned medium assay.** Confluent cultures of MDCK cells in 25-cm<sup>2</sup> flasks were rinsed once with phosphate-buffered saline (PBS) and then either infected with virus (at multiplicities of infection [MOI] of 0.01 to 100) in 1 ml of serum-free MEM or treated with MEM alone for 45 min at 37°C in 5% CO<sub>2</sub>. The cells were then washed with PBS to remove residual virus, 1.5 ml of MEM was added to the cultures, and the mixtures were incubated for various times. The conditioned media were collected and filtered (0.2- $\mu$ m-pore-size filter), and aliquots were tested in the NRK soft agar assay for TGF- $\beta$  activity.

**NRK assay for TGF- $\beta$  activity.** TGF- $\beta$  activity was assessed by determining the colony forming activity of NRK cells, in the presence of epidermal growth factor (EGF) in soft agar as described previously (22). Briefly, 5% Noble agar (Difco, Detroit, Mich.) was diluted 10-fold in 10% calf serum in DMEM, and 0.5 ml of this 0.5% agar dilution was added per well to a 24-well tissue culture plate as a base layer and allowed to solidify. A test sample (0.2 ml) containing EGF (1 ng) was combined with 0.6 ml of 0.5% agar and 0.2 ml ( $2 \times 10^3$  cells) of an NRK suspension in 10% calf serum in DMEM, and 0.5 ml of this 0.3% agar sample solution was added to the cooled base layer. The samples were incubated for 7 days at 37°C in 5% CO<sub>2</sub> and then stained with a 1% solution of neutral red (Sigma Chemical Co., St. Louis, Mo.) in PBS. Colonies greater than 62  $\mu$ m in diameter (>8 to 10 cells) were counted. Experiments were performed in triplicate.

**Activation of latent TGF- $\beta$  by virus in a chemically defined system.** The chemically defined assay was performed as previously described (23). Briefly, various concentrations of virus were incubated with 2 nM purified recombinant latent TGF- $\beta$  (200 ng/ml) in a final volume of 0.5 ml of PBS for 30 min at 37°C. Samples were then assayed for TGF- $\beta$  activity by NRK colony formation in soft agar.

**DNA fragmentation assay.** Fragmentation of cellular DNA into the characteristic apoptotic ladder was assessed as previously described (2). Briefly, approximately  $10^7$  MDCK cells were washed with MEM, infected with virus (MOI of 10) in serum-free MEM or mock infected with MEM alone, and incubated at 37°C in 5% CO<sub>2</sub> for 45 min. After viral attachment, cells were washed with PBS and then incubated for 5 h in 3 ml of MEM at 37°C in 5% CO<sub>2</sub>. DNA was harvested by scraping the cells, centrifuging them to pellet, resuspending them in 600  $\mu$ l of ice-cold lysis buffer (10 mM Tris, 0.5% Triton X-100 [pH 7.5]), and incubating them on ice for 30 min. The lysates were centrifuged for 10 min at 13,000  $\times$  g at 4°C, the supernatants were extracted once with buffered phenol and once with buffered phenol-chloroform, and DNA was ethanol precipitated in the presence of 300 mM NaCl. DNA samples were resuspended in 15  $\mu$ l of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA [pH 7.5]), treated with RNase A (Sigma) to a final concentration of 1  $\mu$ g/ml, and electrophoresed through a 2% SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine). The gel was stained with ethidium bromide to determine fragmentation.

To assess the ability of virus-activated TGF- $\beta$  to induce apoptosis, confluent 25-cm<sup>2</sup> flasks of MDCK cells were rinsed once with PBS and then infected with virus (MOI of 100) in 1 ml of serum-free MEM or mock infected with MEM alone for 45 min at 37°C in 5% CO<sub>2</sub>. The cells were rinsed once with MEM to remove nonadsorbed virus and then incubated for 8 h in serum-free MEM. Conditioned media were collected, filtered (0.2- $\mu$ m-pore-size filter), and then heated at 90°C for 5 min to inactivate the virus. This conditioned media were then added to confluent cultures of MDCK cells in 75-cm<sup>2</sup> flasks and allowed to incubate for 16 h at 37°C in 5% CO<sub>2</sub>. DNA was harvested as described above.

**Western blot (immunoblot) analysis.** Recombinant latent TGF- $\beta$  (3  $\mu$ g) was incubated in PBS alone (30  $\mu$ l), in HCl (0.1 M in PBS), or with influenza virus (190 hemagglutinin [HA] units/30  $\mu$ l) for 30 min at 37°C. Samples were heated for 1 min at 90°C in sample buffer without  $\beta$ -mercaptoethanol and resolved by sodium dodecyl sulfate-12% slab polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose, the proteins were blocked with 1% powdered milk in Tris-buffered saline with 0.1% Tween-20 (TTBS; Sigma) for 30 min at room temperature. The nitrocellulose was probed for the active TGF- $\beta$  homodimer with a rabbit polyclonal antibody that recognizes only active TGF- $\beta$  (R&D Systems, Minneapolis, Minn.) diluted 1:1,000 in TTBS and incubated for 30 min at room temperature. Proteins were detected by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.) following the manufacturer's protocols.

**Infection of mice with influenza virus.** Seven-week-old BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were inoculated with Ty/Ont ( $2.7 \times 10^4$  PFU of virus in 30  $\mu$ l of allantoic fluid) or mock infected (30  $\mu$ l of allantoic fluid) intranasally. The mice were euthanized on days 1, 3, and 6 postinfection and bled for serology, and the lungs were removed for testing.

**Sample preparation.** Serum and lung samples were collected from mice on days 1, 3, and 6 postinfection as follows. Blood was collected from the intraorbital capillary beds and allowed to clot overnight at 4°C. Blood samples were centrifuged at 1,250  $\times$  g for 5 min, and the serum was removed and stored at -20°C. The lungs were harvested from euthanized mice, sealed in stomacher bags containing transport media (PBS containing 50% glycerol, 200,000 U of penicillin G per ml, 400 mg of streptomycin per ml, 1.6 mg of gentamicin per ml, and 10,000 U of nystatin per ml), and stored at -70°C. Prior to use, the lungs were weighed and homogenized in a Stomacher blender (Tekmar Company, Cincinnati, Ohio), and the lung homogenate fluid was removed. Samples were tested

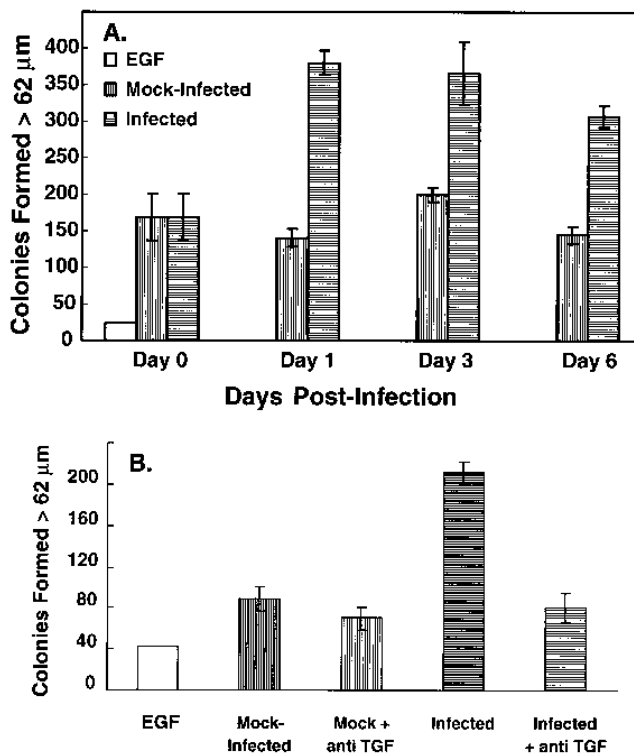


FIG. 1. Infection of mice with influenza virus increases serum TGF- $\beta$  activity. (A) Mice were infected intranasally with 30  $\mu$ l of influenza A virus ( $2.7 \times 10^4$  PFU) or with allantoic fluid from mock-infected eggs. Blood was collected from euthanized mice on days 1, 3, and 6 postinfection, and sera were isolated. Aliquots of sera (100  $\mu$ l) were tested for TGF- $\beta$  activity by the NRK colony-forming soft agar assay. The results for day 0 are those for mice that were not infected and exemplify the baseline of TGF- $\beta$  activity in sera. EGF is the negative control for the soft agar assay. Three mice per condition were tested, and the results are expressed as the means of triplicate determinations; error bars indicate standard deviations. (B) Aliquots of sera (50  $\mu$ l) from mice at 6-days postinfection were incubated in the presence or absence of a neutralizing antibody specific for active TGF- $\beta$  (5  $\mu$ g/ml) and tested for TGF- $\beta$  activity by the NRK colony-forming soft agar assay. Data are expressed as the means of triplicate determinations; error bars indicate standard deviations.

for endogenous TGF- $\beta$  activity by the NRK soft agar assay without further processing.

**Antibodies.** The antibodies specific for influenza virus, including goat polyclonal anti-HA serum and the rabbit polyclonal anti-NA serum, were generous gifts of Robert Webster, St. Jude Children's Research Hospital (Memphis, Tenn.).

**Additional materials.** The purified recombinant latent TGF- $\beta$  was a generous gift of Joanne Murphy-Ullrich, University of Alabama-Birmingham (Birmingham, Al.).

## RESULTS

**TGF- $\beta$  activity increases in mice infected with influenza virus.** We first asked whether TGF- $\beta$  activity increased in vivo in response to influenza virus infection. We infected mice with Ty/Ont (H5N9) virus in allantoic fluid or allantoic fluid alone, collected serum and lung tissue samples, and tested the samples for TGF- $\beta$  activity using the NRK colony-forming soft agar assay, a highly specific and sensitive biological assay for TGF- $\beta$  activity (22). Surprisingly, we observed an increase in both serum and lung TGF- $\beta$  bioactivity in mice, as determined by the NRK soft agar assay (22), after infection with influenza virus (Fig. 1A) (data not shown). TGF- $\beta$  activity remained increased even at day 6 compared with that in mock-infected control mice (Fig. 1A). A neutralizing antibody specific for

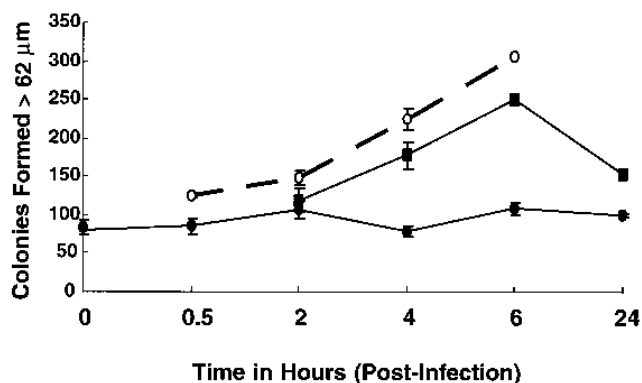


FIG. 2. Influenza virus activates cell-secreted latent TGF- $\beta$ . Confluent cultures of MDCK cells in 25-cm<sup>2</sup> flasks were washed two times with PBS, treated with MEM alone (●) or with MEM plus influenza virus at an MOI of 100 (○) or 0.01 (■), and incubated for 45 min at 37°C in 5% CO<sub>2</sub>. After the 45-min binding period, the cells were washed two times with PBS to remove residual virus, and 1.5 ml of serum-free MEM was added per flask and allowed to incubate from 30 min to 24 h. Conditioned media were collected from the flasks and sterile filtered, and aliquots were tested for TGF- $\beta$  activity by the NRK colony-forming soft agar assay. Data are expressed as the means of triplicate determinations; error bars indicate standard deviations.

TGF- $\beta$  inhibited the increased colony formation observed in the infected serum samples, suggesting that the *in vivo* activity is that of TGF- $\beta$  (Fig. 1b). This is the first report showing an increase in TGF- $\beta$  activity associated with influenza virus infection.

**Influenza virus directly activates latent TGF- $\beta$ .** The increase in TGF- $\beta$  biological activity in response to influenza virus infection was unexpected, since TGF- $\beta$  is secreted by cells only as a biologically inactive molecule, which very few proteins can activate (9). To better understand the mechanism of activation by influenza virus, we examined the activation of latent TGF- $\beta$  by virus in a less complex cell culture system. We infected MDCK epithelial cells with Ty/Ont at an MOI of 0.01 or 100 and tested for TGF- $\beta$  activity at various times postinfection. We used MDCK cells because they support influenza virus replication and secrete latent TGF- $\beta$ . The cellular conditioned media of infected and mock-infected cells were collected and tested for TGF- $\beta$  activity by the NRK colony-forming soft agar assay. Within 30 min postinfection with a high MOI, there was a slight increase in the TGF- $\beta$  activity from the conditioned media of infected cells compared with that of noninfected conditioned medium (Fig. 2). Activity continued to increase through 6 h postinfection, resulting in a threefold induction of activity (Fig. 2). A similar increase in activity was observed when the viral load was decreased, although the levels of activity failed to reach that of the high MOI (Fig. 2). Using a low MOI, we observed a decrease in TGF- $\beta$  activity at 24 h. This is most likely a result of cell death due to infection. To determine the amount of TGF- $\beta$  activated by virus, we compared the number of colonies formed at 6 h postinfection with a recombinant TGF- $\beta$  standard curve in the NRK soft agar assay. These results suggest that influenza virus increases TGF- $\beta$  activity approximately 0.5 ng/ml in MDCK cell conditioned media (data not shown).

The increased TGF- $\beta$  activity induced by influenza virus infection is not due to an egg-associated factor present in allantoic fluid, since allantoic fluid from mock-infected eggs did not increase TGF- $\beta$  activity. Furthermore, we observed no increase in TGF- $\beta$  activity when influenza virus alone was added directly to the NRK assay. These results are not unique to MDCK cells since we determined that influenza virus infec-

tion also activated the latent TGF- $\beta$  secreted by chick embryo fibroblasts, mink lung epithelial cells, and HeLa cells (data not shown). These results suggest that influenza virus infection of cells activates cell-secreted latent TGF- $\beta$ .

The observed increase in TGF- $\beta$  activity in the cell culture system could have resulted from cellular activators, direct activation by the virus, or increased secretion of latent TGF- $\beta$ . To determine the mechanism for activation of latent TGF- $\beta$  by influenza virus, a purified form of recombinant latent TGF- $\beta$  was incubated with sucrose gradient-purified influenza A virus in a chemically defined system (23). The samples were then tested for TGF- $\beta$  activity by the NRK soft agar assay. The relative concentration of purified virus is expressed as HA units. Incubation of purified virus with latent TGF- $\beta$  increased TGF- $\beta$  activity in a dose-dependent manner, resulting in an eightfold increase in activity (Fig. 3A). The direct activation of latent TGF- $\beta$  by virus suggests that activation is independent of increased cell-secreted latent TGF- $\beta$ . Furthermore, incubat-

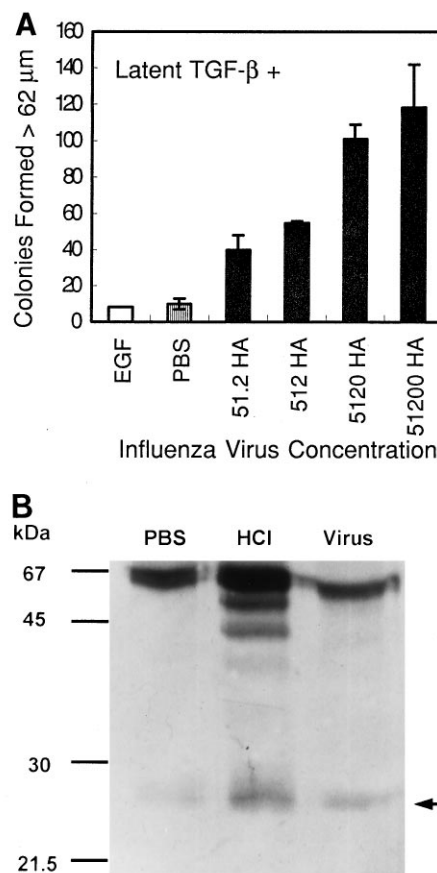


FIG. 3. Purified influenza virus directly activates latent TGF- $\beta$ . (A) Recombinant latent TGF- $\beta$  (200 ng/ml) was incubated with increasing concentrations of purified influenza virus (ranging from 51.2 to 51,200 HA units per 50  $\mu$ l) for 30 min at 37°C in a total volume of 0.5 ml of PBS. Samples were tested for TGF- $\beta$  activity by the NRK soft agar assay. EGF is the negative control for the assay. Data are expressed as the means of triplicate determinations; error bars indicate standard deviations. (B) Recombinant latent TGF- $\beta$  (3  $\mu$ g) was incubated in PBS, in HCl (0.1 M), or with influenza virus (~190 HA units per 30  $\mu$ l) for 30 min at 37°C. Samples were heated for 1 min at 90°C in sample buffer without  $\beta$ -mercaptoethanol and resolved by SDS-PAGE. After being transferred to nitrocellulose, proteins were probed for the active TGF- $\beta$  homodimer with a rabbit polyclonal antibody that recognizes only active TGF- $\beta$ . Bands were detected by enhanced chemiluminescence following the manufacturer's protocols. The arrow indicates the location of active TGF- $\beta$ . Molecular size markers are indicated on the left.

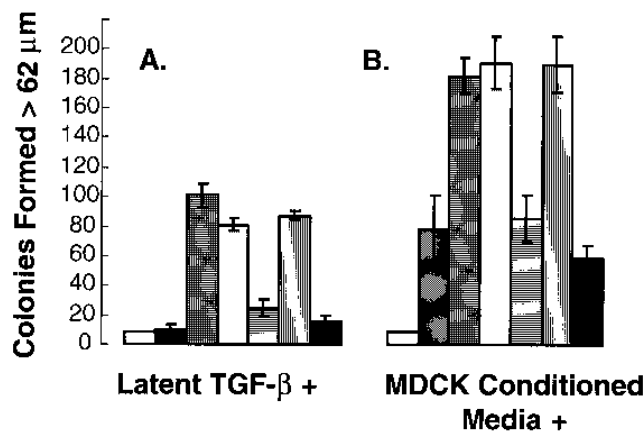


FIG. 4. NA activates latent TGF- $\beta$ . (A) Recombinant latent TGF- $\beta$  (200 ng/ml) (■) was incubated with purified influenza virus alone (~5,120 HA) (□) or with virus plus either goat polyclonal anti-HA serum (25  $\mu$ l) (▨), rabbit polyclonal anti-NA serum (25  $\mu$ l) (▩), normal rabbit serum (25  $\mu$ l) (▧), or rabbit anti-active TGF- $\beta$  antibody (5  $\mu$ g) (■) for 30 min at 37°C in a total volume of 0.5 ml PBS. The samples were tested for TGF- $\beta$  activity by the colony forming soft agar assay. Results are the means of triplicate determinations; error bars indicate standard deviations. (B) Confluent cultures of MDCK cells in 25-cm<sup>2</sup> flasks were washed two times with PBS, treated with MEM alone or MEM with influenza virus (MOI of 100), and incubated for 45 min at 37°C in 5% CO<sub>2</sub>. After the 45-min binding period, the flasks were washed two times with PBS to remove residual virus, and 1.5 ml of serum-free MEM was added per flask without (□) or with goat polyclonal anti-HA (25  $\mu$ l/ml) (▨), rabbit anti-NA polyclonal serum (25  $\mu$ l/ml) (▩), normal rabbit serum (25  $\mu$ l/ml) (▧), or rabbit anti-active TGF- $\beta$  antibody (5  $\mu$ g/ml) (■) and allowed to incubate for 6 h. Uninfected cell conditioned media served as a control (□). Conditioned media were collected, sterile filtered, and tested for TGF- $\beta$  activity by the soft agar assay. Results are the means of triplicate determinations; error bars indicate standard deviations. □, EGF.

ing latent TGF- $\beta$  with either virus or HCl (a known activator) results in the release of the 25-kDa active homodimer from the latent complex as shown by Western blot analysis with an antibody specific for active TGF- $\beta$  (Fig. 3B). The use of Western blot analysis verifies that activation of TGF- $\beta$  by influenza virus is a result of the interaction of virus with latent TGF- $\beta$ . It also shows that activation is independent of previously described cellular factors, suggesting that influenza virus activates latent TGF- $\beta$  via a novel mechanism. Considering these observations, we proposed that a viral protein activates latent TGF- $\beta$ .

**Neuraminidase activates latent TGF- $\beta$ .** Of the two external, membrane-bound glycoproteins on influenza A and B viruses, HA and NA (6), the viral protein most likely to interact with latent TGF- $\beta$  resulting in activation is NA. NA is a functional sialidase, which promotes the release of progeny virus from infected cells by cleaving sialic acid residues at the cell surface (6, 7, 13). Interestingly, Miyazono and Heldin showed that removal of the sialic acid residues on the LAP portion of the latent TGF- $\beta$  complex with purified sialidase results in activation of TGF- $\beta$  (12). We also found that purified sialidase activates MDCK cell-secreted latent TGF- $\beta$  (data not shown). Therefore, we examined the role of influenza virus NA in TGF- $\beta$  activation. We incubated recombinant latent TGF- $\beta$  or MDCK cell-secreted latent TGF- $\beta$  with influenza virus in the presence or absence of polyclonal anti-HA, anti-NA, anti-TGF- $\beta$ , or normal rabbit sera. Samples were assayed for TGF- $\beta$  activity by the NRK soft agar assay. The antibodies alone did not activate latent TGF- $\beta$ . Only the antibodies against NA and TGF- $\beta$  decreased TGF- $\beta$  activity in the NRK assay (Fig. 4). Inhibition occurred in both the cell and the chemically defined

systems, showing that NA interacts with latent TGF- $\beta$  even in a complex cellular milieu. Additionally, the Western blot in Fig. 3b shows a slight shift in the size of the LAP-TGF- $\beta$  complex (band at approximately 65 kDa), which may be suggestive of a cleavage of glycosylation sites. These results demonstrate that the NA present on the viral surface interacts with latent TGF- $\beta$  resulting in activation, possibly through cleavage of the sialic acid residues on latent TGF- $\beta$ .

NA is present on all influenza A and B viruses, although the enzymatic activity differs among the subtypes. We found that several subtypes of influenza A virus (H3N8, H12N5, and H7N7), including human strains (H1N1 and H2N2), and influenza B virus (B/David Breeze) increased TGF- $\beta$  activity in the chemically defined or cell systems (data not shown). These results demonstrate that influenza virus NAs from several different species, including humans, directly activate TGF- $\beta$ .

The ability to activate latent TGF- $\beta$  through NA is not limited to influenza virus; paramyxoviruses also contain NA (5) and activate latent TGF- $\beta$  (data not shown). These studies show that NA activates latent TGF- $\beta$ .

**Virus activated-TGF- $\beta$  and apoptosis.** The above-described data suggests that NA functions as an activator of TGF- $\beta$  in vitro and potentially in vivo. However, it does not elucidate a role for TGF- $\beta$  in influenza virus pathogenesis. An intriguing connection between influenza virus infection and TGF- $\beta$  is the induction of apoptosis by both (2, 8). Our previous studies showed that influenza virus induces apoptosis in MDCK cells (2). TGF- $\beta$  also induces apoptosis in MDCK cells (16). Therefore, to examine the possibility that TGF- $\beta$  is involved in virus-induced apoptosis, we incubated infected MDCK cells in the presence or absence of a TGF- $\beta$ -neutralizing antibody and assayed the cells for DNA fragmentation. The TGF- $\beta$  antibody partially inhibits virus-induced apoptosis (Fig. 5A). In addition, when we collected supernatants from virus-infected MDCK cells, inactivated residual live virus by a short heat treatment, and then tested the supernatants for the ability to induce apoptosis in MDCK cells based on DNA fragmentation (2), we found that sufficient TGF- $\beta$  is activated by the virus infection to induce apoptosis (Fig. 5B). These studies suggest that TGF- $\beta$  activation, potentially by NA, may be important in viral pathogenesis, specifically through apoptosis.

## DISCUSSION

These studies show that the NA glycoprotein of influenza virus directly activates latent TGF- $\beta$  in vitro. Additionally, we observed increased TGF- $\beta$  activity 1 day after infecting mice with influenza virus. Although many mechanisms may be involved in TGF- $\beta$  activation in vivo, our studies suggest that NA may be an additional physiologic activator of TGF- $\beta$ . Viral NA could activate TGF- $\beta$  on entry into the host, during binding to cell-surface receptors, or once the NA is expressed on the surface of infected cells, since latent TGF- $\beta$  is present in the serum and is bound to the surface of cells (9). We propose that activation occurs via the enzymatic cleavage of the sialic acid residues located on the LAP by NA, resulting in the release of the mature TGF- $\beta$  molecule. This is supported by experiments showing that purified sialidase activates latent TGF- $\beta$  (data not shown) (12), the release of the mature TGF- $\beta$  homodimer from the latent complex after incubation with virus, and the inhibition of activation by antibodies against NA.

These studies primarily focus on TGF- $\beta$  activation by NA in vitro. Studies currently in progress will determine whether NA is responsible for the increased activity observed in vivo. However, these studies are technically challenging and may not directly answer the question. The two approaches to address

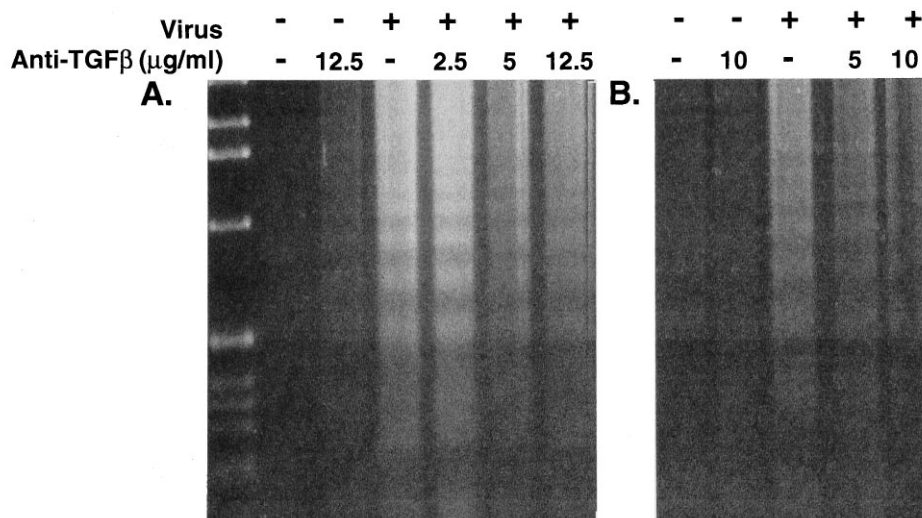


FIG. 5. Role for TGF- $\beta$  in virus-mediated apoptosis. (A) Confluent cultures of MDCK cells in 75-cm<sup>2</sup> flasks were washed two times with PBS, treated with MEM alone or MEM with influenza virus (MOI of 1), and incubated for 45 min at 37°C in 5% CO<sub>2</sub>. After the 45-min binding period, the cells were washed with PBS to remove residual virus and incubated overnight in 3 ml of MEM per flask in the presence or absence of anti-TGF- $\beta$ -neutralizing antibody. We collected DNA and analyzed fragmentation by agarose gel analysis. (B) Confluent cultures of MDCK cells in 25-cm<sup>2</sup> flasks were washed two times with PBS, treated with MEM alone or MEM with influenza virus (MOI of 100) and incubated for 45 min at 37°C in 5% CO<sub>2</sub>. After the 45-min binding period, the cells were washed with PBS to remove residual virus and incubated for 8 h in 3 ml of MEM per flask. Conditioned medium was collected, heat treated for 5 min at 90°C to inactivate the virus, and added to confluent cultures of MDCK cells in 75-cm<sup>2</sup> flasks in the presence or absence of anti-TGF- $\beta$ -neutralizing antibody. The cells were incubated overnight, and then DNA was collected and analyzed by agarose gel for fragmentation.

this question are the use of antibodies specific for NA and the use of influenza viruses that lack NA. However, the proposed role for NA is to facilitate the release of progeny virus from the surface of infected cells by destroying HA receptors and by desialylation of newly synthesized HA and NA (6, 7, 13). Therefore, by interfering with NA's enzymatic activity, we may reduce viral spread, making the interpretation of the results challenging. If a decrease in TGF- $\beta$  activity is observed, it may be a result of reduced spread of the virus. Alternatively, we could immunize the mice with isolated NA and test for increased TGF- $\beta$  activity, although this does not directly address the mechanism during viral infection. Immunization of mice with isolated NA reduces the severity of infection when challenged with the same NA subtype (19, 27). Although these results are attributed only to antibodies against NA, it is possible that NA-activated TGF- $\beta$  also induces a protective immune response. If future studies show that NA activates latent TGF- $\beta$  in vivo, an expanded role for NA during influenza virus pathogenesis would be suggested.

All NA subtypes examined activate TGF- $\beta$ , although the levels of activation correspond with the enzymatic activity of the subtype. High levels of enzymatic activity correlate with a greater increase in TGF- $\beta$  activity. This correlation may be important in host disease. If a virus has strong NA activity, then greater amounts of TGF- $\beta$  may be activated, possibly leading to immunosuppression and organ pathology. This observation may not be limited to influenza viruses, since we showed that a paramyxovirus also activates TGF- $\beta$ , suggesting that TGF- $\beta$  may be important in additional viral infections.

The role for TGF- $\beta$  in vivo is unknown at this time; however, we can propose both potentially beneficial and detrimental roles. First, we propose that TGF- $\beta$  plays a role in the host survival response. On infection of respiratory epithelial cells with influenza viruses, the increased TGF- $\beta$  activity may induce apoptosis in the surrounding cells, thus limiting viral replication. Our studies suggest that virus-activated TGF- $\beta$  is capable of inducing apoptosis in noninfected cells. Future

studies examining the presence of TGF- $\beta$ , influenza virus, and apoptotic cells by double-staining immunofluorescence will address this hypothesis both in vitro and in vivo. Subsequent invasion of the infected site by inflammatory proteins and T- and B-cells may also be induced by the increased levels of TGF- $\beta$ , resulting in viral clearance and resolution.

In contrast, TGF- $\beta$  is immunosuppressive when activated systemically, which may be important in chickens infected with virulent influenza viruses. Virulent avian influenza virus infection in chickens results in severe lymphoid depletion, systemic infection, and death (24, 25). We have previously shown that influenza infection of cultured chicken lymphocytes results in cell death through apoptosis (2). These results are intriguing because the cells do not appear to support productive viral replication. TGF- $\beta$  is found in chickens and appears to have biological activities similar to those seen in mammals (4). It also induces apoptosis in lymphocytes (8). Thus, it is possible that virus-activated TGF- $\beta$  induces apoptosis in chicken lymphocytes, resulting in viral pathogenesis. Recent preliminary studies showed that TGF- $\beta$  activity increases in the sera of infected chickens (unpublished results). Future studies will examine whether TGF- $\beta$  induces apoptosis in chicken lymphocytes in vivo. The TGF- $\beta$  antibody used in our in vitro studies only partially inhibited virus-induced apoptosis and induced DNA fragmentation when used at sufficiently high concentrations. Therefore, future studies will focus on reducing TGF- $\beta$  activity through the use of isoform-specific antibodies and reforming the latent TGF- $\beta$  complex to fully answer the question.

Although we can propose roles for TGF- $\beta$  in influenza virus pathogenesis, the exact role is unclear. To directly examine this, future studies will focus on abrogating increased TGF- $\beta$  activity during influenza virus infection with TGF- $\beta$ -neutralizing antibodies. Alternatively, there are TGF- $\beta$  knockout mice that could be used for infection studies. However, within 2 weeks postweaning the knockout mice died of a massive immune response (3), making interpretation of data difficult.

The study reported here opens a new area to explore, i.e., the biological effects of virus-TGF- $\beta$  interactions. It also opens many new avenues of interest including a greater understanding of the role of NA in viral pathogenesis, the regulation of TGF- $\beta$  by viruses, and the role for TGF- $\beta$  in viral pathogenesis, including apoptosis. The exploration of the role of TGF- $\beta$  in influenza virus infection may lead to a greater understanding of the disease process, potentially leading to novel tools to combat the disease.

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