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Inhibition of the Production of Rat Cytokine-Induced Neutrophil Chemoattractant (CINC)–1, a Member of the Interleukin-8 Family, by Adenovirus-Mediated Overexpression of IκBα

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Cytokine-induced neutrophil chemoattractant (CINC)–1, a counterpart of the human growth-regulated gene product (GRO) of the interleukin-8 family, has been suggested to play critical roles as a mediator of inflammatory reactions with neutrophil infiltration in rats. NF-κB has been speculated to be involved in the production of CINC-1, since the NF-κB–binding domain is important for the CINC-1 promoter activity in several of our reporter assays. In the present study, we examined the effects of an overexpression of IκBα, a specific natural inhibitor of NF-κB, on CINC-1 production. For this purpose, we constructed two recombinant adenoviruses, AxCAIκBα and AxCAmutantIκBα, which express respectively wild IκBα and a mutated nondegradable IκBα in which serine residues 32 and 36 are replaced by alanine residues. Transfecting wild and mutant IκBα by these adenovirus vectors inhibited NF-κB activation and CINC-1 production, which were both caused by IL-1β stimulation in the normal rat kidney epithelial cell line NRK-52E. We also showed that the nondegradable mutant IκBα was approximately 30 times more potent than the wild type in inhibiting CINC-1 production. These findings demonstrate that CINC-1 production with NF-κB activation is primarily regulated by non-phosphorylated IκBα in the cytoplasm.

Key words: CINC-1, GRO, IκBα, NF-κB, recombinant adenovirus.

The accumulation of neutrophils at inflammatory sites is the characteristic response in acute inflammation. This neutrophil infiltration is caused by chemotactic factors including C5a, leukotriene B4, interleukin-8 (IL-8), and its related chemokines. We found a new polypeptide neutrophil chemotactic factor, cytokine-induced neutrophil chemoattractant (CINC), from the normal rat kidney epithelial cell line, NRK-52E (*1*, *2*). On the basis of its amino acid sequence, CINC is a rat counterpart of human growth-regulated gene product (GRO) (*2*, *3*), which is not the counterpart of IL-8 but is a member of the IL-8 family. In spite of previous substantial efforts, rat counterparts of IL-8 have not yet been identified (*4*, *5*), and therefore, in rats, CINC is thought to play a major role as a neutrophil chemoattractant. The IL-8 family acts as a functional chemoattractant for neutrophils in various animal models of inflammation *in vivo* (*6–9*). Our previous studies showed that CINC also produced a marked neutrophil infiltration at inflammatory sites in lipopolysaccharide (LPS)-induced inflammation models in rats (*4*, *10*). Thus, the regulation of CINC and/or IL-8 production is thought to be a critical step in the control of inflammatory reactions associated with neutrophil infiltration.

Recently, Nakagawa *et al.* purified four basic chemokines from a conditioned medium of rat granulation-tissue culture (*11*). Two of the four chemokines were identical to CINC and rat macrophage inflammatory protein–2 (MIP-2) (*12*), and the others were new chemokines related to CINC. These two novel chemokines were named CINC-2α and CINC-2β. Consequently, they have proposed to name CINC and rat MIP-2 as CINC-1 and CINC-3, respectively (*11*). Therefore, we also refer to CINC as CINC-1 according to their nomenclature.

NF-κB is widely accepted to be a ubiquitous transcription factor which is activated by a variety of cytokines and mitogens (*13*) and is thought to be a key regulator of genes involved in inflammation. We have shown that the NF-κB–binding site in the promoter region of CINC is indispensable for the IL-1β–induced CINC promoter activation (*14*), and suggested that CINC production is also controlled by the key gene regulator NF-κB.

NF-κB is primarily a p50/p65 heterodimer in NRK-52E cells (*14*) and is retained in an inactive form associated with IκBα, a specific natural inhibitor of NF-κB, in the

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Abbreviations: AxCAIκBα, recombinant adenovirus expressing IκBα; AxCAmutantIκBα, recombinant adenovirus expressing mutant IκBα where serine residues 32 and 36 were replaced by alanine residues; CINC, cytokine-induced neutrophil chemoattractant; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GRO, growth-regulated gene product; IL-8, interleukin-8; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MOI, multiplicity of infection; PMSF, phenylmethylsulfonyl fluoride.

cytoplasm. After inflammatory stimulation, phosphorylation of IκB leads to its ubiquination and subsequent proteolytic degradation, enabling NF-κB to translocate from the cytoplasm into the nucleus (*15*, *16*). IκBα is phosphorylated upon stimulation at serine residues 32 and 36 located in the N-terminal part of the polypeptide (*16*, *17*). Mutation of these two amino acids has been shown to effectively prevent IκBα phosphorylation, its degradation, and NF-κB activation (*16*, *17*).

These findings prompted us to examine whether the inflammation reactions mediated by CINC-1 could be inhibited by overexpressing IκBα. In the present study, we constructed two recombinant adenoviruses to express wild IκBα and mutant IκBα in which serine residues 32 and 36 were replaced by alanine residues (S32A/S36A), and examined their inhibition activities against CINC-1 production in cultured NRK-52E cells when overexpressed by a technique of gene transfer. We report here that the adenovirus-mediated overexpression of IκBα inhibits NF-κB activation and CINC-1 production in cultured NRK-52E cells.

MATERIALS AND METHODS

Reagents—Human recombinant IL-1β was obtained from R&D Systems (Minneapolis, MN). Dulbecco's modified Eagle's medium (DMEM) and AlamarBlue were purchased from Iwaki Glass (Chiba). Fetal bovine serum (FBS) was obtained from Dainippon Pharmaceutical (Osaka). Protease inhibitors, phenylmetylsulfonyl fluoride (PMSF), leupeptin, aprotinin, and pepstatin were from Boehringer Mannheim (Indianapolis, IN). Polyclonal antibodies raised against MAD-3 (IκBα) and p65 were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Cells—The normal rat kidney epithelial cell line, NRK-52E, and 293 cells were purchased from Flow Laboratories (Tokyo) and Dainippon Pharmaceutical, respectively. The cells were cultured in DMEM supplemented with 10% FBS.

Preparation of Whole Cell, Cytoplasmic, and Nuclear Extracts—Whole cell extracts were prepared by lysing cells in Laemmli buffer (*18*) after washing the cells twice with phosphate-buffered saline. Cytoplasmic and nuclear extracts were prepared as described by Schreiber *et al.* (*19*) with slight modifications. Briefly, cells were harvested, washed in phosphate-buffered saline, resuspended in Buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μg of leupeptin per ml, 10 μg of aprotinin per ml, 2 μg of pepstatin per ml], and incubated for 15 min on ice. Nonidet P-40 was added to a final concentration of 0.6%, and the mixture was mixed vigorously. Nuclei were pelleted by centrifugation at 2,000 ×*g* for 5 min, and the supernatants were used as cytoplasmic extracts. The pelleted nuclei were extracted at 4°C for 30 min with Buffer B [20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μg of leupeptin per ml, 10 μg of aprotinin per ml, 2 μg of pepstatin per ml] prior to centrifugation at 15,000 ×*g* for 20 min at 4°C. The supernatants were used as nuclear extracts. The protein concentration was measured by use of a Bio-Rad protein assay kit with bovine serum albumin as the standard.

Western Blot Analysis—Whole cell, cytoplasmic, or nuclear extracts were used for Western blot analysis. Samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were incubated in blocking solution (Block Ace, Dainippon Pharmaceutical) overnight at 4°C and exposed to 500-fold diluted primary antibodies against IκBα or p65 for 2 h at room temperature. After washing, the membranes were incubated for 1 h at room temperature with 1,000-fold diluted horseradish peroxidase–conjugated donkey anti–rabbit IgG antibody (Amersham, Arlington Heights, IL) followed by enhanced chemiluminescence (ECL) detection (Amersham).

Construction of Recombinant Adenoviruses—The recombinant replication-deficient adenoviruses for the expression of IκBα and mutant IκBα (S32A/S36A) were constructed using cassette cosmids pAxCAwt (*20*). cDNAs of rat wild IκBα and rat mutant IκBα (S32A/S36A) were blunt-ended and inserted into pAxCAwt at the *Swa*I site, so that the inserted cDNAs are driven by the CAG promoter (*21*). The recombinant viruses were prepared according to the method described previously (*22*). Briefly, cosmid DNA was co-transfected with *Eco*T22I-digested DNA-terminal protein complex of Ad5-dlX into 293 cells to generate the recombinant viruses by homologous recombination. The recombinant viruses were designated as AxCAIκBα (for rat wild IκBα expression) and AxCAmutantIκBα [for rat mutant IκBα (S32A/S36A) expression]. After the third propagation in 293 cells, virions were extracted from the 293 cells, purified by double cesium step-gradient purification (*23*), dialyzed against 10% glycerol in phosphate-buffered saline, and stored at –80°C. The titers of recombinant viruses were determined by the modified end-point cytopathic effect assay on 293 cells (*23*) and expressed in plaque-forming units (pfu). Adex1w (*22*), which is the recombinant replication-deficient adenovirus carrying no cDNA insert, was also grown and purified as described above and used as a control recombinant adenovirus.

Viral Infection and Treatment of Cells—NRK-52E cells were seeded at 5×10^4 cells in 48-well culture plates (Costar Corp., Cambridge, MA). On the following day, the cells were infected by recombinant adenoviruses for 1 h at 37°C in 100 μl of culture medium supplemented with 5% FBS. Infected cells were thereafter incubated in a fresh culture medium containing 5% FBS. After 24 h, the cells were stimulated with IL-1β. For Western blot analysis, the cell extracts were prepared after a 30-min stimulation as described above. For the enzyme-linked immunosorbent assay (ELISA), the culture medium was harvested after 5 h of stimulation and stored at –20°C for a later analysis.

Assessment of Cell Viability—NRK-52E cells were infected with Adex1w, AxCAIκBα, or AxCAmutantIκBα at various levels of multiplicity of infection (MOI), or left untreated. After culture for 24 h, AlamarBlue (*24*) was added, and the culture was continued for 2 h. Fluorescence at the excitation wavelength of 560 nm and the emission wavelength of 590 nm was measured on a 48-well plate with CytoFluor (Millipore, Bedford, MA). Viability was expressed by the signal obtained from the cultures infected by the recombinant adenoviruses as a percentage of the signal from the noninfected control cultures.

ELISA for CINC-1—The content of CINC-1 in the culture supernatants was determined by ELISA using a Rat

GRO/CINC-1 ELISA kit (Immuno Biological Laboratories, Gunma) according to the manufacturer's instructions.

RESULTS

IκBα Degradation, NF-κB Activation, and CINC-1 Production Induced by IL-1β Stimulation—Degradation of the inhibitory protein IκB of the cytoplasmic NF-κB/IκB complex is necessary for NF-κB activation (*25, 26*). We have shown that NF-κB in NRK-52E cells is activated by recombinant human IL-1β and is mostly in the form of a p50/p65 heterodimer (*14*). Therefore, we used recombinant human IL-1β here to stimulate NF-κB, since human recombinant IL-1β was shown to have similar activity to rat recombinant IL-1β (27). We examined the levels of IκBα and nuclear p65 by Western blot analysis. Figure 1 shows the time course of IκBα degradation and p65 nuclear translocation in NRK-52E cells following activation by IL-1β. IκBα was degraded time-dependently for 30 min, then recovered almost to the basal level at 60 min (Fig. 1, upper panel). The level of p65 markedly increased at 15 min and was maintained at a high level for at least 60 min (Fig. 1, lower panel). Subsequent analysis of IκBα degradation and p65 nuclear translocation was performed at 30 min after IL-1β stimulation.

We next analyzed the dose-dependency on IL-1β of IκBα degradation, p65 nuclear translocation, and CINC-1 production. Western blot analysis of cytoplasmic and nuclear extracts of IL-1β–treated NRK-52E cells revealed that both IκBα degradation and the level of nuclear p65 increased with the dose of IL-1β (Fig. 2A). Production of CINC-1 from NRK-52E cells was examined by measuring the level of CINC-1 protein by ELISA in the culture supernatants. As shown in Fig. 2B, the CINC-1 level was very low in unstimulated cells, but was increased markedly by treatment with IL-1β. The dose-dependency of CINC-1 production was the same as those of IκB degradation and p65 nuclear translocation, suggesting that CINC-1 production depends on IκBα degradation followed by p65 nuclear translocation.

Assessment of Recombinant Adenoviruses Expressing Wild IκBα and Mutant IκBα (S32A/S36A)—Efficiency of the adenovirus-mediated gene transfer system in NRK-52E cells was first tested by a recombinant adenovirus expressing *Escherichia coli* β-galactosidase (AxCALacZ). Almost 100% of NRK-52E cells were infected with AxCALacZ at an MOI of more than 3, as confirmed by β-galactosidase staining (data not shown), indicating the effectiveness of this gene transfer system. We next constructed two recombinant adenoviruses, AxCAIκBα and AxCAmutantIκBα, carrying wild IκBα cDNA and mutant IκBα (S32A/S36A) cDNA, respectively. The Western blot analysis performed from noninfected, AxCAIκBα-infected, and AxCAmutantIκBα-infected cells with or without IL-1β stimulation revealed increased expression of exogenous IκBα with increasing MOI (Fig. 3). In the analytical conditions, endogenous IκBα was not detected after IL-1β stimulation. More importantly, levels of exogenous IκBα of AxCAmutantIκBα-infected cells after IL-1β stimulation were much higher than those of AxCAIκBα-infected cells. This result was not due to the difference in potency of these viruses to infect NRK-52E cells, since the levels of total IκBα of AxCAIIκBα-infected and AxCAmutantIκBα-infected cells were almost the same in the absence of IL-1β. Thus, these data confirm that AxCAIκBα and AxCAmutantIκBα respectively express wild IκBα and nondegradable mutant IκBα (S32A/S36A).

Effects on Cell Viability after Infection of Recombinant Adenoviruses—NRK-52E cells were infected by Adex1w (recombinant adenovirus carrying no cDNA insert), AxCAIκBα, or AxCAmutantIκBα at various levels of MOI. After 24 h, the cell viability was examined by AlmarBlue assay. As shown in Table I, infection of each recombinant adenovirus had little, if any, effect on the cell viability at an MOI of up to 30 in the present study.

Inhibition of NF-κB Activation by Adenovirus-Mediated Overexpression of IκBα—Effects of AxCAIκBα and AxCA-

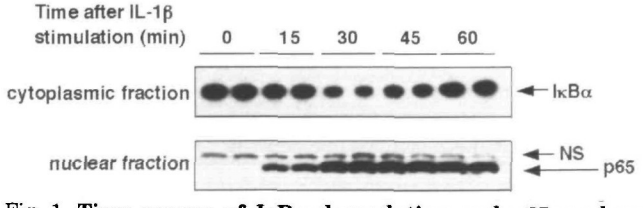

Fig. 1. **Time course of IκBα degradation and p65 nuclear translocation induced by IL-1β in NRK-52E cells.** NRK-52E cells were stimulated with IL-1β (100 units/ml) for the indicated periods. Cytoplasmic and nuclear extracts were prepared after IL-1β stimulation, and 20 μg of each protein was subjected to SDS-PAGE followed by Western blot analysis of IκBα (upper panel) or p65 (lower panel) using the ECL technique as described in "MATERIALS AND METHODS." Positions of IκBα, p65, and nonspecific band (NS) are indicated by arrows. The results are representative of two independent experiments.

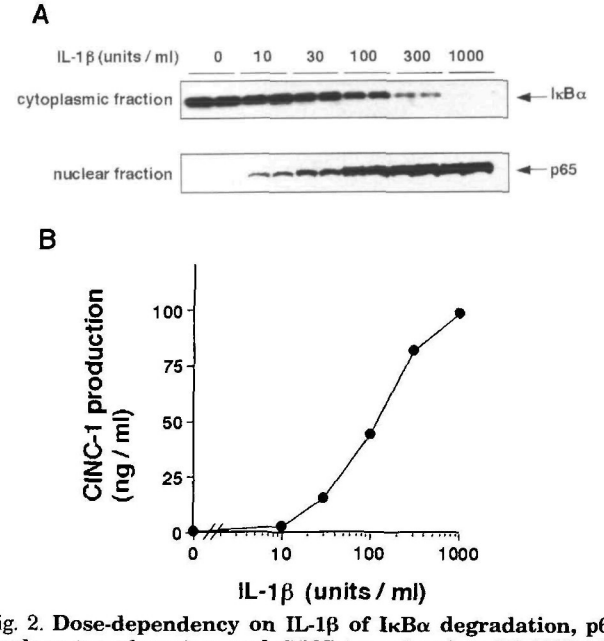

Fig. 2. **Dose-dependency on IL-1β of IκBα degradation, p65 nuclear translocation, and CINC-1 production.** (A) NRK-52E cells were stimulated with the indicated concentrations of IL-1β for 30 min. Western blot analysis of IκBα and p65 was performed as described in Fig. 1. (B) NRK-52E cells were cultured in 48-well plates in 0.3 ml of DMEM supplemented with 10% FBS until reaching confluence. The cells were then stimulated with the indicated concentrations of IL-1β for 5 h. CINC-1 contents in the culture media were determined by ELISA as described in "MATERIALS AND METHODS." The data represented the means ± SE of the results from three independent experiments.

mutantIκBα on NF-κB activation were investigated by studying p65 nuclear translocation. p65 nuclear translocation was visualized by Western blot analysis of the nuclear extracts of Adex1w-infected, AxCAIκBα-infected, or AxCAmutantIκBα-infected cells after IL-1β stimulation. IL-1β strongly induced p65 nuclear translocation in Adex1w-infected cells. AxCAmutantIκBα and AxCAIκBα reduced the level of nuclear p65 induced by IL-1β at an MOI of more than 3 and 10, respectively, when compared with Adex1w-infected cells (Fig. 4). AxCAmutantIκBα inhibited the p65 nuclear translocation more effectively than AxCAIκBα. We also simultaneously analyzed the IκBα degradation in Adex1w-infected, AxCAIκBα-infected, or AxCAmutantIκBα-infected cells. IL-1β induced IκBα degradation in Adex1w-infected cells. However, no apparent IκBα degradation by IL-1β was observed in either AxCAIκBα-infected or AxCAmutantIκBα-infected cells at an MOI of more than 3 (Fig. 4). This result is simply explained by the fact that the amount of degraded IκBα is only a small part of the total amount of overexpressed IκBα.

Inhibition of CINC-1 Production by Adenovirus-Mediated Overexpression of IκBα—In our previous study (*14*), the NF-κB–binding site in the promoter region of CINC-1 was indispensable for CINC-1 promoter activity in response to IL-1β stimulation. The strong inhibition of NF-κB activation in NRK-52E cells by AxCAIκBα or AxCAmutantIκBα observed in the present study suggested that CINC-1 production is reduced by these recombinant adenoviruses. Hence, the effects of AxCAIκBα and AxCAmutantIκBα on CINC-1 production induced by IL-1β were examined by measuring the levels of CINC-1 protein by ELISA in the culture supernatants. As shown in Fig. 5, AxCAIκBα or AxCAmutantIκBα reduced the CINC-1 production resulting from IL-1β stimulation in an MOI-dependent manner. The IC_{50} of AxCAIκBα and AxCAmutantIκBα were estimated to be at an MOI of approximately 9 and 0.3 from the figure, respectively, suggesting that AxCAmutantIκBα is about 30 times more potent than AxCAIκBα in inhibiting CINC-1 production.

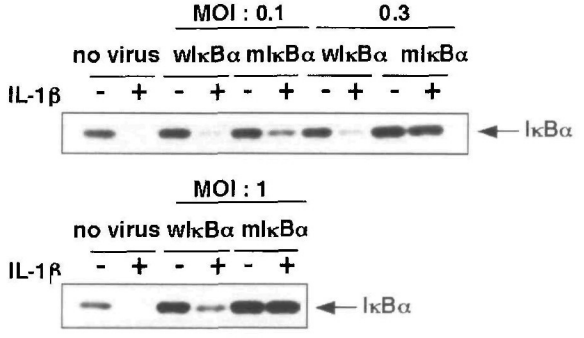

Fig. 3. **Adenovirus-mediated overexpression of IκBα.** NRK-52E cells were infected with AxCAIκBα (wIκBα) or AxCAmutantIκBα (mIκBα) at various levels of MOI or left untreated (no virus) for 24 h, then stimulated with IL-1β (100 units/ml) or media alone for 30 min. Whole-cell extracts were prepared after IL-1β stimulation, and Western blot analysis of IκBα was performed as described above. The results are representative of two independent experiments.

TABLE I. **Effects of infection of recombinant adenoviruses on cell viability.** NRK-52E cells were infected with Adex1w, AxCAIκBα, or AxCAmutantIκBα at various levels of MOI or left untreated. The percentage viability was determined by AlamarBlue assay as described in "MATERIALS AND METHODS." The data represent the means ± SE of the results from four independent experiments.

MOI	Cell viability (% of control)		
	Adex1w	AxCAIκBα	AxCAmutantIκBα
0	100.0±2.0	100.0±2.0	100.0±2.0
0.3	97.6±2.2	101.4±2.5	101.6±3.0
3	93.4±2.7	94.9±1.6	94.6±2.8
30	94.8±4.0	93.4±3.0	95.5±3.7

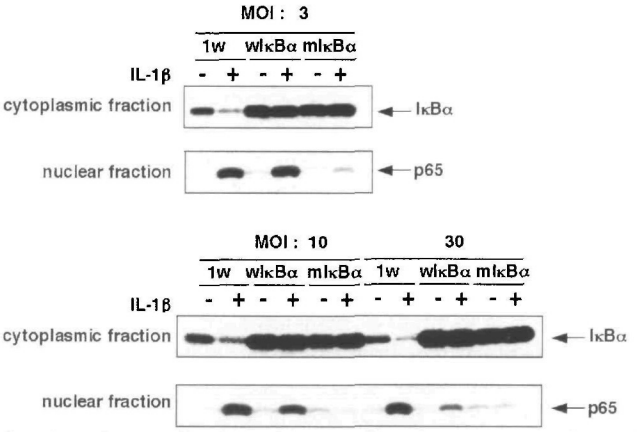

Fig. 4. **Effects of adenovirus-mediated overexpression of IκBα on IκBα degradation and p65 nuclear translocation.** NRK-52E cells were infected with Adex1w (1w), AxCAIκBα (wIκBα), or AxCAmutantIκBα (mIκBα) at various levels of MOI for 24 h. The cells were stimulated with IL-1β (100 units/ml) or media alone for 30 min. Western blot analysis of IκBα and p65 were performed as described in Fig. 1. The results are representative of two independent experiments.

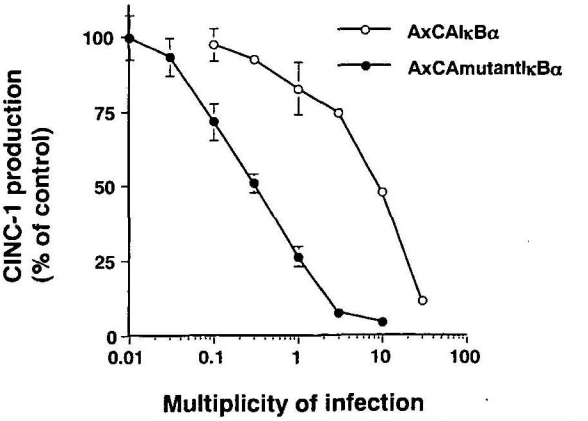

Fig. 5. **MOI-dependency of AxCAIκBα and AxCAmutantIκBα in the inhibition of CINC-1 production by IL-1β stimulation.** NRK-52E cells were infected with Adex1w, AxCAIκBα, or AxCAmutantIκBα at various levels of MOI for 24 h, then stimulated with IL-1β (100 units/ml) for 5 h. CINC-1 contents in the culture media were determined by ELISA as described above. The data were normalized to the Adex1w-infected and IL-1β–treated control (100%) and represent the means ± SE of the results from three independent experiments.

DISCUSSION

Many cellular genes involved in the early process of immune, acute phase, and inflammatory responses are regulated by NF-κB (*28*, *29*), the activation of which is tightly regulated by its endogenous inhibitor, IκB, with which it forms NF-κB-IκB complexes in the cytoplasm. In our previous paper (*14*), we have shown that the NF-κB–binding site in the promoter region of CINC-1 is indispensable for the IL-1β–induced activation of the CINC-1 promoter in reporter assays, suggesting the involvement of NF-κB in CINC-1 production. Moreover, cotransfection of IκBα resulted in a significant reduction of CINC-1 promoter activities in another study using a similar reporter assay system (Ohtsuka, T. and Tsurufuji, S., unpublished observations). Those studies were done with a transient transfection system of 10–20% efficiency. However, in order to evaluate quantitatively the inhibitory effects of IκBα on the expression of the endogenous CINC-1 gene, it is necessary to utilize a nearly 100% efficient transfection system. Since up to 100% of the cells can be infected using recombinant adenovirus expressing IκBα (*30–32*), we investigated the inhibitory effects of IκBα on CINC-1 production in the present study by using a similar adenovirus gene transfer technique. We constructed two recombinant adenoviruses, expressing wild IκBα or mutant IκBα (S32A/S36A), and showed that almost 100% transfection efficiency was obtained with AxCALacZ at an MOI of more than 3 in NRK-52E cells (data not shown). Therefore, an adenovirus-mediated gene transfer system is suitable for the quantitative analysis of regulation of CINC-1 production by IκBα in NRK-52E cells.

Several groups have reported that one disadvantage in using adenovirus vectors is that the viral vectors could induce a local inflammatory response of IL-8 production (*30*, *32*, *33*). Under our experimental conditions, however, neither CINC-1 production nor cytotoxic effects resulted from the infection with the recombinant adenoviruses (Table I), suggesting no sign of an inflammatory response. The difference between our data and others, may be due to differences in the promoters of IL-8 and of CINC-1, virus amounts used, or other factors. Furthermore, IL-1β–stimulated CINC-1 secretion was not influenced in Adex1w-infected cells as compared to that in noninfected cells (data not shown), showing that the infection of the control recombinant adenovirus has no effect on CINC-1 production itself.

The approach using mutant IκBα (S32A/S36A) was thought to be superior to that using wild type IκBα, since the wild type is degradable and must therefore be expressed in much greater amounts to achieve NF-κB inhibition. To our knowledge, however no direct, quantitative comparison of the inhibition of inflammatory reactions by wild type IκBα and mutant IκBα (S32A/S36A) has yet been done. Therefore, we compared the inhibitory activity of AxCAIκBα and AxCAmutantIκBα against CINC-1 production (Fig. 5) and found that the AxCAmutantIκBα is 30 times more potent than AxCAIκBα. This result suggests that AxCAmutantIκBα is an efficient tool for inhibiting NF-κB–dependent inflammatory reactions.

In mock-transfected cells stimulated by IL-1β, almost the total amount of IκBα was degraded. In contrast, in IκBα-transfected cells at an MOI of 3, the amount of IκBα was far higher than its normal level, and therefore no apparent degradation of IκBα was observed in the Western blot analysis. However, p65 nuclear translocation was not affected by the overexpression of IκBα (Fig. 4). This means that the excess amount of wild IκBα did not affect the phosphorylation of IκBα in the NF-κB-IκBα complex. This discrepancy between the p65 nuclear translocation and the IκBα degradation can be explained by the preferential phosphorylation of IκBα which is already present as a complex with NF-κB as compared to free and unbound IκBα. Such preferential phosphorylation of IκBα in the NF-κB-IκBα complex has also been reported by other investigators (*34*). However, as shown in Fig. 4, in the case of IκBα-transfected cells at an MOI of 30, the much greater amount of IκBα significantly inhibited p65 nuclear translocation. This result may suggest that an excess amount of IκBα can override the preferential phosphorylation of IκBα in the NF-κB-IκBα complex or can capture free NF-κB induced by IL-1β before its translocation from the cytoplasm into the nucleus.

Four CINC subtypes have so far been identified in rats, CINC-1, CINC-2α, CINC-2β and CINC-3. Nakagawa *et al.* have suggested that the subtype of CINC appearing at the inflammatory site depends on the rat inflammation model (*35*, *36*). It would be interesting to investigate whether the production of CINC-2α, CINC-2β, and CINC-3 can also be inhibited by overexpression of IκBα.

In summary, our results clearly show that CINC-1 gene expression with NF-κB activation is primarily regulated by IκBα in NRK-52E cells, and that a mutated nondegradable IκBα much more effectively inhibits CINC-1 production than wild IκBα. Further testing the possibility that an adenovirus-mediated overexpression of IκBα down-regulates inflammation in animal models *in vivo* will give more information on the clinical usefulness of adenovirus vectors carrying IκBα in inflammatory diseases.

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