Inhibitor of nuclear factor- κ B induction by cAMP antagonizes interleukin-1-induced human macrophage-colony-stimulating-factor expression

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We have recently reported that interleukin-1 α (IL-1 α) can induce human macrophage colony-stimulating factor (M-CSF) expression through nuclear factor κ B (NF- κ B) activation, and treatment of human pancreatic MIA PaCa-2 cancer cells with forskolin or cAMP attenuated the NF- κ B activation as well as M-CSF expression. In this study, we have further investigated the mechanism of cAMP attenuation. MIA PaCa-2 cells were incubated with forskolin or dibutyryl-cAMP and then stimulated with IL-1 for 1 h. Cell lysates were immunoprecipitated by anti-inhibitory κ B (I κ B) kinase- β (IKK β) antibody and the immune complex assayed for kinase activity using recombinant inhibitor of NF- κ B (I κ B α) as substrate. The levels of IKK β in the respective cellular proteins were measured by subsequent Western blot. The results show that the level of IKK protein remains constant in the presence of cAMP, forskolin and/or IL-1, whereas IKK

activity was robustly stimulated by IL-1. Nonetheless, dibutyryl-cAMP or forskolin did not affect the IKK activation induced by IL-1. This experiment suggests that elevated cAMP has no effect on IKK activity. I κ B α protein level decreased markedly in IL-1-treated cells compared with the untreated. By contrast, cells treated with cAMP or forskolin possessed discernibly higher I κ B α levels. In addition, we observed that forskolin potentiated and prolonged the IL-1-induced I κ B α mRNA levels, whereas it did not stabilize the I κ B α mRNA message. Wholly, these studies indicate that elevated cAMP antagonizes IL-1-induced M-CSF transcription by up-regulating I κ B α gene induction and its consequent attenuation of NF- κ B activation.

Key words: I κ B kinase- β , IKK β , NF- κ B.

INTRODUCTION

An expanding number of reports suggest novel roles for macrophage colony-stimulating factor (M-CSF) other than its first discovered function in myeloid-lineage haematopoiesis [1]. For example, recent studies implied that M-CSF played crucial roles in the pathogenesis of two major public-health concerns: atherosclerosis [2] and osteoporosis [3,4]. Overexpression of M-CSF as well as its receptor in several carcinomas, especially those of reproductive tracts such as breast, ovary, uterus and prostate gland, also implied the importance of M-CSF as an auto/paracrine growth factor in tumour growth. The receptor-mediated mechanism of M-CSF has been investigated extensively. However, only limited information is available on the signal transduction leading to M-CSF expression.

We reported recently that forskolin and dibutyryl-cAMP (DB-cAMP) both attenuated interleukin-1 α (IL-1 α)-induced nuclear factor κ B (NF- κ B) activation in the human pancreatic cancer cell line MIA PaCa-2, from which the *M-CSF* gene was originally cloned, and correlatively reduced M-CSF expression at the mRNA level as well as M-CSF promoter induction [5]. Other inducible genes whose expression was antagonized by cAMP elevation include IL-2 [6], IL-2 receptor [7] and nitric oxide synthase [8]. The common feature in all of these is that NF- κ B-dependent signalling is crucial in the regulation of these genes, so it is probable that cAMP negatively affects one of the signalling components of the NF- κ B activation pathway.

The NF- κ B dimers in most mammalian cells are sequestered in the cytosol by inhibitory κ B (I κ B) isoforms, such as I κ B α , I κ B β or I κ B ϵ [9]. A critical step in the signal-induced activation of NF- κ B is the site-specific phosphorylation of the conserved N-terminal serine residues of I κ B. Specifically, they are Ser-32 and

Ser-36 in I κ B α . This commits I κ B to degradation by the ubiquitin-proteasome pathway, and the freed NF- κ B subunits are then translocated to function in the nucleus. Receptor-mediated signals, such as IL-1, tumour necrosis factor (TNF) and bacterial lipopolysaccharide (LPS), lead to membrane recruitment of signalling proteins, such as TNF-receptor-associated factors 2 or 6 and receptor-interacting protein. These signalling pathways converge at activation of upstream NF- κ B-inducing kinase (NIK) [10]. NIK then phosphorylates and activates a multi-protein I κ B kinase (IKK) complex, which is directly responsible for phosphorylating I κ Bs.

Diverse mechanisms of NF- κ B-activation blockade by drugs, especially anti-inflammatory agents, or chemical and physical agents, have been reported. Aspirin and sodium salicylate bind to IKK β and thus inhibit its kinase activity, supposedly by preventing the ATP binding to IKK β , while sulindac also inhibits IKK β [11,12]. Anti-inflammatory cyclopentenone prostaglandins, which are synthesized in the late stages of inflammatory episodes, modify the IKK subunits and inhibit their kinase activity [13]. Alternatively, the mechanism that forskolin employs to down-regulate induction of LPS-stimulated inducible nitric oxide synthase was attributed to its ability to prevent the degradation of I κ B α and also to induce I κ B α mRNA formation [8].

Here, we hypothesized that intracellular cAMP elevation could either interfere with NF- κ B activation through the NIK/IKK activation axis, or increase cytosolic I κ B availability for complex formation with NF- κ B through I κ B gene induction. Either one would probably cause the decrease in NF- κ B-dependent M-CSF transcription. The present studies have examined how cAMP antagonizes IL-1-induced NF- κ B activation and the subsequent NF- κ B-dependent *M-CSF* gene expression.

Abbreviations used: M-CSF, macrophage colony-stimulating factor; IL, interleukin; TNF, tumour necrosis factor; DB-cAMP, dibutyryl-cAMP; EMSA, electrophoretic mobility-shift assay; NF- κ B, nuclear factor κ B; I κ B, inhibitory κ B; NIK, NF- κ B-inducing kinase; IKK, I κ B kinase; LPS, lipopolysaccharide; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Materials

All standard tissue-culture reagents were obtained from Hyclone (Logan, UT, U.S.A.). Recombinant human IL-1 α (amino acids 117–271) Ro24-5008 was a gift from Hoffmann–LaRoche (Nutley, NJ, U.S.A.). [γ - 32 P]ATP and [α - 32 P]dCTP were purchased from Dupont NEN (Boston, MA, U.S.A.). The mutant NF- κ B gel-shift oligonucleotides, anti-IKK β antibody, purified I κ B α protein (amino acids 1–317) and Protein A–agarose beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). BAY11-7082 and proteasome inhibitor II were acquired from Calbiochem (La Jolla, CA, U.S.A.). FuGene-6 was supplied by Roche (Indianapolis, IN, U.S.A.). Unless otherwise stated, all other biochemical reagents were purchased from Sigma (St Louis, MO, U.S.A.).

Cell culture and treatment

The human pancreatic carcinoma cell line MIA PaCa-2 obtained from the ATCC was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum and 2.5% horse serum. Subculture of cells was done by treatment with 0.05% trypsin/0.02% EDTA and resuspended in fresh DMEM with 10% serum. Cells were cultured at 37 °C in a humidified incubator under 6% CO₂. When 80% confluence was reached, cells were washed twice with PBS before switching to serum-free DMEM. To stimulate the cells, recombinant IL-1 α was added to the cell cultures at a final concentration of 17 ng/ml. Cells were incubated further and harvested at specified times.

Nuclear protein extraction

Nuclear protein extracts were prepared according to the method of Dignam et al. [14]. After incubation under a specific treatment (as detailed in the Results section), (1.5–2) $\times 10^6$ cells were washed twice with PBS before being scraped into buffer A (10 mM Hepes, pH 7.9/1.5 mM MgCl₂/10 mM KCl/0.5 mM dithiothreitol/0.5 mM PMSF) and briefly centrifuged. Cell pellets were consequently resuspended in buffer A, kept on ice for 15 min, 10% Nonidet P40 added, homogenized in a Dounce homogenizer and centrifuged at 10000 *g* for 10 s at 4 °C. Then nuclear pellets were resuspended in extraction buffer (20 mM Hepes, pH 7.9/0.55 M NaCl/1.5 mM MgCl₂/0.2 mM EDTA/0.5 mM dithiothreitol/0.5 mM PMSF/10 mg/ml leupeptin), homogenized and centrifuged at 10000 *g* for 2 min at 4 °C. Samples in the supernatants were collected and stored at –70 °C until use. Concentrations of the nuclear protein samples were determined by BioRad protein assay according to manufacturer's protocol.

Electrophoretic mobility-shift assay (EMSA)

Synthetic oligonucleotides with the sequences corresponding to *cis*-acting elements of the M-CSF promoter are 32 P-labelled at the 5' ends by T4 polynucleotide kinase. Equal amounts (10 μ g) of nuclear extract from each of various cell treatments were incubated in 20 μ l of binding reaction mixture [final concentrations, 40 mM KCl, 15 mM Hepes, pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 5% glycerol and 0.5 mg/ml poly(dI.dC)] for 10 min. Then, equal amounts (60000 c.p.m., approx. 1 ng of DNA) of radiolabelled oligonucleotide probe were added to the reaction mixture, and incubated for an additional 25 min at room temperature. DNA–protein complexes were separated from unbound DNA probe by electrophoresis on native 5% polyacrylamide gels at 140 V in 45 mM Tris/borate

(pH 8.0) containing 1 mM EDTA and then autoradiographed. The following oligonucleotide probe (Biosynthesis, Lewisville, TX, U.S.A.) was used: NF- κ B, 5'-agttgaGGGACTTTCCagc-3'. The upper-case letters indicate the consensus sequence for the NF- κ B-binding site (positions –368 to –377 of the M-CSF promoter).

Plasmids, cell transfection and reporter-gene assay

Luciferase reporter plasmids driven by the 5' region of the human *M-CSF* gene promoter were generated as stated elsewhere [5,15]. In all transfections, 1 μ g of pSV- β -galactosidase were used to monitor the transfection efficiency. MIA PaCa-2 cells were seeded at 2 $\times 10^5$ cells/35 mm plate 24 h before transfection. Cells were washed twice with PBS and switched to serum-free DMEM 6 h before transfection. Transfection of the plasmid DNA mixture into cells was performed by using FuGene-6 according to the manufacturer's protocol (Roche). Transfected cells were stimulated with IL-1 with or without addition of DB-cAMP or BAY11-7082 12 h after transfection, and then they were incubated for a further 12 h until harvesting for the luciferase assay (Promega, Madison, WI, U.S.A.), as per manufacturer's protocol. An assay for β -galactosidase was performed as described previously [16]. Induction of luciferase activity is reported as the mean from two separate experiments performed in triplicate normalized by β -galactosidase activity.

Assay of IKK kinase activity

After IL-1 and forskolin or DB-cAMP treatment, cells were washed with ice-cold 5 mM EDTA in PBS and were lysed on ice in TN buffer (containing 50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 0.5% Nonidet P40, 10% glycerol, 50 mM NaF, 20 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM dithiothreitol, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM EDTA and 1 mM EGTA) by aspiration through a 21-gauge needle. Cells were centrifuged at 10000 *g* for 10 min at 4 °C, and cell lysates were immunoprecipitated using 2 μ g of anti-IKK β antibody and 50 μ l of Protein A–agarose. Aliquots of immunoprecipitate were then incubated in 25 μ l of kinase buffer (containing 20 mM Tris/HCl, pH 7.5, 20 mM MgCl₂, 20 mM β -glycerophosphate, 1 mM PMSF, 1 mM EDTA, 20 mM ATP and 10 μ Ci of [γ - 32 P]ATP) at 30 °C for 30 min. Protein bands were resolved by SDS/PAGE and autoradiographed.

RNA extraction and Northern-blot analysis

Total cellular RNA was isolated using RNastat-60 reagent from Teltest (Friendswood, TX, U.S.A.) using the manufacturer's protocol. Samples containing 15–25 μ g of RNA were fractionated in 1.2% agarose gels and transferred to nylon membranes. The membranes were then hybridized according to standard protocol [16] with either cloned *M-CSF* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA, or with probes generated by PCR for M-CSF or human 18 S rRNA-specific cDNA. The probes were radiolabelled with [α - 32 P]dCTP by random priming using Klenow fragment from Promega, following manufacturer's directions. After hybridization or rehybridization, membranes were autoradiographed.

Data analysis

Densitometry of the autoradiographed bands from Northern blots, kinase assays and EMSA were analysed quantitatively by Alpha Imager™ version 3.23 (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). All values are expressed as means $\pm$ S.E.M. from independent experiments. Two-tailed

paired Student's *t* test was used to determine the significance of changes in densitometric measurements and relative luciferase activity. A significant difference was considered as $P < 0.05$.

RESULTS

NF- κ B blockade and forskolin negatively affected M-CSF transcription in a similar manner

BAY11-7082, a specific inhibitor of inducible I κ B α phosphorylation [17], was used for comparison with cAMP, which we reported earlier to attenuate transcription activity of the *M-CSF* gene promoter. MIA PaCa-2 cells were transfected with a plasmid containing the firefly luciferase cDNA under control of the full-length M-CSF promoter (p570Luc). We detected an approx. 14-fold increase in relative luciferase activity in the transiently transfected MIA PaCa-2 cells treated with 17 ng/ml IL-1 compared with control untreated cells after 12 h (Table 1). Forskolin or BAY11-7082 alone did not cause a significant change in luciferase activity compared with control cells ($P = 0.4174$ and 0.9389 , respectively). The transfected cells appeared normal and the BAY11-7082 concentration used was well within the range (5–20 μ M) used in other reports without any signs of cytotoxicity. Forskolin significantly attenuated IL-1-induced relative luciferase activity from 13.55 ± 1.15 to 8.8 ± 0.41 ($P = 0.012$). In similar fashion, BAY11-7082 distinctly antagonized relative luciferase activity induced by IL-1 (from 12.99 ± 0.94 to 5.79 ± 0.46 , $P = 0.014$).

Effect of cAMP, BAY11-7082 and proteasome inhibitor II on NF- κ B activation

As indicated previously, cells were pre-incubated with 2 μ M DB-cAMP, 10 μ M BAY11-7082 or 1 μ M proteasome inhibitor II for 1 h before they were treated with 17 ng/ml IL-1 for 60 min, then a nuclear protein extract from each treatment was prepared. NF- κ B-binding complex from nuclear extract appeared on the autoradiogram as a sharp single retarded band that was demonstrated earlier to be supershifted with anti-p65 antibody (results not shown). NF- κ B was activated strongly by IL-1 α (Figure 1A, lane 6). A mutant NF- κ B probe that differed from the wild-type NF- κ B-binding site by 1 nt was used with the IL-1-treated extract as a negative control, and no binding was observed (Figure 1A, lane 1). Elevation of intracellular cAMP by pre-treatment of the MIA PaCa-2 cells with 2 μ M DB-cAMP 1 h

Figure 1 NF- κ B activation in IL-1-stimulated MIA PaCa-2 cells is affected by DB-cAMP and BAY11-7082

(A) MIA PaCa-2 cells were harvested and extracted for nuclear proteins 1 h after they were treated with the agents indicated +. Cells were pre-incubated with 2 μ M DB-cAMP, 10 μ M BAY11-7082, or 1 μ M proteasome inhibitor II for 1 h before the IL-1 was added. NF- κ B oligonucleotide probes were incubated with 10 μ g of nuclear extract per lane and resolved on 5% polyacrylamide gel in lanes 2–9. Mutant NF- κ B probe was used with IL-1-treated extract in lane 1 as a negative internal control. Corresponding cytosolic proteins (100 μ g) were resolved on SDS/PAGE, transferred to nitrocellulose membrane, and probed with an antibody against I κ B α (B) and then β -actin (C).

prior to the stimulation by IL-1, significantly attenuated NF- κ B activation (Figure 1A, lane 9). Attenuation of NF- κ B activation by BAY11-7082 or proteasome inhibitor II were both even more pronounced than that for DB-cAMP (Figure 1A, lanes 7 and 8).

Corresponding cytosolic proteins (100 μ g) from the same treatments as above were resolved on SDS/PAGE, transferred to nitrocellulose membrane and probed in a Western blot with an antibody against I κ B α (Figure 1B, apparent molecular mass 37 kDa), which showed the level of I κ B α present in the cytosol of the cells in the specified treatment. This also reflected varying amounts of I κ B α that were not degraded by the ubiquitin-proteasome pathway after phosphorylation by upstream IKK. As expected, IL-1 caused marked degradation of I κ B α (Figure 1B, lane 6) compared with the control in lane 2, and it distinctly activated NF- κ B. By contrast, BAY11-7082, proteasome inhibitor II or DB-cAMP alone each produced a discernibly slight increase in I κ B α protein without causing any change in NF- κ B activation compared with the control (Figure 1B, lanes 3–5 respectively). Furthermore, each of these three compounds effectively preserved the levels of I κ B α protein in the cytosol in the presence of IL-1 (Figure 1B, lanes 7–9 respectively) and significantly antagonized the IL-1-induced NF- κ B activation (Figure 1A, lanes 7–9). Availability of I κ B α in the Western blot was roughly the inverse of the level of NF- κ B activation observed in a particular treatment. The Western blots were stripped and reprobed for β -actin protein with apparent molecular mass of 42 kDa (Figure 1C), which was present equally across every

Table 1 Effects of forskolin and BAY11-7082 on transactivation of IL-1-induced M-CSF promoter-driven luciferase activity

MIA PaCa-2 cells were transiently transfected for 12 h prior to switching to serum-free media containing specified treatments and incubated for a further 12 h, then cells were harvested for reporter-gene assays. Data are given as luciferase activity relative to β -galactosidase activity expressed as the means $\pm$ S.E.M. from two experiments, each of which was performed in triplicate. The asterisks mark significant difference ($P < 0.05$) between transfected cells treated with IL-1 only and cells co-treated with forskolin or BAY11-7082 within each series.

Treatment	Relative luciferase induction
Control	1.0 $\pm$ 0.08
Forskolin (2 μ M)	0.99 $\pm$ 0.12
IL-1 (17 ng/ml)	13.55 $\pm$ 1.15
IL-1 + forskolin	8.81 $\pm$ 0.41*
Control	1.0 $\pm$ 0.3
BAY11-7082 (10 μ M)	0.9 $\pm$ 0.05
IL-1 (17 ng/ml)	12.99 $\pm$ 0.94
IL-1 + BAY11-7082	5.79 $\pm$ 0.46*

Figure 2 Intracellular cAMP elevation did not affect IKK β activity

MIA PaCa-2 cells were pre-incubated in 5 μ M forskolin or 1 μ M DB-cAMP for 1 h prior to addition of 17 ng/ml IL-1 for another 1 h. Cell lysates containing 200 μ g of protein were immunoprecipitated with an antibody directed against IKK β and 50 μ l of Protein A-agarose. Kinase assays containing recombinant I κ B α (amino acids 1–317) as a substrate were resolved by SDS/PAGE (10% gel) and autoradiographed. Fractions of the kinase-assay reactions were analysed by Western blots using polyclonal antibodies against IKK β and I κ B α . + and – signify the presence and absence of the agents in the cell treatments before the immunoprecipitated complexes were acquired. Pre-immune rabbit serum was used instead of anti-IKK β in immunoprecipitation in lane 1. Immune complex from IL-1-treated cells was used in the kinase reaction in lane 8 without the substrate, recombinant I κ B α .

treatment. This result indicated that IL-1-induced protein degradation is specific for I κ B.

Forskolin and DB-cAMP did not affect IKK β activity

IKK β rather than IKK α has been reported to play a central role in cytokine-inducible NF- κ B activation [18]. In order to investigate whether intracellular cAMP elevation could modulate IKK β activity, MIA PaCa-2 cells were pre-incubated in 5 μ M forskolin or 1 μ M DB-cAMP for 2 h before cells were treated with 17 ng/ml IL-1 for 1 h, and subsequently assayed for IKK activity in the anti-IKK β immunoprecipitated complex using recombinant I κ B α (amino acids 1–317) as a substrate. I κ B α is the preferred substrate of IKK β [19]. The immune complex from IL-1-treated cells significantly induced I κ B α phosphorylation compared with that from untreated cells (Figure 2C, lanes 5 and 2). There was no autoradiographed band observed in the kinase reaction of immune complex from IL-1-treated cells without recombinant I κ B α , which was used as an internal negative control (Figure 2C, lane 8). Similarly, the kinase reaction using pre-immune rabbit serum immunoprecipitates did not result in phosphorylation of I κ B α (Figure 2C, lane 1). IL-1 also induced IKK β activity in a dose-dependent manner (results not shown). A one-fifth fraction of each kinase-assay reaction was analysed by Western blots using polyclonal antibodies against IKK β and I κ B α to show that each protein was available equally across all the kinase-assay reactions (Figures 2A and 2B). Forskolin or DB-cAMP by themselves did not induce I κ B α phosphorylation more extensively than the untreated cells (Figure 2C, lanes 3 and 4). In addition, pre-incubation of cells in each of these compounds before IL-1 treatment did not change the extent of IL-1-induced I κ B α phosphorylation (Figure 2C, lanes 6 and 7). Unlike the aforementioned anti-inflammatory agents that also inhibit NF- κ B activation, it suggests that these intracellular cAMP-elevating agents do not modulate the activity of IKK β .

Figure 3 Kinetics of forskolin and IL-1-induced I κ B α mRNA levels

MIA PaCa-2 cells were incubated with forskolin (5 μ M) or IL-1 (17 ng/ml) or were pretreated with forskolin for 1 h and then IL-1 added for the time periods indicated. After cell lysis and RNA purification, Northern blots were hybridized with a cDNA probe for I κ B α , stripped and then probed for GAPDH.

Figure 4 I κ B α mRNA signal-density changes

The graph shows the ratio between the signal density of each I κ B α mRNA and corresponding GAPDH mRNA at the specified time. The data are expressed as means $\pm$ S.E.M. from two independent experiments. ●, cells treated with forskolin; □, IL-1 treatment; ◆, cells pretreated with forskolin before IL-1 stimulation.

Effect of forskolin and IL-1 on I κ B α mRNA levels

The Western blots revealed that in the presence of DB-cAMP (Figure 1B) and forskolin (results not shown), the cytosolic I κ B α protein levels remained high, even though cells were treated with IL-1, which normally induces I κ B α degradation. The findings above did not support our hypothesis that IL-1-induced IKK β activity could be modulated by intracellular cAMP elevation. We then assessed whether forskolin could initiate I κ B α gene expression in MIA PaCa-2 cells. Cells were incubated with forskolin (5 μ M) or IL-1 (17 ng/ml) or pre-treated with forskolin for 1 h and then IL-1 added for 0.5, 1, 2, 4, 6 or 8 h. After cell lysis and RNA purification, Northern blots were hybridized with a cDNA probe for I κ B α . The level of I κ B α mRNA in unstimulated MIA PaCa-2 cells was undetectable (results not shown). In cells treated with forskolin (Figure 3B and Figure 4, ●), I κ B α mRNA peaked 2 h after stimulation, and then the levels declined gradually to the base line by 6 h. By contrast, in the presence of IL-1 (Figure 3A and Figure 4, □), I κ B α mRNA peaked more rapidly at 1 h after stimulation, and the levels remained elevated

Figure 5 Forskolin did not stabilize I κ B mRNA

(A) After MIA PaCa-2 cells were treated with IL-1 with or without forskolin pre-incubation for 1 h, cells were harvested at 20, 40, 60 and 120 min after addition of 5 μ g/ml actinomycin D. Total cellular RNA was extracted and Northern blots were hybridized with a cDNA probe for I κ B α . (B) The percentage of I κ B mRNA signal intensity at the specified time points compared with that before actinomycin D was added (means $\pm$ S.E.M. from three independent experiments).

for up to 4 h. The plot in Figure 4 provides a graphical presentation of the I κ B α mRNA signal-density changes in each treatment, corrected for loading variations by corresponding GAPDH mRNA density at the specified time points after stimulation. In cells pretreated with forskolin before IL-1 stimulation (Figure 3C and Figure 4, $\blacklozenge$), the kinetics of I κ B α mRNA levels looked similar to those in cells treated with IL-1 only, but with forskolin pretreatment I κ B α mRNA levels were significantly higher than for IL-1 alone and the up-regulation was maintained for up to 8 h. Notice that IL-1 was added 1 h after the initial exposure of the cells to forskolin, and that the cells had been in contact with forskolin for 2 h when they were harvested. At that time point, maximal I κ B α mRNA accumulation, induced by IL-1 or forskolin alone, was reached (Figures 3A and 3B). This result essentially explains the finding, which we have observed previously, that DB-cAMP or forskolin had to be added to the cells 30–120 min prior to the stimulus, such as IL-1 or LPS, to provide maximal inhibition of NF- κ B activation (P. J. Kamthong and M.-C. Wu, unpublished work).

After peak accumulation of I κ B α mRNA (60 min when IL-1 was added or 120 min for forskolin alone), 5 μ g/ml actinomycin D was added to inhibit RNA synthesis. Those peak levels of I κ B α mRNA were considered to be 100%. Then cells were collected to isolate total RNA and assayed for I κ B α mRNA by Northern blots at 20, 40, 60 and 120 min after RNA synthesis was halted by actinomycin D. Figure 5(B) shows the remaining

I κ B α mRNA at those time points as a percentage of the peak levels achieved before actinomycin D was added. I κ B α mRNA decayed rapidly regardless of the treatment that induced the expression. Apparent half-lives of the I κ B α mRNA were approx. 30 min. Forskolin, either alone or in combination with IL-1, did not stabilize I κ B α mRNA messages. Since it did not change the rate of I κ B α mRNA decay, these sets of experiments demonstrated the capability of forskolin to induce I κ B α mRNA synthesis and in turn to up-regulate cytosolic I κ B α protein levels.

DISCUSSION

BAY11-7082 inhibits I κ B α phosphorylation and thus avoids its ubiquitin-proteasome-mediated degradation [20]. Similarly, the cell-permeable proteasome inhibitor II directly interferes with proteasome activity. Acting at different consecutive steps of the same pathway, both inhibitors exhibited similar results in antagonizing NF- κ B activation. Both most likely resulted in a high level of cytosolic NF- κ B-sequestering I κ B. Our transfection and EMSA experiments compliment each other and suggest that modulation of I κ B α phosphorylation and the increase in availability of cytosolic I κ B α protein antagonize transcriptional activation of the M-CSF gene in a similar manner to intracellular cAMP elevation.

Similar patterns are observed in the way that arsenite blocks TNF α -induced transcription of IL-8 [21] and the way in which cAMP attenuates IL-1-stimulated M-CSF transcription, as we reported earlier [5]. Arsenite blocks the induced transactivation of NF- κ B but not activator protein-1. Similarly, cAMP antagonizes NF- κ B activation without any effect on activator protein-1. In addition, arsenite has been shown to prevent NF- κ B activation by directly blocking IKK activity and inhibiting the inducible phosphorylation and degradation of I κ B α . This posed the question as to whether cAMP possesses the ability to interfere with activation of the NIK/IKK axis. The kinase activity of IKK β , which is the most downstream protein kinase in this axis, was assessed. The results above indicate that intracellular cAMP elevation does not modulate the activation of NIK/IKK.

At least three AU-rich sequence element (ARE)-like repeats, which normally subject immediate-early mRNAs to rapid degradation, are present in the 3'-untranslated region of I κ B (MAD-3) RNA [22]. Many short-lived mRNAs associated with the transcription and inflammation possess several repetitive AREs in their 3'-non-coding region [20]. Stabilization of these molecules, which are predestined to be rapidly decayed, is a possible way to up-regulate their expression. But it is shown above that forskolin did not affect I κ B α mRNA decay rates; more likely it induced I κ B α transcription to increase the availability of the mRNAs.

The presence of several NF- κ B-binding sites in the promoter region of the I κ B α gene [23] prompts speculation that any stimuli that activate NF- κ B would inevitably induce I κ B α mRNA synthesis. The fact that the transcription rate of I κ B α was increased at least 3-fold 30 min after LPS was added to adherent human donor monocytes [24] supports this notion, and this is more or less similar to what we observed here. If we use the analogy that NF- κ B activation is a 'turning-on' signal, rapid induction of its own inhibitor I κ B α mRNA by NF- κ B may be considered as the machinery to restore the signal to the 'turning-off' state. Enhancement of I κ B α mRNA transcription by cAMP could arguably keep the signal in the turning-off state for longer, hence rendering it able to antagonize NF- κ B activation. Likewise, synthetic glucocorticoid dexamethasone also induces the transcription of I κ B α , causes the newly released NF- κ B to quickly reassociate with newly synthesized I κ B α , and thus mediates its

immunosuppression [25]. Induction of I κ B synthesis is just one mechanism among several other signalling events bestowing modulation at the level and duration of NF- κ B activity, such as phosphorylation of NF- κ B, hyperphosphorylation of IKK and processing of NF- κ B precursors. This mechanism, commanded by the ubiquitous second messenger cAMP through the well-regulated cAMP-dependent protein kinase, potentially provides signal-transduction cross-talk to modulate NF- κ B signalling. In summary, we have provided evidence suggesting that the attenuation by cAMP of IL-1-stimulated M-CSF expression is not by the inhibition of IKK activity. Rather, it is by inducing the up-regulation of I κ B expression, which eventually traps NF- κ B in the cytosolic NF- κ B-I κ B complex and attenuates M-CSF expression.

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