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Adenovirus E3-10.4K/14.5K Protein Complex Inhibits Tumor Necrosis Factor-Induced Translocation of Cytosolic Phospholipase A_2 to Membranes

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We have reported that three adenovirus (Ad) proteins, named E3-10.4K/14.5K, E3-14.7K, and E1B-19K, independently inhibit tumor necrosis factor (TNF)-induced apoptosis in Ad-infected cells. E3-10.4K/14.5K and E3-14.7K also inhibit TNF-induced release of arachidonic acid (AA). TNF-induced apoptosis and AA release are thought to require TNF-activation of the 85-kDa cytosolic phospholipase A_2 ($cPLA_2$). $cPLA_2$ normally exists in a latent form in the cytosol; it is activated by phosphorylation by mitogen-activated protein kinase, and in the presence of agents that mobilize intracellular Ca^{2+}, $cPLA_2$ translocates to membranes where it cleaves AA from membrane phospholipids. We now report that TNF induces translocation of $cPLA_2$ from the cytosol to membranes in Ad-infected human A549 cells and that E3-10.4K/14.5K but not E3-14.7K or E1B-19K is required to inhibit TNF-induced translocation of $cPLA_2$. Ad infection also inhibited TNF-induced release of AA. Under the same conditions, Ad infection did not inhibit TNF-induced phosphorylation of $cPLA_2$ or TNF activation of NFκB. Ad infection also inhibited $cPLA_2$ translocation in response to the Ca^{2+} ionophore A23187 and to cycloheximide, but this inhibition did not require E3-10.4K/14.5K. Ad infection did not inhibit $cPLA_2$ translocation in response to interleukin-1β or platelet-derived growth factor. We propose that E3-10.4K/14.5K inhibits TNF-induced AA release and apoptosis by directly or indirectly inhibiting TNF-induced translocation of $cPLA_2$ from the cytosol to membranes. AA formed by $cPLA_2$ can be metabolized to prostaglandins, leukotrienes, and lipoxyns, molecules that amplify inflammation. E3-10.4K/14.5K probably functions in Ad infections to inhibit both TNF-induced apoptosis and inflammation.

Tumor necrosis factor (TNF) is a major inflammatory cytokine, secreted primarily by activated macrophages and T lymphocytes, that is thought to limit infections by a variety of microorganisms (reviewed in reference 68). In cultured cells infected with different RNA and DNA viruses, TNF inhibits virus replication or induces apoptosis (reviewed in references 21 and 79). Some viruses, in turn, have evolved strategies to block the antiviral effects of TNF (reviewed in references 11, 20, 47, 58, 68, 73, and 76–79). For example, poxviruses secrete TNF receptor homologs that bind TNF and presumably preclude TNF function (reviewed in reference 60).

Human adenoviruses (Ad) encode five proteins that modulate the effects of TNF (reviewed in references 20, 47, and 76–79). In contrast to poxviruses, the Ad proteins remain within the cell and therefore are useful tools to probe the cellular biology of TNF function. TNF induces apoptosis in a minority of tumor cell lines, but most cell types are resistant to TNF. Ad-infected cells are resistant to TNF, but they are lysed by TNF when infected with Ad mutants lacking certain genes (22). The Ad E1A proteins sensitize cells to TNF (7, 17, 59), and three sets of Ad proteins inhibit TNF-induced apoptosis (22–24, 33, 55). Two of these sets are encoded by the Ad E3 transcription unit: a 14,700-kDa protein named E3-14.7K (67) and a complex of two proteins of 10,400 and 14,500 kDa named E3-10.4K/14.5K (64, 65). The third TNF-negating protein, coded by the E1B transcription unit, is E1B-19K (21, 74).

The mechanisms of TNF-induced apoptosis and its inhibition by the Ad proteins are poorly understood. In most cell types, TNF induces apoptosis via the p55 TNF receptor (TNFR1) (reviewed in references 3, 12, 28, 69, 73). TNF-induced apoptosis requires the activation of one or more of the interleukin-1β (IL-1β) converting enzymes that function in apoptosis (reviewed in reference 29). There is also considerable evidence that TNF activation of the 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) is necessary but not sufficient for TNF-induced apoptosis (27, 72; reviewed in references 28 and 41). $cPLA_2$ can be activated by phosphorylation by mitogen-activated protein kinase (MAPK) (1, 14, 50, 52, 54, 56). Studies in vitro have shown that in the presence of submicromolar Ca^{2+}, $cPLA_2$ translocates to membranes where it cleaves arachidonic acid (AA) specifically from the *sn*-2 position of membrane phospholipids (6, 10, 51). In vivo, $cPLA_2$ activity is induced by many agonists, including IL-1β, platelet-derived growth factor (PDGF) (48–50), and TNF (27, 31, 72). TNF-induced release of AA and TNF-induced apoptosis, which require $cPLA_2$, are coordinately inhibited by E3-10.4K/14.5K and E3-14.7K, each able to act independently, in Ad-infected mouse cells (41). E3-14.7K also inhibits TNF-induced AA release and apoptosis in stably transfected mouse (41, 81) and human (30) cells. E1B-19K inhibits TNF-induced apoptosis in transfected human cells (74).

E1B-19K localizes primarily to the nuclear membrane and is considered to be a functional homolog of Bcl-2 (8, 63; reviewed

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in reference 73). Bcl-2 is a modest inhibitor of TNF-induced apoptosis (8, 36). E3-14.7K is a hydrophilic nonmembrane protein (30) that may function at or upstream of the proteolytic events that occur early in apoptosis (71). E3-10.4K/14.5K is an integral membrane protein complex (42, 43) that is localized in many cellular membranes, including the plasma membrane (32, 62, 66). E3-14.5K is phosphorylated on serine (45), and it is O glycosylated (44).

As judged by in vitro studies, agonists that activate cPLA$_2$ to release AA should not only phosphorylate cPLA$_2$ but they should also induce translocation of cPLA$_2$ from the cytosol to membranes in vivo. However, to our knowledge, translocation of cPLA$_2$ to membranes has only actually been shown in response to the Ca^{2+} ionophore A23187 (several cell types), basic fibroblast growth factor (endothelial cells), formylmethionyl (fMet)-Leu-Phe (neutrophils), immunoglobulin E-antigen (mast cells), and transforming growth factor α (keratinocytes) (18, 19, 39, 53, 56, 57). Here we report that TNF, IL-1β, PDGF, and cycloheximide (CHX), as well as A23187, induce translocation of cPLA$_2$ from the cytosol to membranes in A549 human alveolar epithelial carcinoma cells. Interestingly, E3-10.4K/14.5K, but not E3-14.7K or E1B-19K, is required to prevent TNF-induced translocation of cPLA$_2$ to membranes in Ad-infected A549 cells. This may explain how E3-10.4K/14.5K inhibits TNF-induced AA release and apoptosis. Ad infection also inhibited cPLA$_2$ translocation to membranes in response to CHX and A23187, but this inhibition did not require E3-10.4K/14.5K.

MATERIALS AND METHODS

Cells and viruses. A549 cells (American Type Culture Collection) were maintained in Dulbecco's minimal essential medium with 10% fetal calf serum. Viruses were grown in suspension cultures of KB cells, and the titers of CsCl-banded stocks on A549 cells were determined (25). The virus E3 mutants used and the proteins expressed by these mutants are indicated in Table 1 (2, 9, 37, 42–45, 55, 62, 64, 65). The isolation of the A549/14.5K cell line that stably expresses E3-14.5K will be described elsewhere.

Labeling of cells. A549 cells were grown in 35-mm-diameter dishes to 70% confluency, placed in phosphate-free medium for 18 h, and then labeled with 100 μCi of $Na_3{}^{32}PO_4$ (NEN DuPont Research Products, Boston, Mass.) for 6 h. Cells were infected with 60 to 100 PFU per cell in this same medium. At 8 h postinfection (p.i.), human recombinant TNF (250 U/ml, final concentration, was used in all experiments) was added, and the cells were harvested after 30 to 60 min. Some samples were treated with TNF or CHX (25 μg/ml). In the experiment whose results are shown in Fig. 6, cells were treated with 2 μM A23187, IL-1β (1 μg/ml), PDGF (5 ng/ml), or CHX (25 μg/ml) for 1 h.

Analysis of cPLA$_2$ in the membrane fraction. All steps were done at 1 to 4°C. Cells at 8.5 to 9 h p.i. were washed twice with Ca^{2+}-free phosphate-buffered saline, suspended with a cell scraper into 10 volumes of sucrose buffer (0.25 M sucrose, 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM sodium pyrophosphate, 1 mM Na_3PO_4, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 1 mM sodium deoxycholate), and disrupted with 10 strokes of a tight-fitting Dounce homogenizer. This procedure disrupted most cells and nuclei. Remaining whole cells and intact nuclei were removed by centrifugation at 1,000 × *g* for 10 min. The supernatant was centrifuged for 1 h at 100,000 × *g* in a Beckman Optima TLX ultracentrifuge. The pellet, consisting of most cellular membranes, including the nuclear membrane and the endoplasmic reticulum, was used for immunoprecipitation or immunoblot analysis.

All steps for immunoprecipitation of cPLA$_2$ were done at 1 to 4°C. Membrane pellets were solubilized in Luria-Bertani (LB) buffer (20 mM Tris-HCl [7.4], 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na_3PO_4, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 0.5 mM dithiothreitol, 1% Triton X-100) by vigorously pipetting several times, mixing briefly in a Vortex mixer, and rotating for at least 1 h. Remaining insoluble material was removed by centrifugation for 5 min in a microcentrifuge. Protein A-Sepharose beads in LB buffer were preincubated with a rabbit polyclonal antiserum to human cPLA$_2$ (a generous gift from the Genetics Institute, Boston, Mass.) for 1 h and then washed four times with LB buffer. Each membrane fraction was immunoprecipitated with 50 μl of 10% (wt/vol) antibody-containing beads for 1 h, washed four times with LB buffer, and then washed with water. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% polyacrylamide gels (acrylamide: *N*,*N*′-methylenebisacrylamide, 29.2:0.8, wt/wt), as previously described (67).

TABLE 1. E3 Ad mutant phenotypes: E3 proteins expressed[a] and inhibition of TNF-induced translocation of cPLA$_2$ to membranes

Mutant	Phenotype				Inhibition of TNF-induced cPLA$_2$ translocation
	E3-10.4K	E3-14.5K	E3-14.7K	Other E3 proteins	
*rec*700[b]	++	++	++	++	Yes
*dl*748[c]	−	+++++	−	−	No
*dl*753	−	+	++	++	No
*dl*759	±	−	++	++	No
*dl*764	++	−	++	++	No
*dl*763	++	−	−	++	No
*dl*762	++	++	−	++	Yes
*dl*798[c]	+++	−	+	+	No
*pm*760[c]	+++	+++	±	±	Yes
Ad5[d]	++	++	++	++	Yes
Ad5*dl*309	−	−	−	++	No
Ad5*dl*111[e]	−	−	−	++	No
*dl*7001[f]	−	−	−	−	No
*dl*7000	−	−	+++	−	No

[a] The following E3 proteins are expressed by *rec*700, Ad5, and Ad2: 12.5K, 6.7K, gp19K, ADP, 10.4K, 14.5K, and 14.7K. Phenotype symbols are explained in footnote *c*.

[b] *rec*700 is an Ad5-Ad2-Ad5 recombinant which is the parental virus for *dl*748, *dl*753, *dl*759, *dl*764, *dl*763, *dl*762, *dl*798, *pm*760, *dl*7001, and *dl*7000.

[c] E3-10.4K and E3-14.5K are both encoded by E3 mRNA *f*, and E3-14.7K is encoded by E3 mRNA *h* (8, 78). Some of the mutations in the mutants in this table affect alternative splicing of E3 mRNAs such that mRNAs *f* and *h*, as well as other E3 mRNAs, and the proteins they encode, are overproduced or underproduced. Also, some of the deletions mutate the gene so that the protein cannot be produced. For example, *dl*748 greatly overproduces mRNA *f* and underproduces all other E3 mRNAs, and it has a deletion in the gene for E3-10.4K; thus, E3-10.4K is not made, E3-14.5K is greatly overproduced, and the other E3 proteins are barely detectable (64, 65). The relative levels of the E3 proteins synthesized by these mutants are indicated by the number of plus signs. *dl*798 overexpresses E3-10.4K, but it does not express E3-14.5K. *pm*760 overexpresses both E3-10.4K and E3-14.5K.

[d] Ad5 is the parental virus for *dl*309 and *dl*111.

[e] *dl*111 has the same E3 deletion as *dl*309, and it does not express E1B-19K.

[f] *dl*7001 deletes all E3 genes, and *dl*7000 deletes all E3 genes except that for E3-14.7K.

For immunoblots, membrane pellets were solubilized in 2× Laemmli buffer, subjected to SDS-PAGE as described above, and then transferred to Immobilon D (Millipore) membranes for 30 min with a Trans-Blot (Bio-Rad) semidry apparatus. Mouse monoclonal antibody against cPLA$_2$ (a gift from the Genetics Institute) was used at a dilution of 1:1,000. The second antibody, rabbit anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Amersham Life Sciences), was used at a dilution of 1:3,000. cPLA$_2$ was visualized with an ECL kit (Amersham Life Sciences).

Analysis of TNF-induced SDS-PAGE mobility shift of cPLA$_2$. Cells at 8 h p.i. were treated with TNF for 15 min, washed twice with phosphate-buffered saline, and then lysed in boiling hot 2× Laemmli buffer (200 μl of buffer per 30-mm-diameter dish). The cells were subjected to four cycles of boiling and vigorous mixing to reduce viscosity, and then 20 μl was loaded onto a 20-cm-long 7.0% polyacrylamide gel (acrylamide: *N*,*N*′-methylenebisacrylamide, 60:1, wt/wt). The samples were subjected to SDS-PAGE; electrophoresis was terminated 4 h after the tracking dye had run off the bottom of the gel. cPLA$_2$ was detected by immunoblotting as described above.

[^{3}H]AA release. Cells were labeled for 18 h in Dulbecco's minimal essential medium containing 10% fetal calf serum and 0.5 μCi of [5,6,8,12,14,15-^{3}H]AA (180 to 240 Ci/mmol; NEN Dupont) per ml. The cells were washed, infected with Ad, and treated with TNF at 8 h p.i., and then [^{3}H]AA released into the culture supernatant was counted.

EMSA for NFκB. Mock- or virus-infected cells were untreated or treated with TNF at 8 h p.i. After 1 h, cytosol and membrane fractions were prepared. The cytosol was assayed for NFκB binding activity by electrophoretic mobility shift assay (EMSA) as described previously (16). Typically, 2 μl of cell lysate was incubated in 20 μl of HDKE buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 1.0 mM EDTA, 5% glycerol) containing 1 μg of poly(dI-dC) per μl for 10 min at room temperature prior to the addition of 10^5 cpm of ^{32}P-labeled oligonucleotide no. 1. After 10 min, bound DNA was separated from free DNA by electrophoresis through a 4% native polyacrylamide gel and visualized by autoradiography. For the competition experiments, a 100-fold molar excess of unlabeled oligonucleotides was added to the samples for 10 min prior to the addition of the ^{32}P-labeled oligonucleotide.

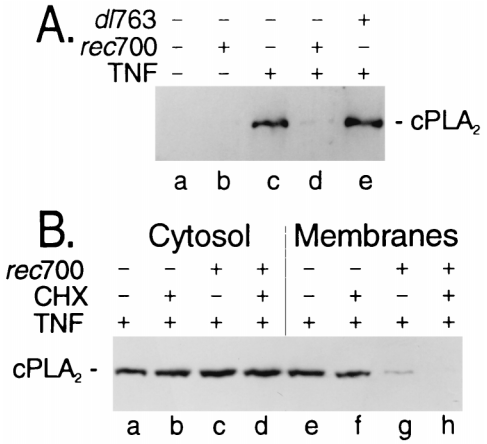

FIG. 1. *rec*700 (wild type) but not *dl*763 (14.5K$^-$ and 14.7K$^-$) inhibits TNF-induced translocation of $cPLA_2$ to membranes. (A) A549 cells were mock infected or infected with *rec*700 or *dl*763 and untreated or treated with TNF for 30 min at 8 h p.i., and then membranes were isolated and probed for $cPLA_2$ by immunoblotting. (B) Same as for panel A, except some cells were treated with CHX (25 μg/ml), and $cPLA_2$ in both the cytosol (lanes a to d) and membrane (lanes e to h) fractions was detected by immunoblotting.

The sequences of the oligonucleotides, with the NFκB motif underlined, are as follows (the oligonucleotides are duplex; only the 5′→3′ strand is shown): oligonucleotide no. 1, 5′-TCGAGGGGAATTCCCGTCGA-3′; oligonucleotide no. 2, 5′-CCGAACATTGCACAATCTGGGAATTCCCCCGA-3′; oligonucleotide no. 3, 5′-CCGAACATTGCACAATCTATCTATCTCCCCGA-3′; oligonucleotide no. 4, 5′-CCGAACACTACAAACTCTGGGAATTCCCCCGA-3′.

RESULTS

Ad E3-10.4K/14.5K protein complex is required to inhibit TNF-induced translocation of $cPLA_2$ to membranes. $cPLA_2$-induced cleavage of AA occurs when $cPLA_2$ is phosphorylated by MAPK and is translocated from the cytosol to membranes in the presence of small amounts of Ca^{2+}. As shown in Fig. 1A, $cPLA_2$ was not detectable by immunoblotting in the membrane fraction from A549 cells that were mock infected (lane a) or infected with *rec*700 (wild-type Ad) (lane b). Treatment with TNF for 30 min caused $cPLA_2$ to translocate to membranes (Fig. 1A, lane c). TNF-induced translocation of $cPLA_2$ was blocked in cells infected with *rec*700 (Fig. 1A, lane d) but not with *dl*763, a mutant that lacks E3-14.5K and E3-14.7K (Fig. 1A, lane e). When both the cytosol and membrane fractions were examined, neither TNF, CHX (which sensitizes cells to TNF), nor *rec*700 markedly affected $cPLA_2$ levels in the cytosol (Fig. 1B, lanes a to d). However, *rec*700 inhibited translocation to membranes when cells were treated with TNF alone (Fig. 1B, lane g) or TNF plus CHX (Fig. 1B, lane h). These results indicate that TNF induces translocation of $cPLA_2$ from the cytosol to membranes; that neither TNF, *rec*700, nor CHX markedly affects the total amount of $cPLA_2$ in the cell; that wild-type *rec*700 blocks TNF-induced translocation of $cPLA_2$ to membranes; that E3 proteins deleted from *dl*763 (14.5K and 14.7K) are required to inhibit TNF-induced translocation of $cPLA_2$; and that E1B-19K, which is expressed by *dl*763 (data not shown), does not block TNF-induced translocation of $cPLA_2$.

The kinetics of TNF-induced translocation of $cPLA_2$ to membranes were examined, in this case by metabolically labeling $cPLA_2$ with $Na_3{}^{32}PO_4$ and then immunoprecipitating ^{32}P-labeled $cPLA_2$ from the membrane fraction. Latent inactive $cPLA_2$ is phosphorylated at multiple sites by unknown kinases (15, 54), so this experiment measures total translocated $cPLA_2$,

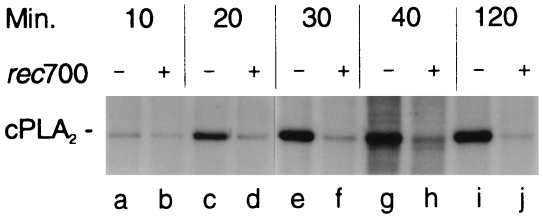

FIG. 2. TNF-induced translocation of $cPLA_2$ to membranes occurs within 20 min, is sustained until at least 2 h, and is inhibited by *rec*700. Cells were labeled with $Na_3{}^{32}PO_4$, infected with *rec*700, and treated with TNF from 8 to 9 h p.i., and ^{32}P-labeled $cPLA_2$ was immunoprecipitated from the membrane fraction.

not simply $cPLA_2$ that has been phosphorylated in response to TNF. (Neither Ad infection nor TNF treatment markedly affected the bulk of total cellular $cPLA_2$ that was metabolically labeled with $Na_3{}^{32}PO_4$ under these conditions; see Fig. 7.) TNF-induced translocation of [^{32}P]$cPLA_2$ was barely detectable after 10 min of TNF treatment, was obvious after 20 min, continued to increase until 120 min (Fig. 2, lanes a, c, e, g, and i), and was still apparent after 6 h (data not shown). Translocation of [^{32}P]$cPLA_2$ was not observed in *rec*700-infected cells at 10 to 120 min following TNF treatment (Fig. 2, lanes b, d, f, h, and j). Thus, in subsequent experiments cells were treated with TNF for 30 to 60 min prior to immunoprecipitating [^{32}P]$cPLA_2$ from the membrane fraction.

Selected Ad mutants were used to further examine the E3 genes responsible for blocking TNF-induced translocation of $cPLA_2$ to membranes (the E3 proteins expressed by these mutants are indicated in Table 1). As expected, TNF-induced translocation of [^{32}P]$cPLA_2$ was not inhibited by *dl*7001, a mutant that lacks all E3 genes (Fig. 3A, lane d). E3-14.7K is one of the proteins that inhibits TNF-induced release of AA and apoptosis (22, 23, 24, 33, 41, 55, 81). However, E3-14.7K is not required to inhibit $cPLA_2$ translocation, because translocation was inhibited with *dl*762 which lacks only 14.7K, and it was not inhibited with *dl*7000 in which 14.7K is the only E3

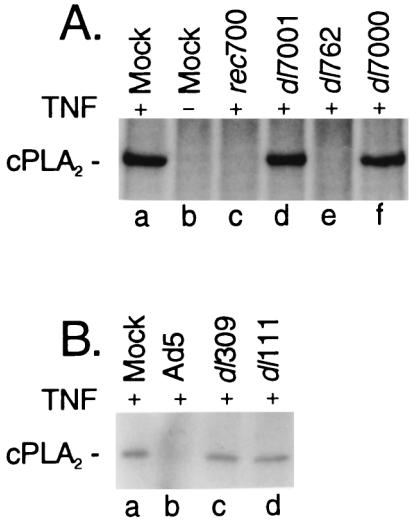

FIG. 3. Inhibition of $cPLA_2$ translocation does not require E3-14.7K, and it occurs with Ad5 as well as *rec*700. (A) Mock- or E3 mutant-infected cells labeled with $Na_3{}^{32}PO_4$ were untreated or treated with TNF at 8 to 9 h p.i., and then [^{32}P]$cPLA_2$ was immunoprecipitated from the membrane fraction. *dl*7001 lacks all E3 genes, *dl*7000 expresses only E3-14.7K, and *dl*762 lacks only E3-14.7K. (B) Same conditions as for panel A. *dl*309 lacks E3-10.4K, E3-14.5K, and E3-14.7K. *dl*111 lacks the same E3 genes as *dl*309, but it also lacks E1B-19K.

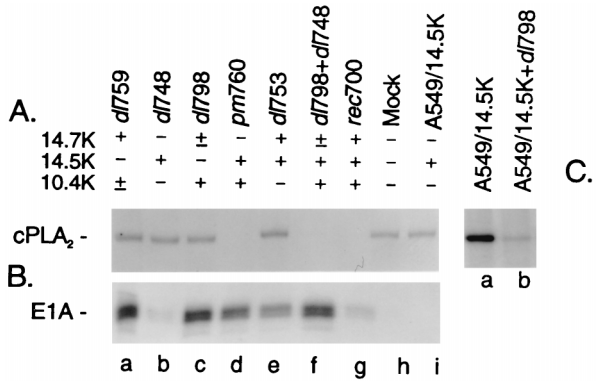

FIG. 4. Both E3-10.4K and E3-14.5K are required to inhibit TNF-induced translocation of cPLA$_2$ to membranes. (A) Mock- or E3 mutant-infected cells labeled with $Na_3{}^{32}PO_4$ were treated with TNF at 8 to 9 h p.i., and then [^{32}P] cPLA$_2$ was immunoprecipitated from the membrane fraction. In lane f, cells were coinfected with *dl*798 and *dl*748 (100 PFU/cell for each mutant). A549/14.5K (lane i) is a clonal line of A549 cells stably expressing E3-14.5K. (B) Immunoprecipitation of ^{32}P-labeled Ad E1A proteins from the membrane supernatant of the extracts used in panel A. (C) Immunoprecipitation of [^{32}P] cPLA$_2$ from the membrane fraction of mock- or *dl*798-infected A549/14.5K cells.

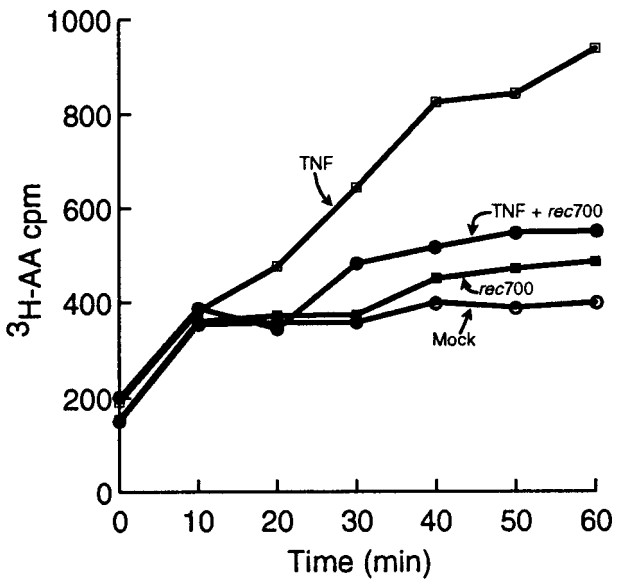

FIG. 5. *rec*700 inhibits TNF-induced release of [^{3}H]AA. Cells were prelabeled with [^{3}H]AA, mock infected or infected with *rec*700, and treated with TNF from 8 to 9 h p.i., and [^{3}H]AA release into the culture supernatant was determined.

protein expressed (Fig. 3A, lanes e and f). As a positive control for infection with these mutants, ^{32}P-labeled Ad E1A proteins were immunoprecipitated from the same extracts as [^{32}P] cPLA$_2$; all mutants expressed the E1A proteins (data not shown).

Ad type 5 (Ad5) inhibited [^{32}P]cPLA$_2$ translocation (Fig. 3B, lane b), establishing that this property is not unique to *rec*700, an Ad5-Ad2-Ad5 recombinant. As expected, inhibition was not observed with the Ad5 mutant *dl*309, which lacks the E3 10.4K, 14.5K, and 14.7K proteins (4) (Fig. 3B, lane c), nor was inhibition observed with *dl*111, which lacks the same E3 genes as *dl*309 and also lacks E1B-19K (2) (Fig. 3B, lane d).

Additional mutants were used to establish more precisely which E3 proteins are required to inhibit TNF-induced translocation of cPLA$_2$. As shown in Fig. 4A, TNF-induced [^{32}P] cPLA$_2$ translocation (lane h) was prevented by *rec*700 (lane g) and *pm*760 (lane d), a mutant that markedly overproduces E3-10.4K and E3-14.5K and underproduces all other E3 proteins (64). Translocation of cPLA$_2$ was not inhibited by *dl*759 or *dl*798, mutants that express E3-10.4K but not E3-14.5K, or by *dl*748 or *dl*753, mutants that express E3-14.5K but not E3-10.4K. *dl*759 and *dl*753 express all other E3 proteins at wild-type levels, so E3 proteins other than E3-10.4K and E3-14.5K do not prevent translocation of cPLA$_2$. When cells were coinfected with *dl*798 (which provides E3-10.4K) and *dl*748 (which provides E3-14.5K), translocation of cPLA$_2$ was blocked (Fig. 4A, lane f); this indicates that *dl*798 and *dl*748 complement and that both E3-10.4K and E3-14.5K are required to inhibit translocation of cPLA$_2$.

As a further test of this conclusion, we examined an A549 cell line (A549/14.5K) stably transfected with the gene for E3-14.5K. These cells were confirmed by immunoprecipitation to express E3-14.5K (data not shown). TNF-induced translocation of [^{32}P]cPLA$_2$ was not prevented in the A549/14.5K cell line (Fig. 4A, lane i). However, when these cells were infected with *dl*798 (10.4K$^+$ and 14.5K$^-$), in which the cells provide E3-14.5K and *dl*798 provides E3-10.4K, cPLA$_2$ translocation was nearly completely blocked (Fig. 5C). This result indicates again that E3-10.4K and E3-14.5K function in concert to inhibit TNF-induced translocation of cPLA$_2$.

If *rec*700 inhibits TNF-induced translocation of cPLA$_2$ to membranes where cPLA$_2$ generates AA, then *rec*700 should inhibit TNF-induced release of AA. To test this, cells were prelabeled with [^{3}H]AA, mock infected or infected with *rec*700, and treated with TNF, and the release of [^{3}H]AA into the medium was determined. As expected, *rec*700 inhibited TNF-induced release of [^{3}H]AA (Fig. 5). We have shown previously that mutants which lack the E3-10.4K, E3-14.5K, and E3-14.7K proteins do not inhibit TNF-induced release of AA (41).

The ability of Ad to inhibit translocation of cPLA$_2$ to membranes induced by several other agonists was examined. As shown in Fig. 6A, TNF, IL-1β, A23187, CHX, and PDGF induced translocation of [^{32}P]cPLA$_2$ to membranes. *rec*700

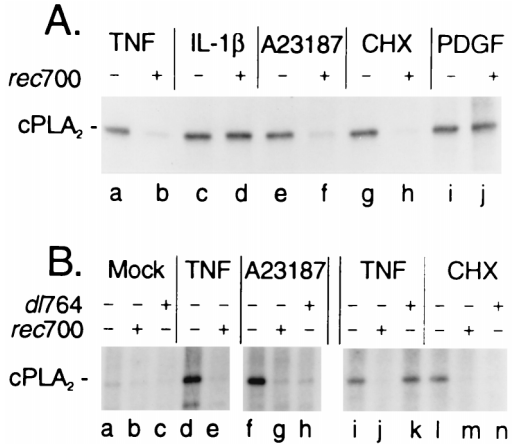

FIG. 6. *rec*700 inhibits cPLA$_2$ translocation in response to TNF, the calcium ionophore A23187, and CHX but not in response to IL-1β or PDGF; only inhibition of TNF-induced cPLA$_2$ translocation requires E3-14.5K. (A) Mock- or *rec*700-infected cells labeled with $Na_3{}^{32}PO_4$ were treated with TNF, IL-1β, A23187, CHX, or PDGF at 8 to 9 h p.i., and then [^{32}P]cPLA$_2$ was immunoprecipitated from the membrane fraction. (B) Same conditions as for panel A. *dl*764 lacks only E3-14.5K.

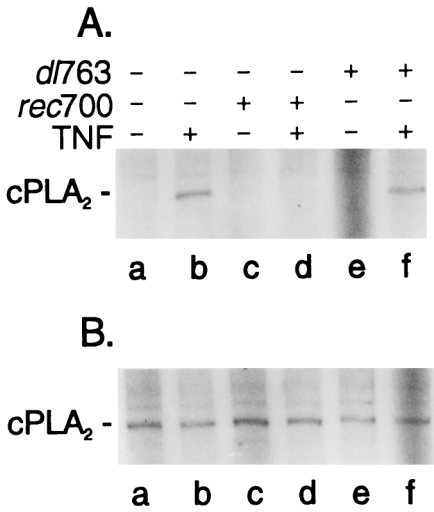

FIG. 7. *rec*700 does not affect the phosphorylation of $cPLA_2$. Cells were labeled with $Na_3{}^{32}PO_4$, infected with *rec*700 or *dl*763 ($14.5K^-$ and $14.7K^-$), and then treated with TNF at 8 to 9 h p.i. Membrane (A) and cytosol (B) fractions were isolated, and ^{32}P-labeled $cPLA_2$ was immunoprecipitated.

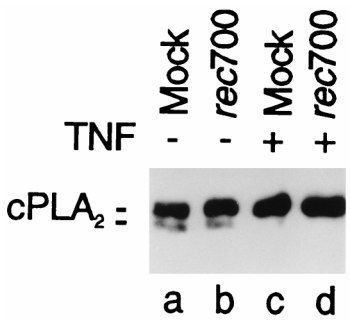

FIG. 8. *rec*700 does not inhibit TNF-induced mobility shift of $cPLA_2$ in SDS-PAGE. Mock- or *rec*700-infected cells were treated with TNF for 15 min at 8 h p.i., and then total cellular $cPLA_2$ was examined by immunoblotting with a 20-cm gel with an acrylamide/bisacrylamide ratio of 60:1. The upper band has been reported to be diagnostic of phosphorylation of $cPLA_2$ on serine 505, an event that increases the activity of $cPLA_2$.

inhibited $cPLA_2$ translocation in response to TNF, A23187, and CHX but not in response to IL-1β or PDGF. Thus, Ad inhibits $cPLA_2$ translocation induced by some but not all agonists. Mutant *dl*764 ($14.5K^-$) inhibited $[^{32}P]cPLA_2$ translocation induced by A23187 (Fig. 6B, lanes f to h) or CHX (lanes l to n) but not that induced by TNF (lanes i to k). Thus, E3-10.4K/14.5K is required to block $cPLA_2$ translocation in response to TNF but not in response to A23187 or CHX.

Ad infection does not inhibit TNF-induced phosphorylation of $cPLA_2$ or activation of NFκB. $cPLA_2$ is phosphorylated at multiple sites (15, 54). To assess how TNF treatment and Ad infection affect the phosphorylation of $cPLA_2$, cells were infected with *rec*700 or *dl*763 ($14.5K^-$ and $14.7K^-$), labeled for 6 h with $Na_3{}^{32}PO_4$, and treated or not treated with TNF, and $cPLA_2$ was immunoprecipitated from the membrane and cytosol fractions. The total amounts of $[^{32}P]cPLA_2$ in the membrane-plus-cytosol extract from each infection appeared to be roughly similar, e.g., the amount of $cPLA_2$ in lane a of Fig. 7B is similar to the sum of the $cPLA_2$ in lanes b of Fig. 7A and B. Thus, neither TNF treatment nor Ad infection markedly affects the bulk of $cPLA_2$ that can be metabolically labeled with $Na_3{}^{32}PO_4$.

Phosphorylation of $cPLA_2$ on serine 505 by MAPK is reported to decrease the mobility of $cPLA_2$ in SDS-PAGE (1, 50, 52). Indeed, two bands of $cPLA_2$ could be resolved on a 20-cm-long gel with a 60:1 ratio of acrylamide to bisacrylamide (Fig. 8, lane b). Most of the $cPLA_2$ in untreated cells migrated as the slower band. TNF treatment shifted all of the $cPLA_2$ to the slower band (Fig. 8, lane c); this shift was not affected by *rec*700 infection (lane d). These results provide indirect evidence that TNF induces phosphorylation of $cPLA_2$, perhaps on serine 505, and that this phosphorylation is not affected by Ad infection. The observation that TNF induces a relatively small fraction of $cPLA_2$ to the slower-migrating form (Fig. 8) is consistent with the observation that TNF does not markedly increase the metabolic labeling of $cPLA_2$ with $Na_3{}^{32}PO_4$ (Fig. 7).

The results in Fig. 8 indicate that Ad infection does not inhibit all aspects of TNF signal transduction. As an alternative means to address this question, TNF activation of NFκB was examined by EMSA at 8 h p.i. As shown in Fig. 9, neither *rec*700 nor *dl*763 ($14.5K^-$ and $14.7K^-$) inhibited the TNF-induced gel shift of a ^{32}P-labeled oligonucleotide containing NFκB binding sites. In this same experiment, TNF-induced translocation of $cPLA_2$ to membranes was completely blocked (data not shown). Thus, inhibition of $cPLA_2$ translocation does not reflect a general inhibition of TNF signal transduction.

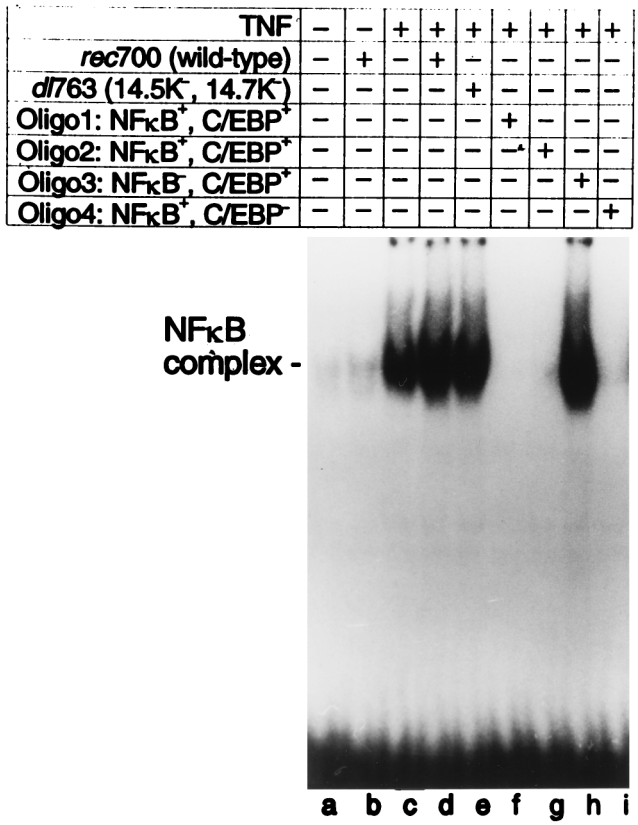

FIG. 9. *rec*700 and *dl*763 do not inhibit TNF activation of NFκB. Mock- or virus-infected cells were treated with TNF from 8 to 9 h p.i. and then fractionated into cytosol and membranes. The cytosol was assayed for NFκB activity by EMSA. In lanes f to i, unlabeled oligonucleotides that do or do not contain binding motifs for the NFκB and C/EBP transcription factors were used (see Materials and Methods). Competition was observed with oligonucleotides no. 1, 2, and 4, which contain the NFκB motif. In the membrane fraction, *rec*700 but not *dl*763 ($14.5K^-$ and $14.7K^-$) inhibited translocation of $cPLA_2$ to membranes (data not shown).

DISCUSSION

Three sets of Ad proteins, E3-10.4K/14.5K, E3-14.7K, and E1B-19K, have been implicated in inhibition of TNF-induced apoptosis in cells infected with Ad mutants. E3-10.4K/14.5K and E3-14.7K have also been shown to inhibit TNF-induced release of AA mediated by cPLA$_2$. TNF activation of cPLA$_2$ activity to generate AA from membrane phospholipids is necessary but not sufficient for TNF-induced apoptosis. For most agonists, it is believed that synthesis of AA by cPLA$_2$ requires that cPLA$_2$ be activated by phosphorylation, perhaps on serine 505 by MAPK, and that cPLA$_2$ translocate to membranes in a Ca^{2+}-dependent manner (6, 10, 19, 50–52, 57). In this report, we have shown that TNF induces translocation of cPLA$_2$ from the cytosol to membranes. We have also established that E3-10.4K/14.5K, but not E3-14.7K or E1B-19K, is required to inhibit TNF-induced translocation of cPLA$_2$ to membranes in human A549 cells infected with Ad mutants lacking these proteins. Thus, E3-10.4K/14.5K may block TNF-induced AA release and apoptosis by preventing translocation of activated cPLA$_2$ to membranes.

In addition to TNF, we have shown that IL-1β, PDGF, and A23187 induce cPLA$_2$ translocation to membranes in vivo. This is expected because these agents induce AA release. We have also shown that CHX induces cPLA$_2$ translocation; CHX does not induce significant AA release (41, 81), so a second event, perhaps phosphorylation, must be required to activate cPLA$_2$. Ca^{2+} is required for cPLA$_2$ to associate with membranes in vitro, and A23187 induces cPLA$_2$ translocation in vivo. Phosphorylation by MAPK is not required for cPLA$_2$ translocation in vivo (57) or in vitro (10, 51). Thus, TNF, IL-1β, PDGF, and CHX probably induce cPLA$_2$ translocation by mobilizing the submicromolar amounts of Ca^{2+} that are required for cPLA$_2$ translocation to occur. Ad infection inhibited cPLA$_2$ translocation in response to A23187 and CHX; we are attempting to identify the Ad genes responsible for this inhibition.

It is unlikely that E3-10.4K/14.5K inhibits the actual cPLA$_2$ translocation step, because Ad infection did not inhibit cPLA$_2$ translocation in response to IL-1β or PDGF. Considering that Ca^{2+} but not phosphorylation is required for translocation of cPLA$_2$, our results could be interpreted to indicate that E3-10.4K/14.5K inhibits TNF-induced increase in intracellular Ca^{2+}. If so, then E3-10.4K/14.5K could function anywhere in the signal transduction pathway from the TNF receptor to this putative TNF-specific Ca^{2+} channel.

There are two receptors for TNF, TNFR1 and TNFR2. Most aspects of signal transduction in nonhematopoietic cells, including apoptosis and activation of NFκB, occur via TNFR1 (reviewed in reference 28). E3-10.4K/14.5K is known to stimulate the endosome-mediated internalization and degradation of the receptors for epidermal growth factor (5, 66), insulin, and insulin-like growth factor 1 (46). We do not know whether E3-10.4K/14.5K also down-regulates TNFR1 or TNFR2. If so, this probably does not explain the ability of E3-10.4K/14.5K to inhibit cPLA$_2$ translocation, because such inhibition was observed in cells where TNF was able to induce phosphorylation of cPLA$_2$ and to activate NFκB. Ad infection also did not inhibit TNF-induced activation of NFκB in mouse L929 cells (30).

Interaction of TNF with TNFR1 induces oligomerization of the receptor and binding of several proteins to the receptor (reviewed in reference 3). TRADD binds the receptor, and FADD, TRAF2, and RIP bind TRADD. TNFR1-TRADD-FADD complexes signal apoptosis, and TNFR1-TRADD-TRAF2 complexes activate NFκB (35). TNFR1-TRADD-RIP complexes induce both apoptosis and NFκB activation (34). The mechanisms by which these protein complexes signal are unknown. One possibility is that sphingomyelinases are activated by TNF to generate ceramide, which acts as a second messenger (reviewed in references 26 and 40). Neutral and acidic sphingomyelinases reportedly are involved in apoptosis and NFκB activation (75). Ceramide activates a ceramide-activated protein phosphatase which may indirectly induce apoptosis (26). Ceramide also activates ceramide-activated protein kinase (40). All of these proteins are associated with membranes, and therefore, inhibition of any of their activities could be the mechanism by which the E3-10.4K/14.5K integral membrane protein complex blocks cPLA$_2$ translocation.

TNF modestly activates MAPK, but it strongly activates the stress-induced pathways where c-Jun kinase and a p38 kinase play a role analogous to that of MAPK (reviewed in references 13 and 38). These stress pathways have been implicated in apoptosis (70, 80). However, it is not known whether these pathways are affected by E3-10.4K/14.5K.

Mice infected in the lung with Ad suffer a modest inflammatory response which is dramatically enhanced with Ad mutants that lack both E3-10.4K/14.5K and E3-14.7K (61). Expression of either E3-10.4K/14.5K or E3-14.7K is sufficient to give a wild-type phenotype. AA generated by cPLA$_2$ can be metabolized to the proinflammatory prostaglandins and leukotrienes. Thus, E3-10.4K/14.5K may inhibit not only apoptosis but also inflammation in Ad infections by inhibiting TNF-induced translocation of cPLA$_2$ to membranes.

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