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Respiratory Syncytial Virus Inhibits Apoptosis and Induces NF-κB Activity through a Phosphatidylinositol 3-Kinase-dependent Pathway*

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Respiratory syncytial virus (RSV) infects airway epithelial cells, resulting in cell death and severe inflammation through the induction of NF-κB activity and inflammatory cytokine synthesis. Both NF-κB activity and apoptosis regulation have been linked to phosphatidylinositol 3-kinase (PI 3-K) and its downstream effector enzymes, AKT and GSK-3. This study evaluates the role of PI 3-K and its downstream mediators in apoptosis and inflammatory gene induction during RSV infection of airway epithelial cells. Whereas RSV infection alone did not produce significant cytotoxicity until 24–48 h following infection, simultaneous RSV infection and exposure to LY294002, a blocker of PI 3-K activity, resulted in cytotoxicity within 12 h. Furthermore, we found that RSV infection during PI 3-K blockade resulted in apoptosis by examining DNA fragmentation, DNA labeling by terminal dUTP nick-end labeling assay, and poly-(ADP-ribose) polymerase cleavage by Western blotting. RSV infection produced an increase in the phosphorylation state of AKT, GSK-3, and the p85 regulatory subunit of PI 3-K. The activation of PI 3-K by RSV and its inhibition by LY294002 was confirmed in direct PI 3-K activity assays. Further evidence for the central role of a pathway involving PI 3-K and AKT in preserving cell viability during RSV infection was established by the observation that constitutively active AKT transfected into A549 cells prevented the cytotoxicity and apoptosis of combined RSV and LY294002 treatment. Finally, both PI 3-K inhibition by LY294002 and AKT inhibition by transfection of a dominant negative enzyme blocked RSV-induced NF-κB transcriptional activity. These data demonstrate that anti-apoptotic signaling and NF-κB activation by RSV are mediated through activation of PI 3-K-dependent pathways. Blockade of PI 3-K activation resulted in rapid, premature apoptosis and inhibition of RSV-stimulated NF-κB-dependent gene transcription.

Respiratory syncytial virus (RSV),[1] a negative-stranded RNA virus of the Paramyxoviridae family, is among the most important respiratory pathogens in children (1). Worldwide, RSV is the most common etiology of bronchiolitis-associated hospitalizations in children less than 2 years old (1–3). RSV is also a significant cause of excess morbidity and mortality in adult patient groups including those with compromised immune status, chronic inflammatory lung disease, and the elderly (4–6). Furthermore, RSV causes long term morbidity and mortality by increasing risk for recurrent wheezing and asthma symptoms throughout childhood (7–11). The mechanisms and signaling pathways activated by RSV that result in airway epithelial cell death and inflammation are not completely understood. In this study, we investigated a novel mechanism through which RSV inhibits apoptotic cell death and regulates the activity of nuclear transcription factor NF-κB.

The earliest pathologic findings in RSV-associated bronchiolitis are airway epithelial necrosis and accumulation of inflammatory cells including neutrophils, lymphocytes, and macrophages (12, 13). Chemokines, including the C-X-C chemokine IL-8, attract neutrophils and lymphocytes to the infected epithelium and contribute to their subsequent activation (14, 15). Our previous work and that of others (16) has demonstrated the direct stimulation of IL-8 synthesis and release from airway epithelial cells by RSV. RSV induces the synthesis and release of IL-8 through activation of multiple transcription factors including NF-κB (16–18). NF-κB is also a critical transcription factor for other inflammatory mediators produced during RSV infection including IL-1α, IL-6, IL-11, RANTES, and intercellular adhesion molecule-1 (19–21). Therefore, the activation of NF-κB is a central determinant of the inflammatory response provoked by RSV infection.

In addition to its role in regulating cytokine synthesis, NF-κB also functions as an important mediator in apoptotic signaling pathways. Inhibition of NF-κB activation has been associated with increased apoptotic cell death initiated by a variety of stimuli including TNF, ionizing radiation, and chemotherapeutic agents (22, 23). NF-κB appears to mediate the suppression of apoptosis through stimulus-specific induction of inhibitor proteins, including IAP (IAP1, IAP2, and XIAP), TRAF, (TRAF1 and TRAF2), Bcl-2 (A1/Bfl-1 and Bcl-x_L), and A20 (24–27). However, the effects of NF-κB on apoptosis are not necessarily anti-apoptotic. In hypoxia-induced epithelial

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[1] The abbreviations used are: RSV, respiratory syncytial virus; PI 3-K, phosphatidylinositol 3-kinase; TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling; PARP, poly(ADP-ribose) polymerase; IL, interleukin; RANTES, regulated on activation normal T cell expressed and secreted; TNF, tumor necrosis factor; MEM, Eagle's minimum essential medium; LDH, lactate dehydrogenase; EMSA, electrophoretic mobility shift assay; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; FITC, fluorescein isothiocyanate; mTOR, mammalian targets of rapamycin; IRF-1, interferon regulatory factor 1; ICE, IL-1β-converting enzyme; PI, phosphatidylinositol; PBS, phosphate-buffered saline.

death (28), reovirus-induced epithelial death (29), and activation-induced T-cell death (30, 31), NF-κB activity promotes apoptosis. Overall, the pro-apoptotic or anti-apoptotic effects of NF-κB appear to be determined by both the type of stimulus and the cell type. The impact of NF-κB up-regulation by RSV with respect to apoptosis has not been categorized in airway epithelial cells.

NF-κB activity is regulated at multiple levels both in the cytoplasm, where it is sequestered in an inactive state, and in the nucleus following translocation. The NF-κB superfamily of transcriptional activators form homodimers or heterodimers composed of subunits of the Rel family (p65/RelA, p50/NF-κB1, p52/NF-κB2, RelB, and c-Rel), which are sequestered in the cytoplasm by the inhibitor proteins IκBα and IκBβ. NF-κB is released and translocates to the nucleus when IκB is phosphorylated by IκB kinase and targeted for degradation (32). Specific NF-κB subunits including p65/RelA may be further regulated by phosphorylation in the transactivation domain (33–35). Finally, NF-κB activity may also be determined by the ability of the individual subunits to associate with basal transcription factors including TFIID/TBP (36, 37).

The lipid kinase, phosphatidylinositol 3-kinase (PI 3-K), has been implicated in the regulation of diverse cellular functions including proliferation, metabolic regulation, and apoptosis (38, 39). In addition to its direct role in apoptosis and metabolic signaling, there is evidence that PI 3-K may play a role in NF-κB regulation. PI 3-K has been shown to mediate NF-κB up-regulation during IL-1 and bradykinin stimulation of cultured epidermoid and airway epithelial cells, respectively (40, 41). Whereas the precise mechanism of NF-κB up-regulation by PI 3-K remains to be identified, the effects may be partially mediated through AKT (protein kinase B). The enzyme activity of AKT, a serine/threonine kinase, is regulated by the phosphoinositide products of PI 3-K both directly by binding of these lipids to its pleckstrin homology domain (42, 43) and indirectly through subsequent phosphorylation by the phosphoinositol lipid-dependent, upstream kinase PDK-1 (44–46). AKT has been found to play a role in cell survival and apoptosis through its downstream effects on the apoptosis-related proteins BAD and caspase 9 (47). Furthermore, AKT has been shown specifically to contribute to NF-κB regulation through association with and activation of IκB kinase during TNF signaling in 293 cells and platelet-derived growth factor signaling in primary fibroblasts (48, 49). Together, these findings are consistent with the observation that that PI 3-K inhibition can prevent the downstream activation of both AKT and NF-κB in pervanadate-stimulated T cells (50). Additionally, AKT may contribute to NF-κB regulation through p65/RelA phosphorylation as observed in HepG2 cells with IL-1 stimulation, an effect that did not appear dependent on IκB kinase activation and IκB degradation (35). Therefore, the PI 3-K/AKT pathway provides multiple potential links between survival or apoptosis signaling and NF-κB regulation.

We have undertaken these studies to define further the mechanisms of inflammatory gene induction and cell death during RSV infection. Because cultured airway epithelial cells display little or no cytotoxic effects early in the course of RSV infection, we hypothesized that RSV activates cell survival and gene transcription pathways that maintain cell viability until mature viral production has been accomplished. In A549-cultured airway epithelial cells, we have found that simultaneous RSV infection and PI 3-K blockade by chemical inhibition with LY294002 results in premature and exaggerated cell death in comparison to RSV alone. Furthermore, the death produced by concurrent RSV infection and LY294002 has features of apoptosis including characteristic DNA fragmentation and poly-(ADP-ribose) polymerase (PARP) cleavage. RSV infection produces an early activation of PI 3-K as measured directly by PI 3-K activity assays and indirectly by phosphorylation of the PI 3-K p85 regulatory subunit. Additionally, the RSV-induced PI 3-K activity correlates with phosphorylation of the downstream effectors, AKT and GSK-3, at regulatory-specific residues. The phosphorylation was blocked by pretreatment with LY294002. The role of the PI 3-K/AKT pathway in preserving cellular viability during infection was further supported by the observation that constitutively active AKT attenuated the cytotoxicity and apoptotic effect of chemical PI 3-K blockade. Finally, PI 3-K inhibition by LY294002 or AKT inhibition by transfection of a dominant negative AKT blocked RSV-induced NF-κB transcriptional activity. These observations suggest that RSV activates the PI 3-K/AKT survival pathway, which promotes cellular survival and contributes to NF-κB activation.

EXPERIMENTAL PROCEDURES

Reagents—PI 3-K p85 monoclonal antibody and LY294002 were purchased from Calbiochem. Serine 473-phospho-AKT and serine 21/9-phospho-GSK-3 antibodies were purchased from Cell Signaling Laboratories, (Beverly, MA). Phosphotyrosine PY20, GSK-3, AKT, PARP, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture—A549 cells, a tumor cell line with properties of normal airway epithelial cells (16, 51), were obtained from American Type Culture Collection (ATCC, Manassas, VA) and incubated at 37° in 5% CO_2. The cells were cultured in Eagle's minimum essential medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 40 mg/ml gentamicin and had been subcultured by harvesting in 0.12% trypsin no more than 20 times from stock originally designated at pass 70. To minimize effects of exogenous growth factors or cytokines in our system, we reduced the supplemented serum concentration to 0.5% 24 h prior to and during all experiments with RSV infection. This serum supplement concentration slowed, but did not stop, cell division and produced no evidence of cytotoxicity for 72 h.

Virus, Plasmids, and Adenoviral Vectors—RSV, strain A2, was obtained from Advanced Biotechnologies Inc. (Columbia, MD) and was used directly as supplied for all experiments. The viral preparation was tested to have a $TCID_{50}$ titer of $\sim 1 \times 10^9$ in Hep-2 cells at 7 days. Sterile vials of the RSV preparation were supplied in MEM supplemented with 10% fetal calf serum, stored at −135 °C and rapidly thawed at 37 °C immediately prior to use. The RSV was diluted to a final concentration of 1:1000 of original stock for all experimental treatments. A replication-defective adenovirus vector encoding a constitutively active murine AKT (Ad.myr-AKT) was generously provided by K. Walsh (Boston). The vector contained epitope tagged enzyme (HA-AKT) with the c-*src* myristoylation sequence fused in sequence to the N terminus of the coding sequence. The construction and activity of the vector has been described previously (52). The vector was amplified without modification in 293 cells, purified by CsCl gradient centrifugation, and stored at −80 °C in 10 mM Tris buffer containing 20% glycerol. Virus particle titers were determined by A_{260} measurement for DNA and were $\sim 10^{13}$ DNA particles/ml. Control adenovirus vector containing β-galactosidase (Ad.LacZ) was purchased commercially (Vector Core Laboratory, University of Iowa, Iowa City). Plasmid vectors containing a hemagglutinin-tagged, murine α-AKT mutant (K179M) inserted into a pCMV6 parental vector were generously provided by A. Toker (Boston, MA). This mutation is a substitution of lysine with methionine at position 179 in the ATP-binding site resulting in complete inhibition of the kinase activity. This mutation has been characterized previously (53, 54).

Cell Viability and Death Assays—Cell death and cytotoxicity were measured by two different methods: lactate dehydrogenase assay to quantify cellular lysis and an ethidium homodimer/calcein combined fluorescent assay to quantify membrane integrity and cell viability. For the lactate dehydrogenase assay cell culture, supernatants were aspirated, and the remaining adherent cells were lysed by addition of 0.1% Triton X-100 directly to each tissue culture well. Following incubation at 4 °C for 30 min, the supernatants and lysates were centrifuged to remove debris. After addition of phosphate buffer (0.1 M, pH 7.40), NADH (0.3 mM), and sodium pyruvate (0.6 mM), absorbance kinetics

were measured at 340 nm. LDH activity in the supernatant was normalized to total LDH measured in supernatant plus lysate for each sample and expressed as percent of total activity, (*i.e.* % LDH activity = LDH activity supernatant/LDH activity supernatant + LDH activity cell lysate).

For the death and viability combined assay a commercially available kit, the LIVE/DEAD® Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR) was used in a 96-well microplate format. A549 cells were seeded in 96-well tissue culture plate at 20,000 cells/well and infected with adenoviral vector constructs as described below. After 36 h, the media were replaced with MEM at 0.5% and the cells cultured overnight. The cells were then exposed to 50 μM LY294002 or solvent control for 1 h followed by infection with RSV. Twelve or twenty four hours post-infection, the A549 cells were stained with 8 μM ethidium homodimer (EthD-1) for 15 min, and the fluorescence of EthD-1 bound to DNA in damaged cells was measured using 540 ± 10 nm excitation filter and 620 ± 10 nm emission filter on a Victor$^{2®}$ (EG&G Wallac, Gaithersburg, MD) microplate reader. The same cells were subsequently stained with 4 μM calcein acetoxymethyl ester (calcein-AM) for 30 min, and the fluorescence of calcein was measured using 485 ± 8 nm excitation filter and 620 ± 10 nm emission filter. Cell death and cell viability were expressed as relative fluorescence intensity of EthD-1 and calcein, respectively, after subtraction of background fluorescence from wells containing the fluorescent dyes in culture media and no cells.

TUNEL Analysis and Flow Cytometry—Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) analysis for DNA fragmentation was carried out using an Apo-Direct™ kit obtained from PharMingen (San Diego, CA). Briefly, A549 cells were grown to 80% confluence in 100-mm tissue culture dishes, incubated 24 h in MEM supplemented with 0.5% fetal calf serum, and exposed to LY294002 (50 μM) or control solvent Me_2SO (1 μl/ml) for 1 h. RSV was added and the cells incubated for an additional 6 h. Cells were washed once with PBS and harvested by trypsinization. Cells in the washes and supernatants were pelleted and combined with the adherent fractions. The cells were fixed in 1% paraformaldehyde for 15 min and stored in 70% ethanol at −20 °C until staining and analysis. Cells were labeled with FITC-conjugated deoxyuridine triphosphate nucleotides and propidium iodide according to manufacturer's instructions and analyzed by flow cytometry (FACScan™, Becton Dickson, San Jose, CA) using CELLQuest software, (Becton Dickson).

DNA Fragmentation Analysis—Cellular DNA was isolated using Easy-DNA™ obtained from Invitrogen (Carlsbad, CA). Briefly, A549 cells in 100-mm tissue culture dishes were exposed to LY294002 and RSV as above, harvested by scraping, and washed once with PBS. Following cell lysis, DNA was extracted by phenol/chloroform partition and precipitated in ethanol. The DNA was then resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and purified by RNase treatment (10 μg/ml) at 37 °C for 30 min. DNA fragmentation was assessed by gel electrophoresis in 1.7% agarose containing 3.0 μg/ml ethidium bromide.

Western Analysis—Following experimental exposure, the cells were washed in sterile PBS and harvested by scraping into lysis buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5 M phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate, all from Roche Molecular Biochemicals). Detached cells in supernatants and wash were pelleted at 300 × *g* for 3 min and combined with the scraped lysate. Complete cell disruption was carried out by sonication for 15 s, and insoluble debris was pelleted by centrifugation at 15,000 × *g* for 10 min. The protein concentration in the lysate supernatant was measured by Bradford assay normalized to bovine serum albumin. Equal protein weights were mixed 1:1 with sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, and 1.25 M Tris, pH 6.8), separated by SDS-PAGE, and transferred to a nitrocellulose membrane by electroblotting. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus, Amersham Biosciences). Following development of phospho-AKT and phospho-GSK-3 bands, bound immunoglobulins were removed from the membranes by washing for 1 h at room temperature in ImmunoPure IgG Elution Buffer (Pierce), and the membranes were reprobed for total AKT and GSK-3.

PI 3-K Immunoprecipitation—Adherent and detached cells were harvested following the same procedures for the Western blot protocol. 200 μg of total cell protein was cleared by incubating for 2 h with 1 μg/sample of rabbit IgG and 10 μl/sample of GammaBind-Sepharose. After centrifuging, the supernatants were transferred to a tube containing 2.5 μg/sample of anti-p85 antibody bound to GammaBind-Sepharose and rotated at 4° overnight. For study of the PI 3-K phosphorylation status, the immunoprecipitated protein was washed three times with high salt buffer (0.5 M Tris, pH 7.4, 0.50 M NaCl, and 1% Nonidet P-40) and twice with lysis buffer (see Western analysis). The protein was then released with 2× Western sample buffer (above), incubated at 95 °C for 5 min, separated by SDS-PAGE, and immunoblotted for phosphotyrosine.

PI 3-Kinase Activity Assay—Activity was assayed by measuring the formation of PI 3-[^{32}P]phosphate by immunoprecipitated PI 3-kinase preparations. Immunoprecipitation using an antibody to the p85 regulatory subunit of PI 3-kinase was performed as above. Following isolation, the bound protein was washed three times with buffer I (phosphate-buffered saline containing 1% Nonidet P-40 and 100 μM Na_3VO_4), three times with buffer II (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, and 100 μM Na_3VO_4), and finally three times with buffer III (Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4). After pelleting, the immunoprecipitates were resuspended in 50 μl of buffer III with the addition of 10 μl of 100 mM $MgCl_2$ and 10 μl of PI (2 μg/ml). The PI was prepared by drying down an aliquot of chloroform-diluted stock under nitrogen, adding Tris, pH 7.5, with 1 mM EGTA to the tube, and sonicating on ice until the lipid was dispersed in suspension. It was then added immediately to the assay tube. The samples were equilibrated at room temperature for 5 min prior to the addition of 10 μl of radiolabeled substrate ATP (440 μM ATP with 30 μCi/10 μl of [γ-^{32}P]ATP). Following gentle agitation for 10 min, the reaction was terminated by the addition of 20 μl of 8 N HCl and 160 μl of chloroform:methanol (1:1). The radiolabeled lipids were extracted, concentrated, and separated by thin layer chromatography using silica gel plates (pretreated with 10% w/v potassium oxalate) in a solvent system of chloroform:methanol:water:NH_4OH (60:47:11:2.2, v/v). Incorporation of ^{32}P into PI was detected by autoradiography, and activity was quantified on a Bio-Rad Molecular Imager FX using Bio-Rad software.

Nuclear Protein Preparation—Following experimental treatments, cells were washed in PBS, scraped in lysis buffer (10 mM Hepes, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5 M dithiothreitol, 10 mg/ml phenylmethylsulfonyl fluoride, 100 mM TLCK, 5 mg/ml leupeptin, and 2 mg/ml pepstatin), and incubated on ice for 15 min. Cells were lysed by addition of 10% Nonidet P-40 and centrifuged at 4 °C and 14,000 rpm. The nuclear pellet was resuspended in buffer (50 mM Hepes, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% v/v glycerol, 0.5 M dithiothreitol, 10 mg/ml phenylmethylsulfonyl fluoride, 100 mM TLCK, 5 mg/ml leupeptin, and 2 mg/ml pepstatin), incubated on ice for 20 min, and centrifuged at 14,000 rpm for 5 min. Supernatants containing nuclear proteins were stored at −70 °C until analysis by EMSA for NF-κB translocation or Western blotting for PARP protein.

Electrophoretic Mobility Shift Assay—A549 cells were exposed to treatment conditions and harvested, and nuclear protein was extracted as described above. A consensus NF-κB oligonucleotide (5′-AGTTGAGGGGATTTTCCCAGGC-3′, Promega, Madison, WI) was labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) (55). Binding reactions using 7.5 μg of nuclear protein were carried out as described previously (55). Protein-DNA complexes were separated by electrophoresis on 5% polyacrylamide gels followed by autoradiography for visualization.

NF-κB-dependent Reporter Assay—NF-κB-dependent gene expression was measured using a luciferase reporter gene driven by NF-κB as described previously (37). Briefly, A549 cells were transiently transfected with a pUC vector containing four tandem copies of the κ enhancer (κB_4) (CLONTECH, Palo Alto, CA). In some experiments, cells were also transfected with either a control LacZ plasmid or a kinase-dead AKT plasmid. Transfection of 1 μg of DNA was carried out using SuperFect transfection reagent (Qiagen, Valencia, CA) or LipofectAMINE (Invitrogen), according to manufacturers' instructions. Twenty four hours after transfection, the cells were treated for 1 h with solvent control Me_2SO (1 μl/ml) or LY294002 (50 μM) followed by RSV (or by RSV alone in the kinase-dead AKT experiments). Cells were harvested 6 h later, and luciferase activity normalized to total cell protein was measured as described previously (37).

Statistical Analysis—One-way analysis of variance with multiple comparisons and paired *t* tests were performed for all statistical parameter calculations.

RESULTS

LY294002 Exposure Prior to RSV Infection Produces Premature Cell Death—RSV protein synthesis can be detected as early as 2 h post-inoculation in CV-1 cells (56). Significantly increased viral mRNA, proteins, and mature virus particles appeared 16–24 h after infection in A549 cells (57, 58). Despite this previously reported early consumption of cellular synthetic machinery and metabolic demand, we were unable to observe

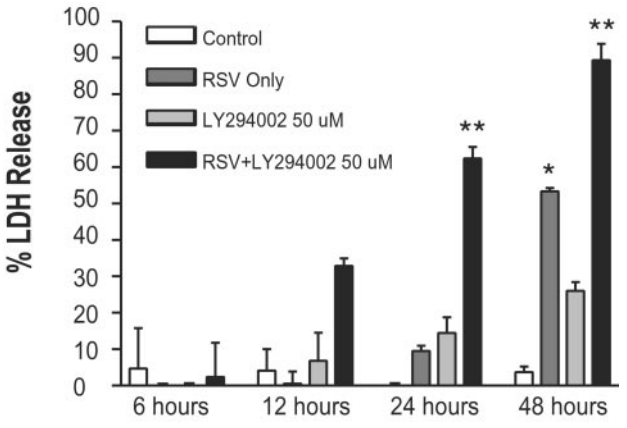

FIG. 1. **PI 3-K blockade results in an early increase in cytotoxicity associated with RSV infection.** A549 cells were pretreated for 1 h with control solvent Me_2SO (1 μl/ml) or LY294002 (50 μM) followed by RSV infection. Cells and culture supernatants were harvested at the indicated time points, and percent LDH activity was calculated in the supernatants according to methods outlined under "Experimental Procedures." Data are mean ± S.E. of three separate experiments. *, $p < 0.005$, RSV only 48 h *versus* 24 h. **, $p < 0.005$ RSV + LY294002 *versus* RSV only at 24 and 48 h.

morphologic changes consistent with cytotoxicity and cell death (plaque formation, cell fragmentation, and cell lift-off) until 24–48 h following infection in A549 cells (data not shown). Lactate dehydrogenase assays performed over the course of RSV infection confirmed the lack of significant cytotoxicity or loss of membrane integrity until late time points following infection (%LDH release 53.3% at 48 h *versus* 9.5% at 24 h, $p < 0.005$, Fig. 1). On the basis of these observations, we have hypothesized that RSV activates intracellular signaling pathways early in the course of infection to promote cell survival until completion of significant viral protein synthesis or production of mature viral particles.

PI 3-kinase is an upstream effector in a well defined pathway of cell survival that includes AKT and GSK-3 (47, 59). To evaluate the role of PI 3-kinase in promoting cell survival during RSV infection, we simultaneously infected cells and blocked PI 3-K activity with LY294002, a relatively specific inhibitor of PI 3-K (60). Following 1 h of pretreatment with LY294002, RSV infection produced cellular morphologic criteria of death including cell lift-off and fragmentation as early as 8–12 h following inoculation (data not shown). Pretreatment of A549 cells with LY294002 prior to infection increased LDH release compared with RSV infection alone within 12 h of infection, (RSV + LY294002 − 32.9% *versus* RSV alone − 0.5%, $p = 0.08$, Fig. 1). This trend became statistically significant at 24 h (62.4% *versus* 9.5%, $p < 0.005$). These data suggest that chemical blockade of PI 3-K activity during RSV infection results in premature cell death.

LY294002 Treatment Prior to RSV Infection Leads to Apoptotic Cell Death—DNA fragmentation analysis by agarose gel electrophoresis was carried out to determine whether RSV produced characteristics of apoptotic cell death in the presence of LY294002. Pretreatment of cells with LY294002 for 1 h produced a dose-dependent DNA laddering pattern (indicative of internucleosomal DNA cleavage) within 6 h of infection (Fig. 2*A*). PARP is a nuclear-associated enzyme, which is targeted for proteolytic cleavage by cysteine proteases during apoptosis. PARP is cleaved from an approximate 112-kDa whole protein into 85- and 24-kDa fragments by activated caspase-3 and -7 enzymes. Thus, detection of the cleaved fragments serves as a marker of activation of apoptotic biochemical pathways. By Western blot analysis of harvested total nuclear protein, we were unable to demonstrate that RSV infection alone resulted in PARP cleavage at 6 or 24 h post-inoculation (data not shown). In agreement with the DNA fragmentation analyses, however, pretreatment of A549 cells by LY294002 resulted in the appearance of cleaved PARP within 6 h of infection (Fig. 2*B*).

To confirm these results, we performed TUNEL staining and flow cytometric analysis of A549 cell controls, RSV-infected cells, LY294002-treated cells, and simultaneous RSV- and LY294002-treated cells. In these experiments, we detected deoxyuridine triphosphate incorporation into DNA strand breaks consistent with apoptosis after 6 h of infection (Fig. 2*C*). Data from three separate experiments demonstrated no statistically significant difference in the mean percent of apoptotic cells between the control group (0.37%), RSV alone (0.08%), or LY294002 groups (2.93%). However, there was a significant increase in mean percent of apoptotic cells between the RSV alone and the RSV-infected plus LY294002 group (19.86 *versus* 0.08%, $p < 0.005$). Together, these results suggest that PI 3-K or a phosphatidylinositol kinase-related enzyme is a necessary transducer of survival signaling during early RSV infection.

RSV Activates PI 3-K and AKT Inhibits GSK-3—Kinase assays and immunoblot assays for the phosphorylated isoforms of PI 3-K, AKT, and GSK-3 were performed to evaluate the role of RSV in enzyme activation or inhibition. PI 3-K is activated following tyrosine phosphorylation of its p85 subunit (61). AKT, the downstream target of PI 3-K, is activated subsequent to phosphorylation of its Ser-473 and Thr-308 residues. GSK-3 is constitutively active in cells and becomes inactivated by phosphorylation of residues Ser-21 and Ser-9 by AKT. Fig. 3*A* shows activation of PI 3-K by RSV at 30 min post-infection by kinase activity assay using PI as a substrate. This activation was blocked by pretreatment of the cells with LY294002. PI 3-K activity correlated in a time-dependent manner with tyrosine phosphorylation of the p85 regulatory subunit of PI 3-K (Fig. 3*B*). Fig. 4, *A* and *B*, shows concurrent increases in AKT and GSK-3 phosphorylation following infection. RSV infection did not alter total cellular levels of p85, AKT, or GSK-3 protein as assayed by immunoblotting for total enzyme protein. LY294002 pretreatment abolished phosphorylation of both AKT and GSK-3 by RSV (Fig. 4*C*). These results demonstrate that RSV activates PI 3-K and its downstream kinase, AKT, in a time-dependent manner. Furthermore, RSV infection produces a time-dependent increase in phosphorylation and thus inhibition of GSK-3.

Constitutively Active AKT Blocks the Pro-apoptotic Effects of LY294002—The appearance of apoptosis after RSV infection with LY294002 correlated with absence of specific activity and regulatory phosphorylation of PI 3-K, AKT, and GSK-3. Fig. 5 demonstrates the preventive effect of constitutive AKT activity on the cytotoxicity observed during concurrent RSV infection and LY294002 treatment. Fig. 5*A* shows increased amounts of phosphorylated AKT in A549 cells, after infection with Ad.myrAKT (52). There was no increase in phosphorylated AKT in the cells infected with the control, Ad.LacZ. Fig. 5*B* contains representative photomicrographs of cells infected with control or constitutively active AKT adenovirus vectors and subsequently exposed to LY294002 and RSV for 6 h. The fluorescent staining was done at 6 h after infection to evaluate an early time point. The visible decrease in viable cells stained with green fluorescent calcein and increase in dead cells stained with red fluorescent ethidium homodimer was reduced by constitutively active AKT. In Fig. 5*C*, we used automated fluorescence measurements to evaluate the same conditions at 24 h. These results demonstrate that as far out as 24 h the constitutively active AKT still prevented cell death induced by RSV and LY294002. Furthermore, by DNA fragmentation

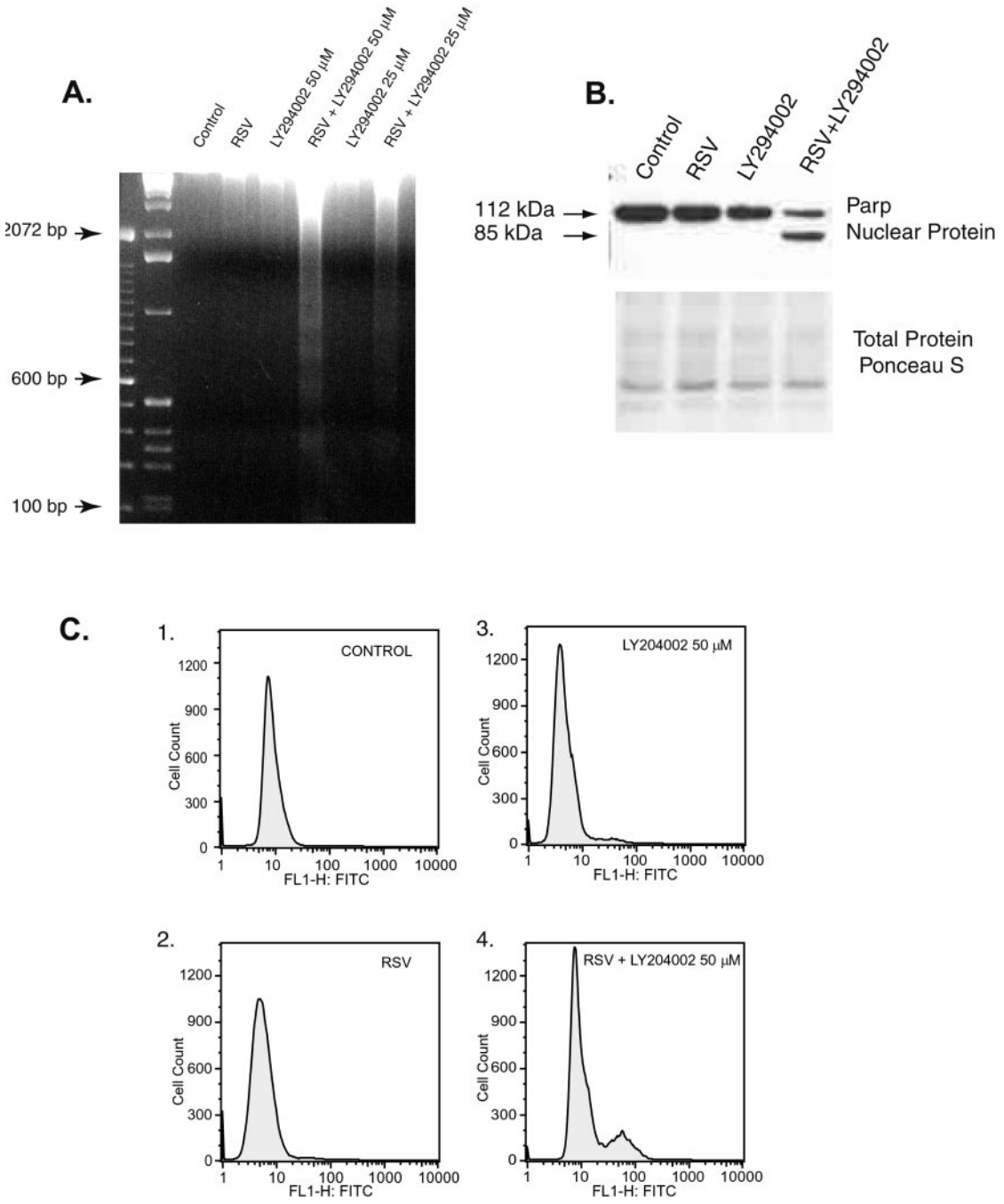

FIG. 2. **PI 3-K blockade during RSV infection produces cell death with characteristic apoptotic features.** 2×10^6 A549 cells per sample were pretreated with LY294002 followed by RSV infection for 6 h. Cells were harvested and analyzed for markers of apoptosis. *A*, photograph of agarose gel with separated DNA stained with ethidium bromide and visualized by UV transillumination. DNA was extracted as described under "Experimental Procedures," and molecular size markers were run in the *left lanes.* This gel is representative of four separate experiments. *B*, PARP Western blot for proteolysis detection. Nuclear protein was isolated and separated by SDS-PAGE. Immunoreactive bands for PARP protein and cleavage fragments were developed using a chemiluminescent substrate as described under "Experimental Procedures." Total protein on the membrane was stained with Ponceau S solution and photographed to demonstrate equal protein loading. The blot is representative of three separate experiments. *C*, flow cytometric analysis of TUNEL-labeled cells to detect apoptosis. Following experimental treatments, harvest, and FITC-conjugated dUTP DNA labeling, cells were sorted and counted by flow cytometry as described under "Experimental Procedures." Apoptotic cells demonstrate an increase in FITC-dUTP DNA labeling and are represented by the appearance of a distinct peak of increased FITC labeling intensity seen in *C*, *panel 4*, RSV plus LY294002. These data are representative of three separate experiments.

analysis and PARP cleavage analysis, constitutively active AKT blocked the induction of apoptosis by combined RSV infection and LY294002 (Fig. 5, *D* and *E*). Together, these data suggest that RSV infection leads to activation of a PI 3-K-AKT cell survival pathway and that blockade of this pathway coincides with cell death through apoptosis.

PI 3-K Activation Contributes to NF-κB Activity during RSV Infection—We and others (16, 62) have shown previously that RSV infection of A549 cells induces nuclear translocation of NF-κB and up-regulation of NF-κB-dependent protein synthesis. The initial steps in activation of NF-κB by RSV involve the phosphorylation and degradation of the regulatory proteins IκBα and IκBβ (63, 64). This allows dissociation of the NF-κB subunits and their subsequent nuclear translocation. However,

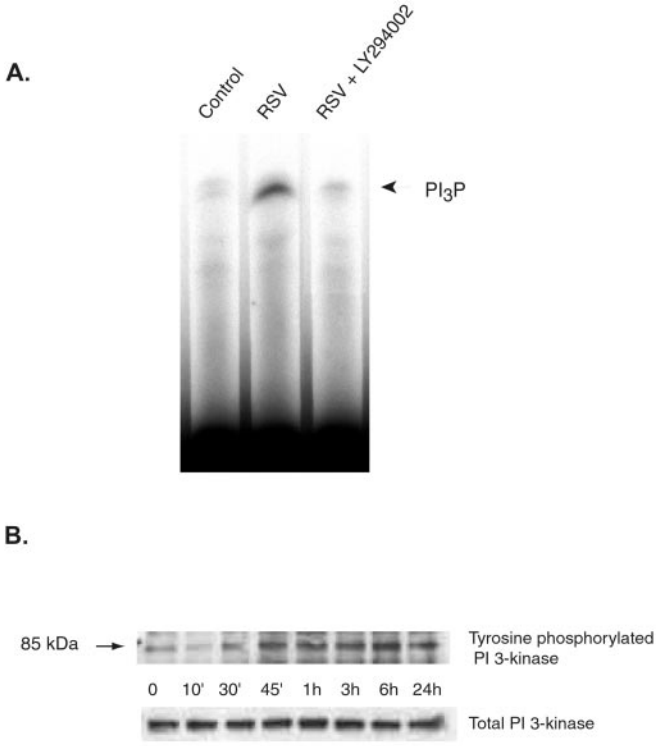

FIG. 3. **RSV infection results in an increase in PI 3-K activity and phosphorylation of the p85 regulatory subunit.** A549 cells were infected with RSV in the presence or absence of LY294002 and harvested at the indicated time points. *A*, autoradiogram demonstrating kinase activity using phosphatidylinositol as substrate for γ-^{32}P incorporation by immunoprecipitated PI 3-K. The samples were preincubated with LY294002 for 30 min and then infected with RSV for 30 min. *B*, Western blot of phosphorylated p85 subunit. The p85 subunit of PI 3-K was immunoprecipitated from cell protein isolates and separated by SDS-PAGE. The blot was probed for phosphotyrosine. The same total cell lysates were also separated by SDS-PAGE and probed for p85 to demonstrate unchanged total p85 protein levels.

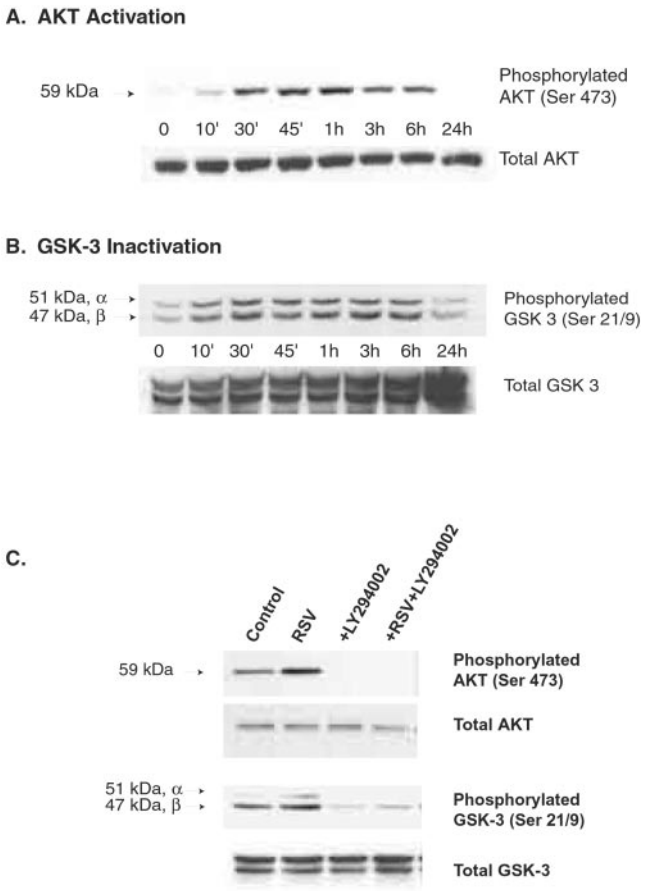

FIG. 4. **RSV infection causes increased phosphorylation of AKT and GSK-3 at regulatory sites.** Total cell protein was isolated at the indicated time points and separated by SDS-PAGE. *A* and *B*, immunoblotting was performed with antibodies specific for serine 473-phosphorylated AKT and serine 21/9-phosphorylated GSK-3. Following development of immunoreactive bands, IgG was eluted from the membranes as described under "Experimental Procedures," and the same blots were re-probed for total AKT and GSK-3. These blots are representative of four separate experiments. *C*, cells were preincubated with control Me_2SO solvent (1 μl/ml) or LY294002 (50 μM), infected with RSV, and harvested 1 h later. Total cell protein was separated by SDS-PAGE. Immunoblotting was performed for phosphorylated and activated AKT and phosphorylated and inhibited GSK-3. Following development with chemiluminescent substrate, IgG was eluted from the blots as described under "Experimental Procedures," and the blots re-probed with antibodies for total AKT and GSK-3.

additional factors including protein phosphorylation can also modulate NF-κB activity. To determine the role of PI 3-K activity in NF-κB up-regulation by RSV, we have examined the effect of LY294002 on the nuclear translocation and transcriptional activity of NF-κB. LY294002 appeared to have little effect on NF-κB nuclear translocation by RSV (Fig. 6*A*). However, by using an NF-κB-driven luciferase gene reporter assay, we were able to demonstrate that LY294002 significantly decreased NF-κB-dependent gene transcription during RSV infection (Fig. 6*B*). The role of AKT as a downstream mediator of PI 3-K activity during RSV infection was examined through utilization of a previously characterized, kinase-dead AKT isoform (K179M) (53, 54). The inhibitory effect of transfecting this plasmid-based mutant on RSV-stimulated NF-κB activity is shown in Fig. 6*C*. These data suggest that although RSV-stimulated NF-κB nuclear translocation functions independently from PI 3-K, its transcriptional activity depends on PI 3-K or its downstream effector AKT.

DISCUSSION

Our observation that RSV does not cause significantly increased cell cytotoxicity until 48 h following inoculation suggested that the virus promotes cellular survival during the early course of infection. Our initial experiments showed that LY294002 pretreatment caused a significant and premature increase in the cytotoxicity associated with RSV. By DNA fragmentation analysis, TUNEL analysis, and PARP cleavage analysis, we further demonstrated apoptosis during RSV infection in the setting of LY294002 treatment. Infection of the cultured cells with an adenoviral vector containing a constitutively active AKT enzyme prevented the cytotoxicity and apoptosis observed under these conditions. By direct kinase activity assays, we have shown that RSV activates PI 3-K within 30 min of infection, an effect that parallels the changes in the phosphorylation status of the PI 3-K p85 regulatory subunits, AKT and GSK-3. From these experiments we have concluded that RSV activates both pro- and anti-apoptotic pathways in A549 cells. The anti-apoptotic effects of RSV during the first hours of infection appear to be mediated through PI 3-K or possibly the downstream mediators, AKT and GSK-3. Under our experimental conditions, the inhibition of the PI 3-K pathway resulted in unopposed pro-apoptotic effects and rapid host cell apoptosis.

Previous studies have demonstrated induction of IL-1β-converting enzyme (ICE) and the transcriptional activator, interferon regulatory factor 1 (IRF-1), within 7 h of infection by RSV in A549 cells (65). Although both ICE and IRF-1 have been

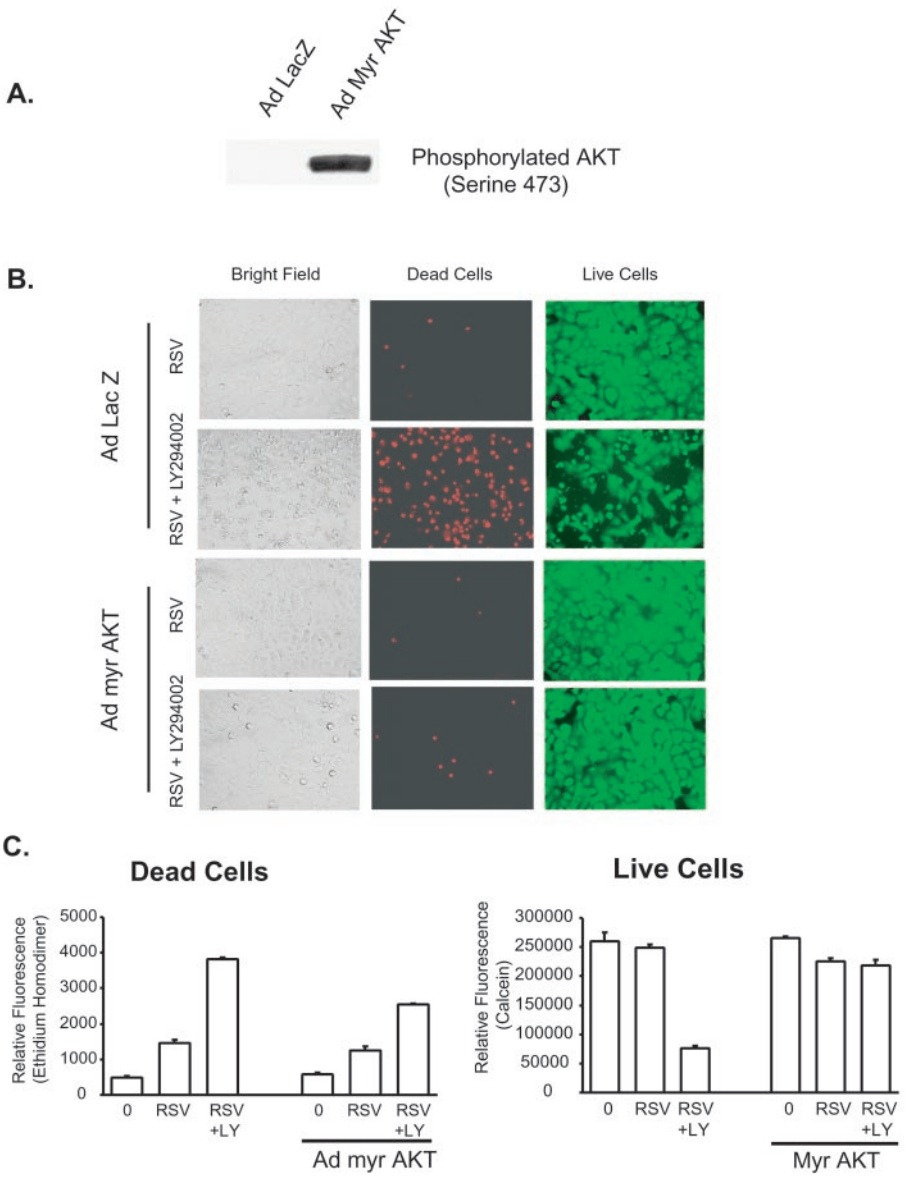

FIG. 5. **Constitutively active AKT prevents LY294002-induced cytotoxicity and apoptosis.** A549 cells were infected with an adenovirus vector containing a myristoylated, constitutively active AKT isoform or a control LacZ, 36 h prior to infection with RSV as outlined under "Experimental Procedures." *A*, Western blot for phosphorylated AKT (36 h after adenovirus vector infection). *B*, photomicrographs of A549 cells exposed to LY294002 and infected with RSV in the presence of constitutively active AKT (Ad.myr-AKT) or control vector, (Ad. LacZ) (6 h RSV infection). Sequence includes white-light microscopy (*left*), ethidium homodimer stain for cells without intact membranes (*center*), and calcein AM stain for intact cells (*right*). *C*, quantitative assay of cell death, ethidium homodimer (*Dead Cells*) (24 h RSV infection). A549 cells were plated in 96-well microtiter plates and infected with Ad. myr-AKT or control Ad.LacZ for 36 h. Subsequently cells were exposed to LY294002 (1 h pretreatment) followed by RSV infection. 24 h later the cells were stained and relative fluorescence quantified using a microtiter plate reader as outlined under "Experimental Procedures." *D*, photograph of agarose gel demonstrating DNA fragmentation (6 h RSV infection). *E*, PARP immunoblot of nuclear protein isolated from vector and RSV-infected cells (6 h RSV infection).

associated with pro-apoptotic signaling in other studies (66), the investigators were unable to demonstrate activation of caspase-3 or apoptosis at 36 or 48 h post-infection. RSV has also been found to induce expression of other apoptosis-associated proteins including functionally active Fas (CD95) in A549 cells (67). This study, in contrast to the previous findings, provided evidence that RSV alone resulted in apoptotic cell death at 72 h following infection; this effect was further increased by cross-linking Fas with anti-Fas antibodies (67). RSV may confer a survival advantage and actively inhibit apoptosis in human peripheral blood monocytes and cord blood monocytes following 24–48 h of infection (68). Our data suggest that RSV infection of A549 cells produces early anti-apoptotic signaling which, when inhibited, results in RSV-driven apoptosis within 6 h. Our conclusions that RSV possesses both apoptotic and anti-apoptotic properties are consistent with prior observations that RSV induces Fas, ICE, and IRF-1 but does not necessarily result in apoptosis, even within the same cell line. In our system, it is possible that the early effect of PI 3-K activation decreases over time as viral protein synthesis consumes cellular resources and induces cellular response genes. In fact, the phosphorylation of PI 3-K, AKT, and GSK-3 in our experiments was observed to decrease by 24 h post-infection. Thus the early anti-apoptotic effect of PI 3-K activation by RSV in A549 cells may represent a transient response that may not confer apoptosis protection at late time points.

Although the existence of the PI 3-K/AKT pathway of cell survival has been carefully investigated in cytokine and TNF signaling, very little previous work has examined the effects of activation of PI 3-K during active viral infection on cell survival. Here we demonstrated increased kinase activity of PI 3-K and phosphorylation of the regulatory sites on PI 3-K, AKT, and GSK consistent with activation (PI 3-K, AKT) and inhibition (GSK-3) by RSV. Epstein-Barr virus glycoprotein gp350 has been shown to activate TNF-α gene transcription through pathways involving PKC and PI 3-K; however, the effects of this viral gene product on cell cycle and survival were not described (69). Another viral product, the polyomavirus middle T antigen, has been shown to activate constitutively AKT through a PI 3-K-dependent pathway resulting in transformation and tumorigenesis (70). Finally, during infection of erythroid cells, the Friend spleen focus-forming virus has been shown to induce cellular transformation through PI 3-K and AKT-dependent pathways (71). Unlike these previous observations, however, activation of PI 3-K by RSV appears to have a different and novel functional significance including the main-

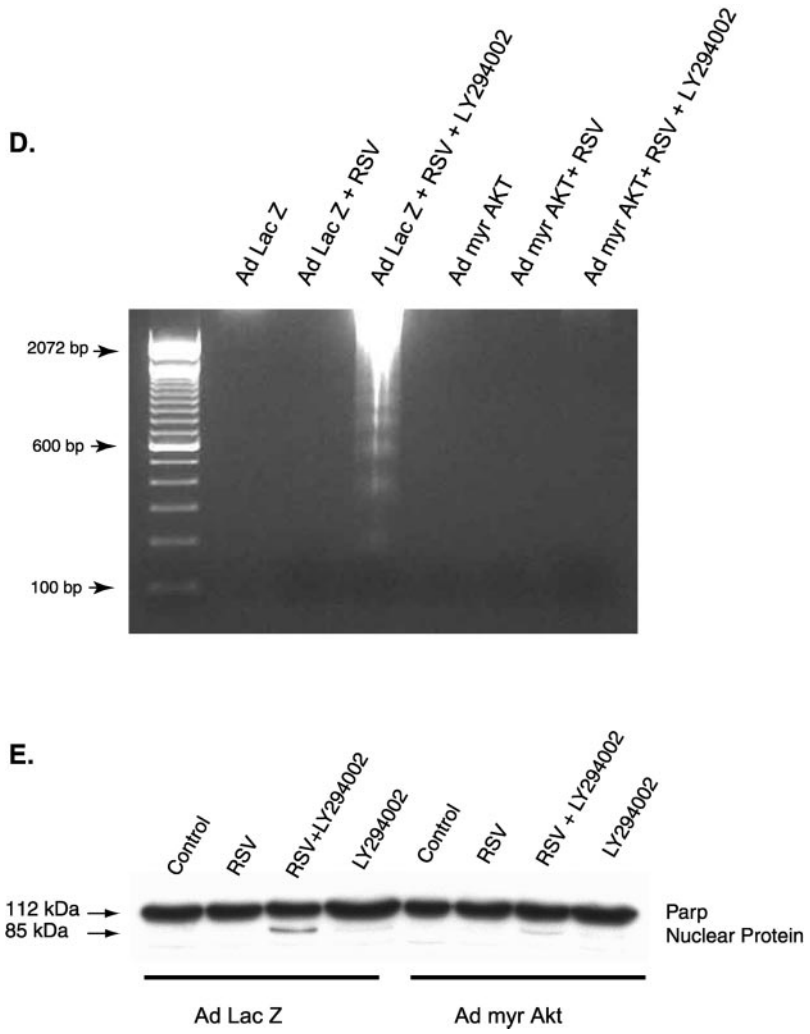

FIG. 5—*continued*

tenance of cell survival during acute infection and the regulation of cell synthetic response through NF-κB.

LY294002 inhibits phosphatidylinositol (PI) kinases by competitively occupying the ATP-binding site and blocking kinase activity (72). In addition to blocking the p85/p110 PI 3-kinases, however, this compound and the related compound wortmannin have been shown to variably inhibit the activity of other members of the subfamily of PI kinase-related kinases including the targets of rapamycin (TORs), (73), DNA-dependent protein kinases, and related enzymes, as reviewed in Ref. 74, and recently SMG-1 (75). Thus, the effects of LY294002 by itself are not sufficient to establish PI 3-K as the mediating target in human airway epithelial cells. Although the effects of LY294002 on survival could have been mediated through mammalian TOR (mTOR)-dependent pathways, this is unlikely because mTOR is both inhibited by LY294002 (73) and is a direct downstream target of PI 3-kinase/AKT signaling (76, 77). If mTOR was the primary survival factor blocked by LY294002, then constitutively active AKT should not have maintained cellular viability following LY294002. Our observations that RSV infection leads to activation and specific regulatory phosphorylation of immunoprecipitated p85/p110 PI 3-K and also that constitutively active AKT prevented LY294002-induced apoptosis suggest that the pathway involving p85/p110 and AKT is responsible for mediating cellular survival during early RSV infection.

The mechanism of activation of NF-κB by RSV has been shown previously to involve regulation of phosphorylation or degradation of the inhibitor peptides IκBα and IκBβ. Bitko and Barik (63) have presented data which demonstrated that the early activation of NF-κB in A549 cells by RSV coincided with the phosphorylation and degradation of IκBα, while at later time points under-phosphorylated IκBβ appeared to contribute to persistent NF-κB activation. The role of IκBα in RSV-mediated NF-κB up-regulation was further established by the observation that the simultaneous transfection of an adenovirus vector containing a mutated, nondegradable form of IκBα and RSV infection blocked NF-κB activation and RANTES production in BEAS2B, normal human bronchial epithelial cells and A549 cells (20). Feidler and Wernke-Dollries (64) have also presented data from A549 cells showing that IκBα is phosphorylated and degraded within 24 h of RSV infection. In these studies, however, blockade of proteolysis of IκBα was not able to reverse completely RSV-mediated NF-κB activation (64). Thus, RSV activates NF-κB through IκB phosphorylation and degradation, but additional factors may be involved in regulating and maintaining NF-κB activity.

In addition to its role in preservation of cell viability and interference with apoptosis, we have reported evidence that the PI 3-K/AKT pathway is involved in the regulation of NF-κB activity. LY294002 abolished the induction of NF-κB-driven gene transcription during RSV infection. This occurred despite the lack of effect of LY294002 on nuclear translocation and DNA binding of NF-κB as measured by electromobility shift assays. This observation is in contrast to previous studies in A549 cells, which have shown that wortmannin and LY294002

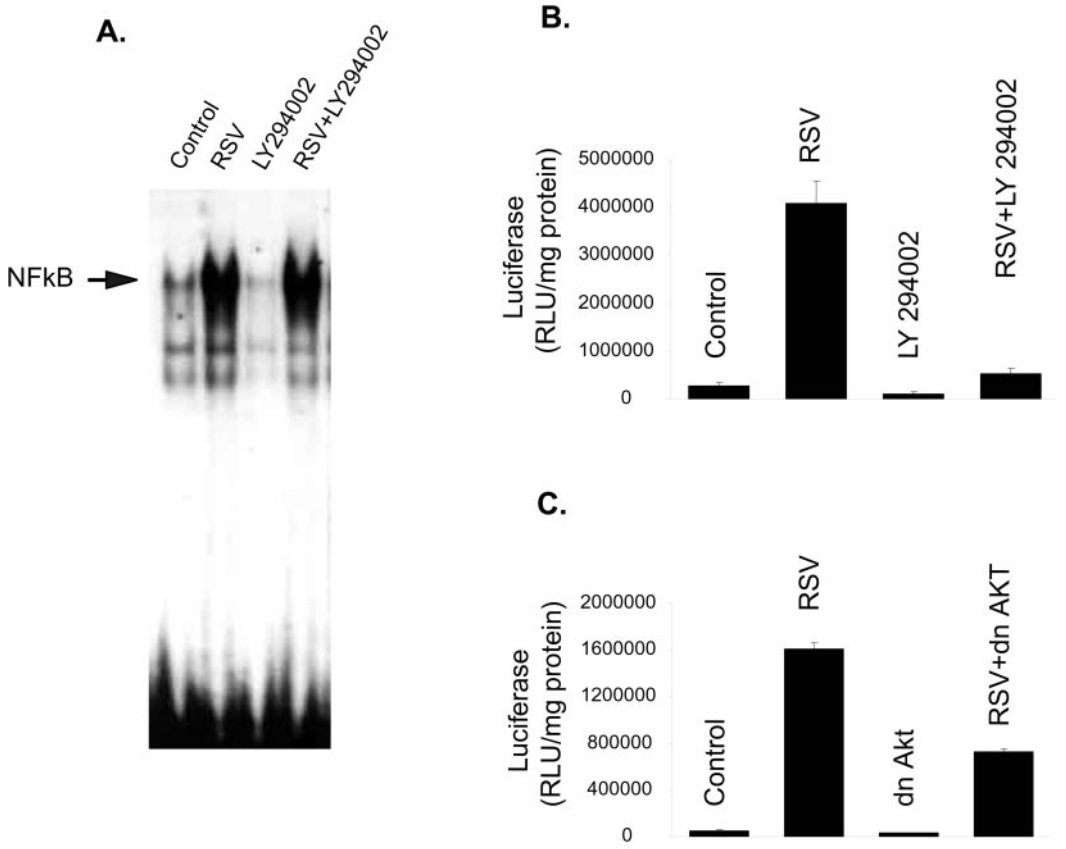

FIG. 6. **NF-κB-dependent gene transcription is inhibited by PI 3-K blockade despite lack of effect on nuclear translocation.** *A*, EMSA for NF-κB nuclear translocation. Cells and controls were pretreated with LY294002 or solvent followed by RSV infection and harvested 3 h later. Nuclear protein was isolated and EMSA performed as indicated under "Experimental Procedures." *B*, NF-κB-driven luciferase reporter assay. A549 cells were transfected with a plasmid containing the luciferase gene driven by the κ enhancer site. Cells were then pretreated with LY294002 or control conditions followed by RSV infection. Protein was harvested at 6 h following infection and assayed for luciferase activity as described under "Experimental Procedures." Luciferase activity was normalized to total protein concentration in the cell lysates. *C*, A549 cells were simultaneously transfected with the NF-κB luciferase plasmid and control plasmid containing bacterial β-galactosidase or kinase-dead AKT. Quantitative data are mean values with standard error measurements of three separate experiments.

blocked NF-κB activation by bradykinin at the level of nuclear translocation (41). These different results suggest the possible existence of more than one pathway in A549 cells through which PI 3-K activity can modulate NF-κB-dependent gene transcription. In addition to sequestration in the cytoplasm, the transcriptional activity of the p65/RelA NF-κB subunit can be mediated by phosphorylation at its C-terminal transactivation domain (33, 34). Evidence from HepG2 cells stimulated with IL-1 suggests that AKT can mediate phosphorylation of the p65/RelA subunit (35). We have observed that a dominant negative AKT isoform was also able to block RSV-mediated NF-κB transcriptional activity. Therefore, we can postulate that RSV not only stimulates the nuclear translocation of NF-κB through IκB-mediated effects, which has been shown by other authors, but also that it may impose an additional level of regulation through a phosphorylation pathway involving PI 3-K and AKT.

During PI 3-K blockade, RSV infection results in the early apoptosis of A549 cells. This effect could be mediated by the loss of anti-apoptotic signaling at the level of the downstream kinases AKT or GSK-3 or, alternatively, as the result of failure of NF-κB activation. We have shown that AKT was serine-phosphorylated in a PI 3-K-dependent mechanism during RSV infection. Furthermore, constitutively active AKT prevented the cytotoxicity and apoptosis observed with PI 3-K blockade. Phosphorylation and activation of AKT have been implicated in protection of cells from apoptosis through multiple potential mechanisms, including phosphorylation of the pro-apoptotic Bcl-2 family protein Bad (78), phosphorylation and inactivation of the cysteine protease caspase 9 (79), and phosphorylation and inactivation of forkhead transcription factors (80). As above, AKT also may regulate NF-κB activity, which then may have distinct anti-apoptotic effects (48, 49). Furthermore, AKT is involved in phosphorylation and inactivation of GSK-3. GSK-3 has been found to have apoptotic effects in staurosporine or heat shock stress of the neuroblastoma cell line SH-SY5Y (81). Additionally, inhibition of GSK-3 has been shown to promote survival and inhibit apoptosis caused by PI-3K blockade in both Rat-1 and PC12 cells (82). Finally, the anti-apoptotic effects of RSV could be mediated through NF-κB-driven gene transcription, possibly including induction of synthesis of Bcl-2 proteins. Although the precise mechanism of the anti-apoptotic effect of PI 3-K-AKT–GSK-3–NF-κB pathway in RSV infection remains to be elucidated, the demonstration of apoptosis during its inhibition establishes the functional role of this pathway.

In summary, we have shown that RSV inhibits apoptosis and activates NF-κB through a PI 3-K-dependent pathway (Fig. 7). The prevention of apoptosis in airway epithelial cells may function to preserve host cell integrity until the replication phase of the virus is completed. The activation and regulation of NF-κB by RSV has been shown previously to be of critical importance in mediating the inflammatory response to infection. The precipitation of apoptosis and the inhibition of NF-κB transactivation through blockade of PI 3-K suggest that activation of the PI 3-K pathway itself is a critical determinant of the disease manifestation of RSV infection.

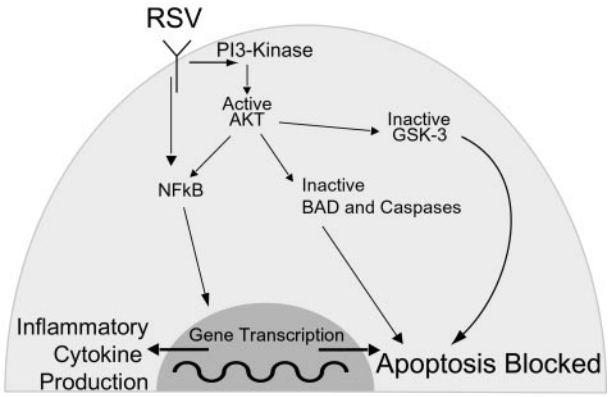

FIG. 7. **RSV activates PI 3-K leading to inhibition of apoptosis and inflammatory cytokine production.** We have shown that blockade of PI 3-K by the chemical inhibitor LY294002 results in rapid cell death through apoptosis. Constitutively active AKT prevented the cytotoxicity induced by LY294002. LY294002 blocks the induction of NF-κB-dependent gene transcription by RSV, an effect duplicated by kinase-dead AKT. Therefore, PI 3-K AKT signaling provides a critical pathway in RSV-mediated gene transcription and cell death.

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