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Akt/Protein Kinase B Activation by Adenovirus Vectors Contributes to NFκB-Dependent CXCL10 Expression

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In gene therapy, the innate immune system is a significant barrier to the effective application of adenovirus (Ad) vectors. In kidney epithelium-derived (REC) cells, serotype 5 Ad vectors induce the expression of the chemokine CXCL10 (IP-10), a response that is dependent on NFκB. Compared to the parental vector AdLuc, transduction with the RGD-deleted vector AdL.PB resulted in reduced CXCL10 activation despite increasing titers, implying that RGD-α_V integrin interactions contribute to adenovirus induction of inflammatory genes. Akt, a downstream effector of integrin signaling, was activated within 10 min of transduction with Ad vectors in a dose-dependent manner. Akt activation was not present following transduction with AdL.PB, confirming the importance of capsid-α_V integrin interactions in Ad vector Akt activation. Inhibition of the phosphoinositide-3-OH kinase/Akt pathway by Wortmannin or Ly294002 compounds decreased Ad vector induction of CXCL10 mRNA. Similarly, adenovirus-mediated overexpression of the dominant negative AktAAA decreased CXCL10 mRNA expression compared to the reporter vector AdLacZ alone. The effect of Akt on CXCL10 mRNA expression occurred via NFκB-dependent transcriptional activation, since AktAAA overexpression and Ly294002 both inhibited CXCL10 and NFκB promoter activation in luciferase reporter experiments. These results show that Akt plays a role in the Ad vector activation of NFκB and CXCL10 expression. Understanding the mechanism underlying the regulation of host immunomodulatory genes by adenovirus vectors will lead to strategies that will improve the efficacy and safety of these agents for clinical use.

Adenovirus (Ad) vectors are an attractive vector system for gene therapy and vaccination. Ad vectors efficiently activate host antiviral innate and adaptive immune responses (23, 39). This feature of Ad vector biology may be detrimental or desirable, depending on the intended application of these agents. The interplay between Ad vectors and the host innate immune system is receiving more attention, since it is increasingly clear that the innate response to Ad vectors plays an important role in determining the efficiency and inflammatory consequences of in vivo adenovirus-mediated gene transfer (31, 40). An understanding of the molecular basis underlying the inflammatory response to Ad vectors is therefore essential to improve the efficacy and safety of these agents for gene therapy.

Ad vectors activate host innate immune responses in vivo and in vitro. The activation of host inflammatory genes is mediated by the adenovirus capsid independent of viral gene transcription. This is illustrated by the similar inflammatory responses induced by UV/psoralen-inactivated, helper-dependent, and first-generation Ad vectors in vivo (9, 25). Ad vectors interact with and activate numerous different cell types, including leukocytes and endothelial and epithelial cells. In nonhematopoietic cells, studies in our laboratory show that Ad vectors activate NFκB and a number of signaling pathways such as ERK and p38 during viral cell entry, which results in the upregulation of immunoregulatory genes, including those for cytokines, chemokines, and adhesion molecules (7, 20, 33). CXCL10 (IP-10) is a C-X-C chemokine that is rapidly upregulated in models of Ad vector-induced inflammation (24). CXCL10, along with its receptor CXCR3, is actively involved in Th1 immune responses (36). $CXCL10^{-/-}$ mice have impaired immune responses as manifested by reduced T-cell proliferation to antigen stimulation and reduced inflammatory cell infiltrates. $CXCL10^{-/-}$ mice also exhibit a reduced immune response to a neurotropic mouse hepatitis virus infection as manifested by decreased $CD4^+$ and $CD8^+$ lymphocyte trafficking to the brain and reduced levels of inflammatory cytokine/chemokine production (13). A recent study has confirmed that CXCL10 plays a pivotal role in the recruitment of T cells into the liver after adenovirus infection (3). The induction of CXCL10 thus serves as a useful marker of cellular activation and the host immune response to Ad vectors in vitro and in vivo.

Ad vector entry in nonhematopoietic cells first occurs through a high-affinity interaction between the Ad fiber knob region and the coxsackievirus-adenovirus receptor (CAR) (6). The binding of the fiber knob to CAR is thought not to trigger signaling events but rather to disrupt the integrity of host cell junctions to facilitate the spread of progeny virions (34). Following initial binding, RGD (arginine-glycine-aspartate) motifs in the Ad penton base protein bind to α_V integrins, which facilitates virus internalization (38). Our previous studies have shown reduced induction of host inflammatory genes by CAR-ablated and RGD-deleted tropism-modified Ad vectors in epithelium-derived and endothelial cells in vitro (20, 33). Ad vector-induced signal transduction and chemokine gene expression correlated with reduced cellular entry but still occurred in the absence of CAR and integrin binding. These results suggested that activation of host inflammatory mecha-

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nisms occurred in a postinternalization step of Ad vector cell entry. However, in vivo studies with mice also demonstrated reduced induction of inflammatory genes by RGD-deleted Ad vectors, implying a more significant role for capsid RGD-integrin interactions in the host response to Ad vectors (20).

Binding of capsid RGD motifs to α_V integrins not only facilitates virus internalization but also triggers several integrin-induced signaling pathways, including the phosphoinositide-3-OH kinase (PI3K) pathway (26, 28). Akt is the cellular homologue of the transforming oncogene of the AKT8 oncovirus (vAkt) and lies downstream of PI3K (5, 12). Akt contains an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal hydrophobic and proline-rich domain. PI3K catalyzes the transfer of phosphate from ATP to the D-3 position of the inositol ring of membrane-localized phosphoinositides (PI3,4P and PI3,4,5P). The binding of these lipids to the Akt PH domain translocates cytoplasmic Akt to the plasma membrane and renders Akt accessible to phosphorylation at T-308 and S-473 (12). The PI3K-Akt pathway has been demonstrated to positively regulate NFκB (27, 30). Recent studies show that infection of airway epithelial cells by respiratory syncytial virus or serotype 5 Ad vectors activates the PI3K-Akt pathway and postpones apoptosis by positively regulating NFκB activity (14, 32). In murine NIH 3T3 fibroblasts, Akt utilizes IκB kinase beta and p38 to stimulate the transactivation potential of the RelA/p65 subunit of NFκB (21). Finally, the induction of the chemokine CCL2 (MCP-1) during platelet-derived growth factor signaling is dependent on Akt (1). These results imply that in addition to promoting the expression of genes involved in cell survival, Akt may also play a role in cytokine/chemokine gene regulation.

As part of a continuing effort to determine the regulatory mechanisms of Ad vector-dependent inflammatory gene expression, we have examined the role of Akt in the inflammatory response to Ad vectors. In this paper, we show that Ad vectors rapidly activate Akt within minutes of transduction in an RGD-dependent manner. Furthermore, Akt activation contributes to the Ad vector induction of CXCL10 through its effects on NFκB. These data increase our understanding of the regulation of Ad vector-induced inflammation and identify Akt as a potential regulator of host inflammatory gene expression.

MATERIALS AND METHODS

Adenovirus vectors. The type 5 E1-E3-deleted adenoviruses expressing β-galactosidase (AdLacZ) and luciferase (AdLuc) under the control of the cytomegalovirus (CMV) promoter were propagated in 293 cells and purified as previously described (4). AdL.PB* and AdL.F* are tropism-modified E1-E3-deleted Ad5-based vectors carrying the luciferase transgene in the E1 region under the control of the CMV promoter (29, 37) (kindly provided by T. Wickham and GenVec Inc.). AdL.PB* contains a deletion of the RGD motif in the penton base, and AdL.F* has the mutations R412S, A415G, E416G, and K417G in the AB loop of the fiber protein. The cDNA encoding bovine dominant negative AktAAA was provided by J. R. Woodgett (35). AktAAA has K179A, T308A, and S473A substitutions, rendering it unable to bind ATP or be activated. A recombinant Ad vector expressing AktAAA under the control of the CMV promoter was constructed using the AdEasy system as per the manufacturer's protocol (Stratagene, La Jolla, CA). AdLacZ was rendered transcription defective by UV-psoralen treatment as previously described (10). Sucrose vehicle buffer (10 mM Tris pH 7.4, 2 mM $MgCl_2$, 150 mM NaCl, 3% sucrose) was used for controls.

Ad vector particle number was determined by optical density measurement at 260 nm (22). Multiplicity of infection (MOI) was expressed as particles per cell and calculated by dividing the total particle number by the cell number used in transductions. Vectors were screened for replication-competent adenovirus by plaque assay on HeLa cells and remained consistently $<1:10^{10}$ particles. All vectors contained <0.1 endotoxin unit of endotoxin/ml as per the *Limulus* amebocyte lysate assay (11).

Cell culture. The immortalized epithelium-derived DBA/2 kidney cell line REC (7) was maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). For all studies, cells were preincubated in DMEM containing 1% FCS 16 h prior to transduction with Ad vectors. Ad vector transductions at specified titers were performed in six-well plates with cells that were 70 to 80% confluent ($\sim 1 \times 10^6$ cells/plate). Cells were transduced in 1 ml of DMEM containing 1% FCS for 90 min, followed by incubation in fresh medium for the remainder of the experimental period. The experimental time periods began immediately after the addition of vectors to cells. Cells were preincubated with the PI3K inhibitor Wortmannin at 10 or 30 nM or with Ly294002 at 10 μM (Calbiochem, La Jolla, Calif.) 30 min prior to transduction with Ad vectors.

Immunoblotting. REC cells were stimulated with Ad vectors for specified time periods and lysed directly with lysis buffer (20 mM Tris pH 7.4, 1% NP-40, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM Na_3VO_4, 20 mM NaF) (2). Lysate protein concentration was determined with the Bradford assay. Equivalent amounts of protein (20 μg) were separated on a sodium dodecyl sulfate–10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. Membranes were blocked with milk proteins by using 10% skim milk in Tris-buffered saline–Tween 20 for 1 h at room temperature and incubated overnight with a 1:1,000 dilution of antibodies against phosphorylated Akt (Ser-473) or Akt (Cell Signaling Technology, Beverly, MA). Membranes were washed and then incubated for 1 h with a 1:7,500 dilution of peroxidase-conjugated anti-rabbit immunoglobulin. Proteins were visualized with ECL-Plus substrate reagent (Amersham, Piscataway, N.J.). Quantification of the density ratio of the phosphorylated Akt to total Akt within the same sample was performed using Quantity One software (Bio-Rad).

RNase protection assay. REC cells were processed for total RNA extraction by using RNeasy (QIAGEN, Chatsworth, CA) per the manufacturer's protocol. RNase protection assays were performed with the RiboQuant RNase protection assay system (BD PharMingen, San Diego, CA) per the manufacturer's protocol, using the mCK5c probe set or single probes for murine CXCL10 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a housekeeping control. Autoradiographs were analyzed by phosphorimaging with a Personal FX phosphorimager and Quantity One software (Bio-Rad, Hercules, CA) Fold induction was determined as the mRNA density ratio of CXCL10 to GAPDH within the same sample.

Luciferase reporter assays. The construct pGL3-IP-10(−533), containing a 533-bp fragment upstream of the transcription start site of the mouse CXCL10 gene, was described previously (7). To specifically study the effects of Akt activation by Ad on transcription factor NFκB, a luciferase reporter plasmid containing five sequential NFκB binding sites upstream of a minimum promoter element, NFκB-Luc (Stratagene), was used. For luciferase assays, cells were transfected with 2 μg of plasmid DNA by using Lipofectamine (Invitrogen, Carlsbad, CA) and incubated for 24 h. For vector stimulation studies, Ad vectors at the specified titer were added to the cells in a 1-ml volume and incubated at 37°C for 90 min. The medium was removed and replaced with fresh prewarmed DMEM containing 1% fetal bovine serum, and the cells were incubated for 6 or 24 h before luciferase assays were performed. Cells were washed with phosphate-buffered saline, harvested by scraping, and centrifuged into a pellet, followed by resuspension in 300 μl of lysis buffer (1% Triton X-100; 25 mM glycylglycine, pH 7.8; 15 mM MgSO4; 4 mM EGTA; 1 mM dithiothreitol). Samples were centrifuged at 14,000 rpm for 10 min, and 50 μl of supernatant was added to 300 μl of assay buffer (25 mM glycylglycine pH 7.8, 15 mM K_2PO_4 pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 2 mM ATP) in polystyrene tubes. The luciferase activity of each sample was measured in a luminometer following the addition of 100 μl of luciferin (0.3 mg/ml) (Promega). Values are expressed as relative light units (RLU) per milligram of total protein as determined by the Bradford assay.

Statistical analysis. All experiments were performed at least three times. Values were expressed as means ± standard deviations. Results were analyzed for statistical variance using an unpaired Student *t* test.

RESULTS

Role of capsid RGD in adenovirus vector induction of CXCL10. Our previous studies have shown that wild-type and tropism-modified Ad vectors induce the expression of CXCL10 in REC

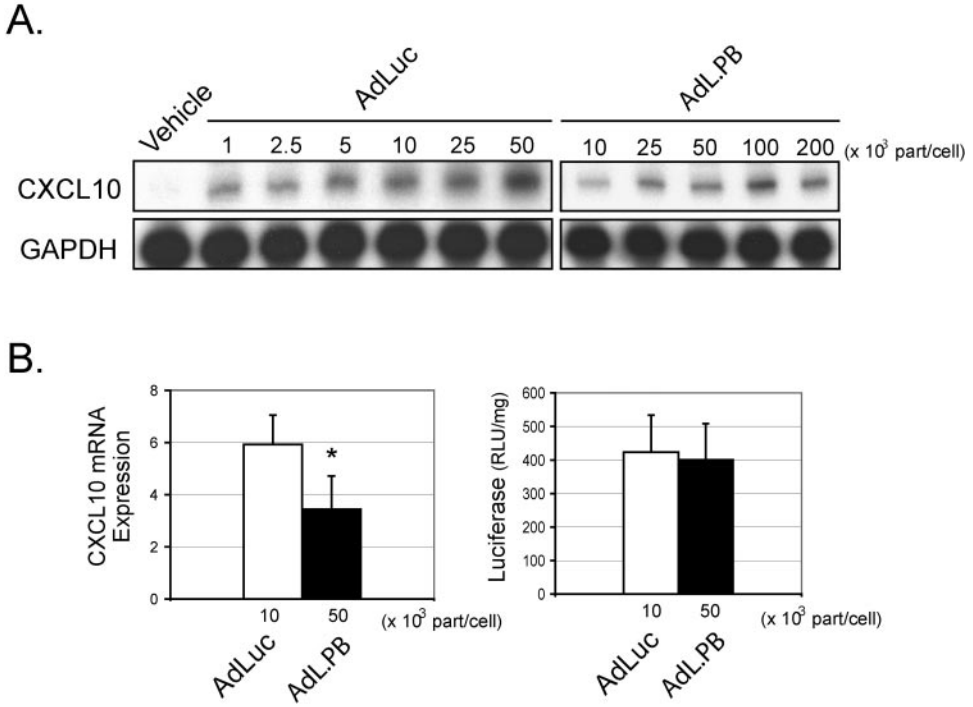

FIG. 1. CXCL10 induction by adenovirus vectors. (A) CXCL10 mRNA expression at 6 h in response to increasing MOI of AdLuc and the RGD-deleted vector, AdL.PB* (RNase protection assay). (B) Comparison of CXCL10 mRNA expression (RNase protection assay) (*, $P < 0.05$; $n = 3$) and transduction (luciferase activity [RLU/milligram, 10^4]) between AdLuc (MOI of 10×10^3 particles/cell) and AdL.PB* (MOI of 50×10^3 particles/cell). Error bars indicate standard deviations.

cells (7). The tropism-modified Ad vector AdL.PB* is deleted of the RGD motif on the penton base and therefore does not bind to α_V integrins (37). To determine if AdL.PB* activated equivalent levels of CXCL10 mRNA as its parental vector AdLuc, REC cells were transduced with an increasing titer of AdL.PB*. Increasing the AdLuc MOI (1×10^3 to 50×10^3 particles/cell) efficiently induced the expression of CXCL10 mRNA at 6 h in REC cells as determined by RNase protection assay (Fig. 1A). In contrast, despite very high titers (MOI of 10×10^3 to 200×10^3 particles/cell), AdL.PB* consistently induced less CXCL10 than AdLuc. Since AdL.PB* transduces cells less effectively than wild-type capsid Ad vectors, CXCL10 expression levels for Ad vector titers that result in similar levels of cellular transduction were compared. REC cells transduced with 10×10^3 particles/cell of AdLuc and 50×10^3 particles/cell of AdL.PB* express similar levels of the luciferase transgene as determined by luciferase assay. However, at these titers AdLuc induced more CXCL10 mRNA than the RGD-deleted vector ADL.PB* (Fig. 1B). These results suggest that while CXCL10 induction by Ad vectors can occur in an RGD-independent manner, an RGD-dependent mechanism that contributes to CXCL10 expression exists.

Adenovirus vectors activate Akt. Ad vectors activate PI3K, a process that is required for optimal cell entry (19). Activated PI3K catalyzes the transfer of phosphate from ATP to the D-3 position of the inositol ring of membrane-localized phosphoinositides (15). The binding of these lipids to Akt's PH domain translocates cytoplasmic Akt to the plasma membrane and results in Akt activation by phosphorylation (16). Previous studies have demonstrated Ad vector activation of Akt occurring at longer than 12 h, a process dependent on viral gene transcription (14). To determine whether Ad vectors could induce an earlier activation of Akt, REC cells were incubated with 10×10^3 particles/cell AdLacZ or vehicle and total cell lysates harvested at predetermined time points. Since Akt activation can be manifested by inducible phosphorylation of Akt at Thr-308 and Ser-473 (2), immunoblotting was carried out to detect phosphorylated Akt in total cell lysates with a phospho-Ser-473-Akt antibody. Compared to that in vehicle-treated cells, Akt phosphorylation was increased following AdLacZ transduction as early as 10 min and remained activated at 90 min (Fig. 2A). The amount of total cellular Akt detected with an Akt antibody remained the same at all time points. These results indicate that Ad vector transduction rapidly activates signaling via Akt.

To study whether the increased Akt phosphorylation induced by AdLacZ vector was dependent on the input titer, a dose-response experiment was performed. REC cells were transduced with increasing titers of AdLacZ and total cell lysates harvested at 30 min. The Akt phosphorylation level increased with increasing Ad vector titer, with a maximal activation at a multiplicity of infection of 50×10^3 particles/cell (Fig. 2B). These results show that the degree of Akt phosphorylation correlates directly with input Ad vector titer.

Adenovirus vector activation of Akt requires capsid RGD. The previous experiments demonstrate that AdLacZ rapidly activates Akt in REC cells. To determine whether viral transcription was required for Akt activation, AdLacZ was rendered transcription defective by UV/psoralen treatment (UV/Ps-Ad). REC cells were transduced with 10×10^3 particles/cell of UV/Ps-Ad and cell lysates analyzed for phosphorylated Akt by immunoblotting at 30 min. UV/Ps-Ad induced a similar

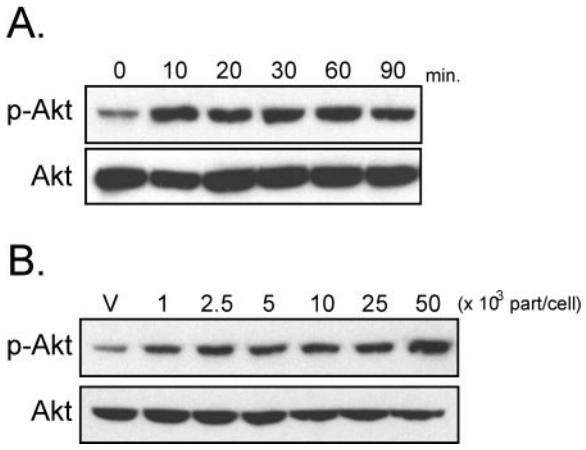

FIG. 2. Akt activation by adenovirus vectors. (A) Time course of phosphorylated and total Akt (immunoblot) in REC cells following transduction with AdLacZ at an MOI of 10×10^3 particles/cell. (B) Immunoblot of phosphorylated and total Akt following transduction with increasing MOIs of AdLacZ at 30 min. Representative samples from experiments performed at least three times are shown.

degree of Akt phosphorylation as transcription-competent AdLacZ, suggesting that the adenovirus particle alone is sufficient to activate Akt (Fig. 3). Consistent with this result, AdLuc induced a similar degree of Akt phosphorylation as AdLacZ, confirming that the adenovirus particle activates Akt independent of viral transcription or the encoded transgene.

The induction of PI3K by adenovirus is dependent on the capsid penton base protein, since purified penton base protein, but not the fiber protein, can activate PI3K to the same degree as intact adenovirus particles (19). The early, transcription-independent activation of Akt by Ad vectors suggests that the adenovirus capsid proteins also mediate this response. To determine the Ad vector-cell interaction that underlies Akt activation, tropism-modified Ad vectors were again employed. AdL.F* carries the mutations R412S, A415G, E416G, and K417G in the AB loop of the fiber protein, which ablates its ability to bind CAR, but maintains the normal complement of penton RGD motifs. REC cells were transduced with 10×10^3 particles/cell of AdL.F* or AdL.PB*, and the cell lysates were harvested at 30 min after transduction and analyzed by immunoblotting. As shown in Fig. 3, at this titer Akt phosphorylation was marginally increased by these two capsid-modified Ad vectors. Since REC cells express both CAR and α_V integrins, the reduced Akt activation by AdL.F* and AdL.PB* may reflect reduced cellular binding and subsequent transduction compared to the wild-type vector AdLuc, as shown previously (33). Therefore, to account for this possibility, experiments were next performed to assess the activation of Akt at vector titers that result in comparable transduction of AdLuc, AdL.F*, and AdL.PB*. REC cells were transduced with increased titers of AdL.F* (100×10^3 particles/cell) and AdL.PB* (50×10^3 particles/cell), and again cell lysates were analyzed at 30 min for phosphorylated Akt (Fig. 3). AdL.F* at the increased titer induced Akt phosphorylation similarly to the wild-type vector AdLuc. In contrast, AdL.PB* at the higher titer failed to activate Akt. These results indicate that early Akt activation by Ad vectors is RGD dependent and likely occurs via α_V integrins.

Inhibition of PI3K and Akt decreases adenovirus vector-induced CXCL10 expression and NFκB activation. The previous experiments demonstrated that Akt activation by Ad vectors requires penton base RGD-α_V integrin interaction and suggest a direct link to PI3K. To examine the role of PI3K in the Ad vector activation of Akt, PI3K activity was suppressed with a PI3K inhibitor, Ly294002. As shown in Fig. 4A, treatment of REC cells with Ly294002 (10 μM) resulted in reduced baseline phosphorylation of Akt in REC cells, consistent with previous studies (17). To determine whether PI3K is involved in Akt activation by Ad vectors, REC cells were again treated with Ly294002 (10 μM) prior to Ad vector transduction. Following incubation with AdLacZ (MOI of 10×10^3 particles/cell), the level of Akt phosphorylation was largely suppressed in Ly294002-treated cells, confirming that the activation of Akt by Ad vectors was dependent on PI3K (Fig. 4A).

Signaling via PI3K and Akt results in numerous downstream effects, including the activation of NFκB-regulated genes (12). To test the potential role of Akt in CXCL10 regulation, PI3K

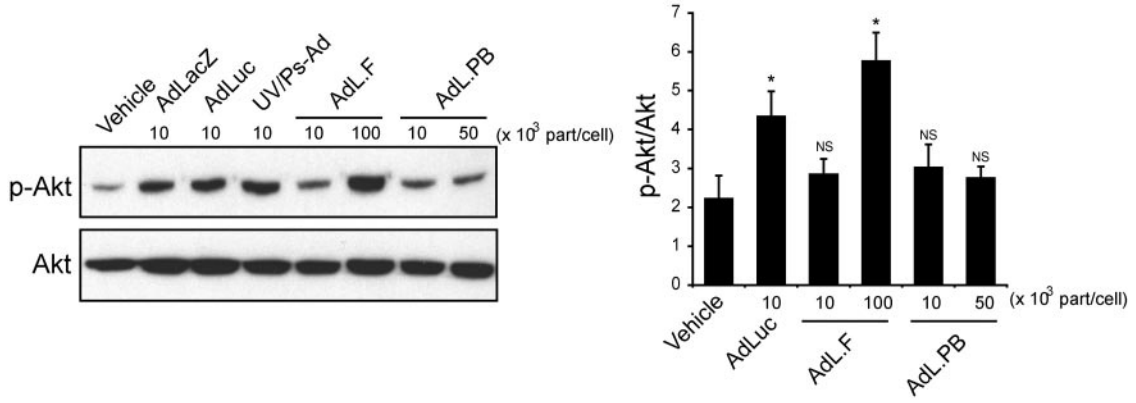

FIG. 3. Role of capsid and transgene in Akt activation by adenovirus vectors. Immunoblotting for phosphorylated and total Akt showing the effect of transgene/viral transcription 30 min following transduction with AdLacZ, AdLuc, and UV/psoralen-inactivated AdLacZ (MOI of 10×10^3 particles/cell). Tropism-modified vectors AdL.F* and AdL.PB* demonstrate differential Akt activation with increasing MOI. Immunoblots are representative of experiment performed three times (for phosphorylated Akt/Akt, vehicle versus treatment group, *, $P < 0.001$; NS, not significant; $n = 3$ to 8). Error bars indicate standard deviations.

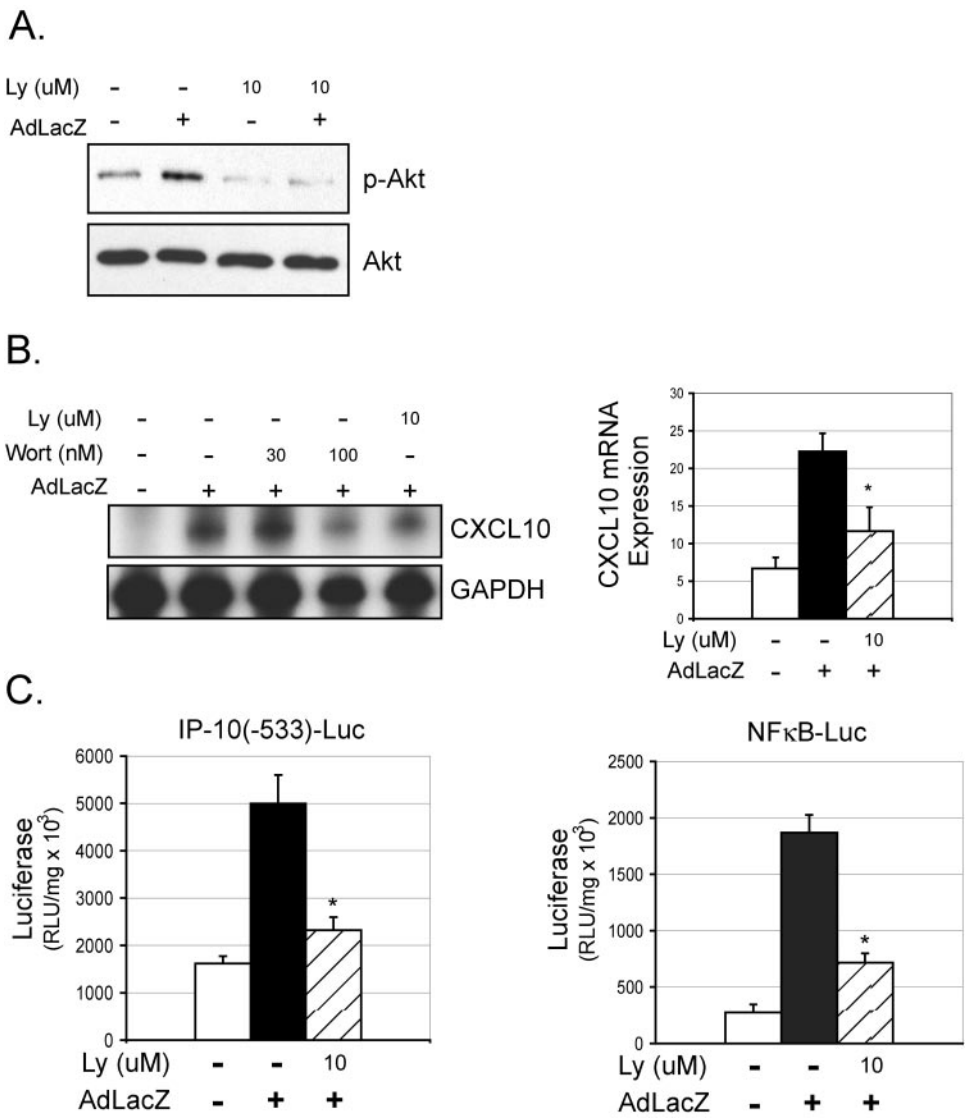

FIG. 4. Effect of PI3K inhibition on adenovirus vector induction of CXCL10 and NFκB activation. (A) Immunoblot for phosphorylated and total Akt following transduction with AdLacZ at 30 min. Ly294002 (10 μM) blocks adenovirus activation of Akt. (B) Effect of Ly294002 and Wortmannin on Ad vector induction of CXCL10 mRNA at 6 h (RNase protection assay) (for +Ly294002 versus −Ly294002, ∗, $P < 0.01$; $n = 5$). (C) Inhibition of PI3K with Ly294002 (10 μM) blocks AdLacZ transcriptional activation of the murine CXCL10 and NFκB minimal promoters at 6 h (luciferase activity [RLU/milligram]) (∗, $P < 0.01$; $n = 3$). Error bars indicate standard deviations.

activity was inhibited by Ly294002 (10 μM) and a second PI3K inhibitor, Wortmannin (30 and 100 nM). REC cells were then transduced with AdLacZ (MOI of 10×10^3 particles/cell), and the expression of CXCL10 mRNA was determined at 6 h. Compared to untreated cells, Wortmannin and Ly294002 significantly reduced AdLacZ induction of CXCL10 mRNA (Fig. 4B). Our previous studies show that NFκB mediates the Ad vector induction of CXCL10 (7). To determine if Ad vector-induced signaling via PI3K affected the transcriptional regulation of CXCL10 and NFκB activation, luciferase reporter experiments were conducted. The luciferase construct pGL3-IP-10(−533) contains the promoter fragment 533 bp upstream of the murine CXCL10 gene transcriptional start site, and NFκB-Luc contains five sequential NFκB binding sites upstream of a minimum promoter element. First, pGL3-IP-10(−533) and NFκB-Luc were transiently transfected into REC cells. To block PI3K and the downstream activation of Akt by Ad vectors, cells were incubated with Ly294002 (10 μM) and then transduced with AdLacZ (10×10^3 particles/cell) or vehicle treated. At 6 h, AdLacZ activated both CXCL10 and NFκB minimal promoters fivefold compared to vehicle-treated cells (Fig. 4C). Pretreatment with Ly294002 effectively inhibited, but did not completely suppress, the Ad vector transcriptional activation of CXCL10 and NFκB promoters. Taken together, these results show that the Ad vector induction of CXCL10 and NFκB activation occurs in part through PI3K.

Akt contributes to the adenovirus vector induction of CXCL10 and NFκB activation. The previous results imply that Akt via PI3K is involved in the Ad vector induction of CXCL10. Since

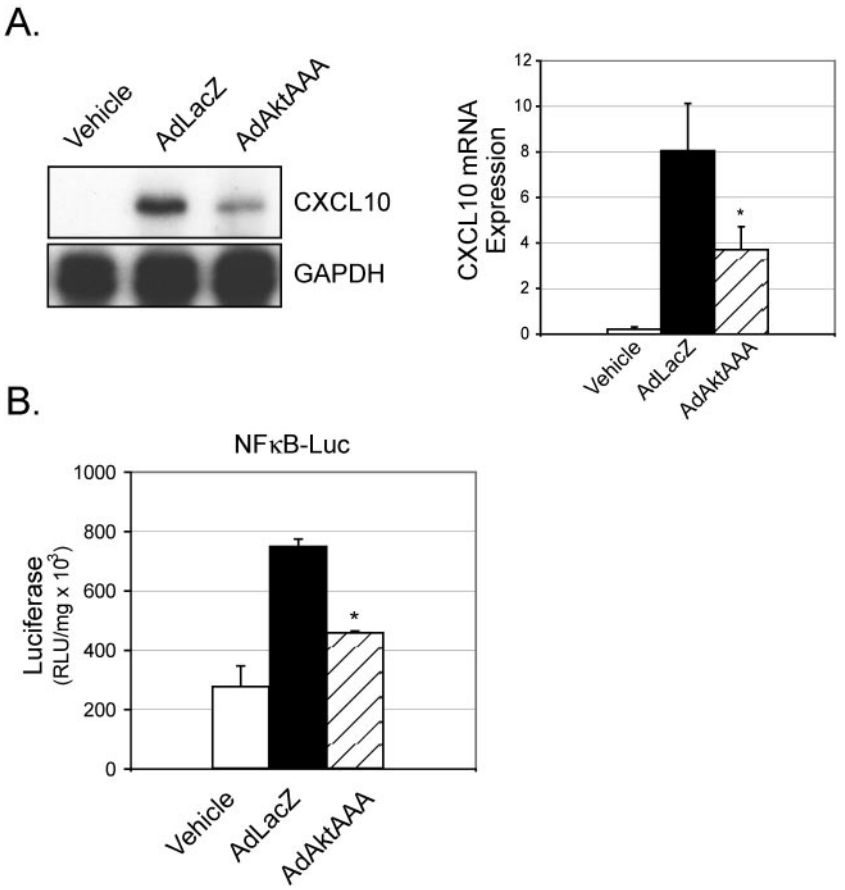

FIG. 5. Effect of Akt on CXCL10 expression and NFκB activation. (A) RNase protection assay for CXCL10 mRNA expression at 24 h following transduction with AdLacZ or AdAktAAA (MOI of 10×10^3 particles/cell) (for AdLacZ versus AdAktAAA, *, $P < 0.01$; $n = 5$). (B) Effect of AdAktAAA or AdLacZ (MOI of 10×10^3 particles/cell) on the transcriptional activation of the NFκB minimal promoter at 24 h (luciferase activity [RLU/milligram]) (for AdLacZ versus AdAktAAA, *, $P < 0.01$; $n = 3$). Error bars indicate standard deviations.

signaling via PI3K is also essential for adenovirus cell entry, the reduction in CXCL10 expression may also reflect a reduction in Ad vector transduction that would also reduce the activation of inflammatory genes (33). To address this possibility, the direct effect of Akt on CXCL10 expression was determined in the context of an Ad vector expressing the dominant negative AktAAA transgene. AktAAA contains triple alanine substitutions at positions K179, T308, and S473. AktAAA functions in a dominant negative manner, since the ATP binding site (K179) and two inducible phosphorylation sites (T308 and S473) that are required for Akt kinase activation (35) are removed. REC cells were transduced with 10×10^3 particles/cell of AdAktAAA or AdLacZ and incubated for 6 and 24 h. Total RNA was harvested and analyzed for CXCL10 expression by RNase protection assay. At 6 h, both AdLacZ and AdAktAAA induced similar levels of CXCL10 mRNA. This was not unexpected, since delayed and/or insufficient AktAAA expression at 6 h would not antagonize early capsid-induced Akt effects (data not shown). Since Ad vectors also activate Akt in a transcription-dependent manner that occurs late after cell entry (14), the incubation period following transduction was extended. At 24 h both AdLacZ and AdAktAAA induced CXCL10 mRNA above that for vehicle-treated cells. Compared to AdLacZ, adenovirus-mediated AktAAA overexpression significantly reduced CXCL10 mRNA expression (Fig. 5A).

To verify that the effect of Akt on CXCL10 mRNA expression correlated with NFκB activation, REC cells were transfected with NFκB-Luc, followed by transduction with AdLacZ or AdAktAAA (10×10^3 particles/cell). At 24 h, both AdLacZ and AdAktAAA increased luciferase activity compared to that in vehicle-treated cells, confirming NFκB-dependent transcription. However, in cells overexpressing dominant negative AktAAA, transcriptional activation of the NFκB minimal promoter was significantly reduced compared to that with AdLacZ (Fig. 5B). Taken together, these data show that Akt exerts a positive regulatory effect on Ad vector induction of CXCL10 and NFκB.

The preceding studies illustrate Akt activation by Ad vectors that directly affects CXCL10 expression and NFκB activation. Next, to confirm that the early capsid-mediated activation of Akt also plays a role in CXCL10 transcriptional activation, the effect of Akt on Ad vector activation of the CXCL10 promoter was determined. REC cells were transfected with pGL3-IP-10(−533) and then vehicle treated or transduced with a low titer of AdLacZ or AdAktAAA (1×10^3 particles/cell) to minimize basal activation of the promoter. Cells were incubated

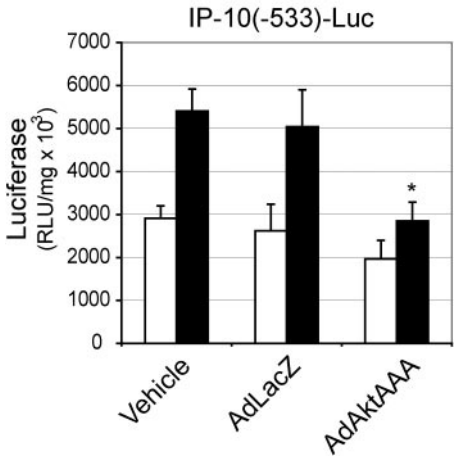

FIG. 6. Role of Akt in early adenovirus vector CXCL10 transcriptional activation. Luciferase assay showing the effect of AdAktAAA or AdLacZ pretreatment (MOI of 1×10^3 particles/cell) on the transcriptional activation of the CXCL10 promoter by AdLacZ (MOI of 10×10^3 particles/cell) (closed bars) or vehicle (open bars) at 6 h (AdLacZ-induced luciferase activity [RLU/milligram]) (for pretreatment with AdLacZ versus AdAktAAA, *, $P < 0.01$; $n = 6$).

for 24 h and subsequently stimulated with 10×10^3 particles/cell of AdLacZ or vehicle. At 6 h following the second transduction, REC cell luciferase activity was determined. At baseline, REC cells transduced with the lower titer of AdAktAAA displayed slightly less luciferase activity than vehicle- and AdLacZ-treated cells; the differences were not statistically significant (Fig. 6). Following the second transduction with AdLacZ, luciferase activity increased significantly at 6 h in vehicle- and AdLacZ-pretreated cells. In contrast, AdLacZ activation of the CXCL10 promoter was blunted in REC cells previously transduced with AdAktAAA, confirming that early activation of Akt contributes to the transcriptional activation of CXCL10 by Ad vectors (Fig. 6).

DISCUSSION

Our previous studies have demonstrated that Ad vectors significantly induce the expression of proinflammatory chemokines in vitro and in vivo, a response that is dependent on NFκB (7, 24). In this study, we shed further light on the mechanism of inflammatory gene expression induced by Ad vectors in nonhematopoietic cells. These results show that although Ad vectors can activate CXCL10 independent of RGD interactions with cell surface integrins, RGD-dependent activation of cellular signaling contributes to NFκB activation and CXCL10 expression. The rationale for this study stems from the observation that RGD-deleted vectors, while capable of inducing inflammatory genes in vitro and in vivo, do not induce a similar level of chemokine gene expression as wild-type capsid vectors when differences in transduction are considered. This is particularly evident at lower multiplicities of infection. We have previously demonstrated a reduction in early chemokine gene expression by RGD-deleted vectors in vivo (20). Although reduced Ad vector internalization likely plays a role, these data suggest that RGD-dependent Akt activation may also enhance early NFκB-dependent gene expression and the innate immune response. Akt-mediated inflammatory gene expression may represent a protective host defense mechanism to counterbalance the adenovirus-induced cell survival mechanisms used to enhance viral infection, a possible indicator of cell stress.

Hunninghake and coworkers recently demonstrated the activation of Akt by adenovirus and Ad vectors (14). In their study, Akt activation occurred via PI3K and protected airway epithelial cells from tumor necrosis factor alpha-mediated apoptosis. Consistent with our results, they reported biphasic Akt activation by adenovirus (12). While the mechanism of late Akt activation (beginning 12 to 24 h postinfection) is probably mediated by the expression of adenovirus E4 proteins (41), in this study we demonstrate that RGD-dependent capsid-cell interactions are responsible for early signaling via Akt. Our data also confirm that the fiber-CAR interaction is dispensable for Ad vector induction of Akt. The reduced Akt activation by the fiber-CAR mutant at a lower MOI probably resulted from the lack of the primary and high-affinity fiber knob-CAR interaction and an associated reduction in RGD-α_V integrin interaction. The subsequent activation of Akt with a higher titer of AdL.F* supports the conclusion that capsid RGD plays an important role in Ad vector-induced cell signaling pathways. Given the known biology of integrin signaling (18), it is not surprising that adenovirus capsid RGD motifs activate Akt signaling via integrins and PI3K. More importantly, these experiments suggest that it is the interaction of RGD-α_V integrins, not the transduction per se, that triggers early Akt signaling, since a similar degree of transduction achieved by AdL.PB* at high titer still fails to activate Akt during entry. This is consistent with a recent study performed with dendritic cells. Philpott et al. demonstrated that RGD-dependent PI3K activation by adenovirus was necessary for dendritic cell maturation and tumor necrosis factor alpha expression (28).

Akt was shown to exert a positive effect on NFκB activation and the induction of CXCL10 by Ad vectors. We have previously shown that the Ad vector induction of the chemokines CXCL10 (IP-10) and CCL5 (RANTES) depends on NFκB (7, 8). Akt is known to positively regulate NFκB in numerous cellular models, including infection with respiratory syncytial virus (27, 30, 32). Therefore it is not surprising that Akt can influence expression of CXCL10, an NFκB-regulated gene. Early PI3K signaling was demonstrated in this study to contribute to the transcriptional activation of CXCL10. Since PI3K is also required for adenovirus internalization (19), it is possible that PI3K inhibition reduced mechanisms of gene upregulation that are dependent on viral internalization rather than signaling through Akt (see below). Our data support a direct effect of Akt on inflammatory gene regulation. First, in cells expressing AktAAA, early Ad vector-induced CXCL10 transcriptional activation was impaired. Second, consistent with the biphasic nature of Akt activation induced by transcriptionally competent adenovirus vectors, late Akt activation contributed to NFκB activation and CXCL10 expression. In contrast to the potential effects of PI3K inhibition, adenovirus-mediated expression of dominant negative AktAAA and the subsequent effects on Akt, CXCL10 expression, and NFκB activation would occur after and thus independently of cell entry.

The rapid induction of NFκB and CXCL10 by Ad vectors also involves mechanisms independent of integrins and Akt.

Both Ly294002 and dominant negative AktAAA failed to completely suppress the Ad vector induction of NFκB-dependent gene expression. This is consistent with our previous finding that Ad vector activation of p38 mitoten-activated protein kinase and ERK signal transduction pathways in a postinternalization step of viral entry also contributes to CXCL10 expression (33). Thus, in nonhematopoietic cells, Ad vectors stimulate host inflammatory genes by activating at least two signal transduction pathways at different steps during virus-cell attachment and entry: the PI3K-Akt pathway, activated through capsid-dependent binding to cell surface integrins, and the mitoten-activated protein kinase pathway, activated postinternalization (33).

Host antiviral immune responses continue to be an obstacle for a wide application of Ad vectors in gene therapy. The development of newer generations of Ad vectors devoid of all viral coding sequences and thus viral replication has shown promising progress in the elimination of the antigen-specific immune responses. The innate arm of antiviral immunity remains a critical barrier to overcome, since it significantly reduces the efficiency and safety of Ad vectors for therapeutic purposes. This study increases our understanding of the molecular mechanisms underlying the cellular response to Ad vectors that is required to develop strategies to modulate Ad vector-induced innate responses.

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