IxβB Interacts with the Retinoid X Receptor and Inhibits Retinoid-dependent Transactivation in Lipopolysaccharide-treated Cells

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To elucidate the molecular action of the NFκB inhibitor IxβB, we isolated a number of IxβB interactors using the yeast two-hybrid system. These include the retinoid X receptor (RXR), whose interaction with IxβB is significantly stimulated by the RXR ligand 9-cis-retinoic acid, as shown in the yeast system as well as the glutathione S-transferase pull down assays. RXR is a nuclear protein, whereas IxβB accumulates in the nucleus only in cells stimulated with lipopolysaccharide or other inducers that result in prolonged activation of NFκB. Consistent with this, cotransfection with IxβB specifically repressed the 9-cis-RA-induced transcriptional activities of RXR in an lipopolysaccharide-dependent manner. These results suggest a novel IxβB-mediated antagonism between the signaling pathways of NFκB and RXR.

The transcription factor NFκB is important for the inducible expression of a wide variety of cellular and viral genes (1, 2). NFκB is composed of homo- and heterodimeric complexes of members of the Rel/NFκB family of polypeptides. In vertebrates, this family comprises 50, 65 (RelA), c-Rel, p52, and RelB. These proteins share a 300-amino acid region, known as the Rel homology domain, which binds to DNA and mediates homo- and heterodimerization. This domain also is the target of the IxβB inhibitors, which include IxBα, IxBβ, IxBγ, Bcl-3, p105, and p100 (3). In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IxB proteins. Treatment of cells with various inducers results in the degradation of IxB proteins. The bound NFκB is released and translocates to the nucleus, where it activates appropriate target genes. IxBα is degraded in response to all of the known inducers of NFκB, whereas IxBγ is degraded only when cells are stimulated with inducers such as lipopolysaccharide (LPS) and interleukin-1 that cause persistent activation of NFκB (4).

Following degradation of the initial pool of IxβB in response to LPS or interleukin-1, newly synthesized IxβB accumulates in the nucleus as an unphosphorylated protein that forms a stable complex with NFκB and prevents it from binding to newly synthesized IxBα (5–7).

To understand the molecular action of IxβB, we exploited the yeast two-hybrid system (8) to isolate a series of cDNAs encoding proteins that specifically interact with IxβB. Interestingly, these include retinoid X receptor (RXR), a member of the nuclear hormone receptors that comprise a large family of ligand-dependent transcription factors, bind as homodimers or heterodimers to their cognate DNA elements, and regulate genes involved in critical aspects of cell proliferation, differentiation, and homeostasis (9). Herein, we show that the RXR-IxβB interactions are stimulated by the RXR ligand 9-cis-RA and that cotransfection with IxβB specifically represses the 9-cis-RA-induced transcriptional activities of RXR in an LPS-dependent manner. These results are consistent with a novel IxβB-mediated antagonism between the signaling pathways of NFκB and RXR.

EXPERIMENTAL PROCEDURES

Plasmids—Polymerase chain reaction-amplified fragments encoding IxBα, Bcl-3, p50, and p65 were cloned into EcoRI and Sall restriction sites of the LexA fusion vector pEG202PL or EcoRI and Xhol restriction sites of the B42 fusion vector pG4–5 (8). B42/IxBβ1, IxBβ2, and IxBβ3 were isolated as independent isolates of Trypt in the yeast two-hybrid screening as described (10). Plasmids encoding LexA fusions to glucocorticoid receptor, RXR, TR, and TR459 as well as T7 vectors to express RXR-LBD, TR-LBD, TR459 and TR-ABC are as described (11–13). To express GST fusions, polymerase chain reaction-amplified fragments encoding full-length IxBβ and IxBβΔ1 were cloned into EcoRI and Xhol restriction sites of pGEX4T (Pharmacia Biotech Inc.). For mammalian expressions, full-length IxBα and IxBβ were constructed into the CDM8 vector. The expression vector for RXR, the reporter construct TREgal-CAT, and the transfection indicator construct pRSV-β-gal are as described (12, 13). The expression vector for Gal4–VP16 and the reporter construct Gal4-TKLuc were as described (14).

Yeast Two-hybrid Screening of cDNA Library—Candidate IxβB interacting clones were isolated from a mouse liver cDNA library (13) using the yeast two-hybrid system as described (8), with slight modifications. Approximately 10⁶ primary yeast transformants of a derivative of EGY48 expressing the LexA-IxBβ1A were generated from an initial transformation with the cDNA library with selection for Trypt auxotrophy. Library transformed cells were pooled and selected for Leu auxotrophy and expression of the LexA-β-galactosidase construct. A number of leucine-independent colonies harboring cDNAs encoding candidate LexA-IxBβ1A interactors were obtained. The cDNA library plasmids were recovered from appropriate yeast strains, propagated in Escherichia coli, and reintroduced into EGY48 derivatives expressing LexA alone, LexA-IxBβ1A, or other LexA chimeras to confirm specific interaction. Finally, identities of isolated cDNAs were determined by DNA sequencing.
Interactions between various components of the NFκB complex in yeast

The indicated B42 and LexA plasmids were transformed into a yeast strain containing an appropriate β-galactosidase reporter gene. At least six separate transformants from each transformation were transferred to indicator plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, and reproducible results were obtained using colonies from a separate transformation. W, white colonies (no interaction); B, blue colonies after 1 day (strong interaction); LB, blue colonies after 3 days (weak interaction).

TABLE I

Interactions between various components of the NFκB complex in yeast

| Ligand | LexA/GR | LexA/RXR | LexA/TR | LexA/TR459 |
|--------|---------|---------|---------|------------|
| B42/-  | -       | +       | +       | +          |
| B42/Bcl-3 | W W W W | W W W W | W W W W | W W W W |
| B42/1xββ | W W W B | W W B W | W W B W | W W B W |
| B42/ββΔ1 | W W B B | W B W B | W B W B | W B W B |
| B42/ββΔ2 | W W L B | W B B B | W B B B | W B B B |
| B42/ββΔ3 | W W L B | W B B B | W B B B | W B B B |

Yeast Two-hybrid Test—For the yeast two-hybrid tests, plasmids encoding LexA fusions and B42 fusions were cotransformed into Saccharomyces cerevisiae EGY48 strain containing the β-gal reporter plasmid, SH18–34 (8). Plate and liquid assays of β-gal expression were carried out as described (10, 13). Similar results were obtained in more than two similar experiments.

GST Pull Down Assays—The GST fusions or GST alone was expressed in E. coli, bound to glutathione-Sepharose-4B beads (Pharmacia), and incubated with labeled receptors or lucerase expressed by in vitro translation by using the TNT-coupled transcription-translation system, with conditions as described by the manufacturer (Promega, Madison, WI). Specifically bound proteins were eluted from beads with 40 mM reduced glutathione in 50 mM Tris (pH 8.0) and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described (12).

Cell Culture and Transfections—CV1 cells were grown in 24-well plates with medium supplemented with 10% charcoal-stripped serum for 24 h and transfected with 150 ng of β-galactosidase expression vector pSVβ-β-gal and 100 ng of a reporter gene TREpal-CAT along with 10 ng of RFXs and increasing amounts (10–200 ng) of IκBβ expression vectors. For control experiments, Gal4-TR-Luc and Gal4-VIP16 (14) replaced TREpal-CAT and XR, respectively. Total amounts of expression vectors were kept constant by adding decreasing amounts of the CDMS expression vector to transfections containing increasing amounts of the IκBβ vector. After 12 h, cells were washed and refed with Dulbecco’s modified Eagle’s medium containing 10% charcoal-stripped fetal bovine serum. After 12 h, cells were left unstimulated or stimulated with 2 μg/ml LPS either in the presence or the absence of 10 nM 9-cis-RA. Cells were harvested 24 h later, and CAT or luciferase activity was assayed as described (15), and the results were normalized to the β-galactosidase expression. Similar results were obtained in more than two similar experiments.

RESULTS AND DISCUSSION

Genetic Selection for IκBβ Interacting cDNA Clones in Yeast—The validity of the yeast two-hybrid system (8) in isolating IκBβ interactors was tested by examining interaction properties of IκBβ with various components of the NFκB complex in yeast. As expected from the published results (1–4), IκBβA1, a N-terminal deletion mutant of IκBβ (depicted in Fig. 1), interacted specifically with p65 but not with p50. Similarly, p50 interacted with p65, Bcl-3, and p50, whereas p65 did not interact with Bcl-3 or p50 (Table I). In addition, Bcl-3 readily interacted with IκBβ and IκBβA1. Full-length IκBβ was a transcriptional activator in yeast when fused to a heterologous DNA binding domain, whereas IκBβA1 was transcriptionally inert. Thus, we used IκBβA1 as a bait to isolate IκBβ interactors in the yeast two-hybrid system. One of the strongest IκBβ interactors isolated from a mouse liver cDNA library (13) encoded the RXR sequences from the third cysteine residue of the first zinc finger motif within the DNA-binding domain to the C termini. This result is consistent with our previous report in which IκBβ (initially referred to as Trip9) was first identified as one of a series of TR or RXR interacting proteins (10). These results immediately raised a possibility of a novel, IκBβ-mediated cross-talk between NFκB and nuclear receptor signaling pathways.

Ligand-stimulated Interactions of IκBβ and RXR/TR—The interaction of IκBβ with receptors was further characterized using the yeast two-hybrid system and GST pull down assays. As shown in Table II, the full-length IκBβ and three N-terminal deletion mutants (depicted in Fig. 1) corresponding to independent isolates of Trip9 (10) all interacted strongly with TR or RXR interacting proteins (10). These results immediately raised a possibility of a novel, IκBβ-mediated cross-talk between NFκB and nuclear receptor signaling pathways.

2 S. Y. Na and J. W. Lee, unpublished observations.
with LexA/TR459, a mutant TR-LBD that lacks AF-2 function but retains wild type affinity for thyroid hormone (T3) (10, 11). This mutation blocked interaction with all of the IxB proteins, suggesting that the interaction interface may involve the AF-2 domain of nuclear receptors and the LXXLL motifs in the ankyrin repeats of IxB.

To further characterize these interactions in vitro, GST fusions to IxB and IxBΔ1 were expressed, purified, and tested for interaction with various in vitro translated receptor constructs. These include the ligand binding domains of RXR and TR (RXR-LBD and TR-LBD), the ABC domains of TR (TR-ABC), and full-length TRs deleted or point-mutated for the AF2 domain (TR4 and TR459, respectively). As shown in Fig. 2, IxB and IxBΔ1 interacted weakly with TR-LBD and RXR-LBD in the absence of ligand. In agreement with the yeast results, however, interactions of IxB with TR-LBD or RXR-LBD were significantly enhanced in the presence of each cognate ligand. IxBΔ1 behaved similarly. In contrast, the AF2 mutants TR4 and TR459 as well as TR-ABC showed relatively weak and hormone-independent interaction with IxB and IxBΔ1. Thus, these results confirm the importance of the AF-2 for the ligand-dependent interactions and also suggest an additional ligand-independent interaction interface at the N-terminal ABC domains.

Cotransfections of IxB Repress the 9-cis-RA-induced Transcriptional Activities of RXR in an LPS-dependent Manner—To assess the functional consequences of these interactions, IxB was cotransfected into CV1 cells along with an RXR expression vector and a reporter construct controlled by TREpal, which is transactivated by RXR-RXR homodimers as well as various receptor heterodimers (18). Increasing amounts of cotransfected IxB had no significant effect on transcriptional activities of the TREpal-driven reporter, either in the presence or the absence of 9-cis-RA (Fig. 3). Similarly, RXR did not affect the ability of either IxB or IxBΔ1 to inhibit transactivation by p65.3 However, addition of 2 μg/ml LPS specifically inhibited 9-cis-RA-induced transcription in an IxB dose-dependent manner, with cotransfection of 200 ng of IxB decreasing transcriptional activities near background levels (Fig. 3). In contrast, cotransfection of IxB did not affect the transcriptional activity of Gal4-VP16, either in the presence or the absence of LPS, as assessed using the Gal4-TKLuc reporter construct (14) (data not shown). Similarly, IxB did not significantly affect β-galactosidase expression of the transfection indicator construct pRSV-β-gal in the presence or the absence of LPS or 9-cis-RA (data not shown). These results are consistent with the proposal that IxB translocation into the nucleus is dependent on stimulation by chronic inducers such as LPS (5–7) and suggests that only this nuclear IxB is capable of interacting with nuclear receptors. These results along with the yeast and in vitro interaction results suggest that this nuclear IxB may mask the AF-2 domain of nuclear receptors from interacting with receptor coactivators. Alternatively, IxB bound to the AF-2 domain may have more direct inhibitory interactions with the transcriptional machinery. Consistent with this, LexA/IxB was a transcriptional activator in yeast, whereas LexA/IxBΔ1 was not, suggesting the existence of an autonomous transactivation domain at the N terminus of IxB (amino acids 1–173).2 In addition, full-length IxB also showed specific binding to a novel transcription cofactor we recently isolated.3

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3 M. Chung and D. D. Moore, unpublished observations.
4 H. J. Kim, S. K. Lee, S. Y. Na, H. S. Choi, and J. W. Lee, manuscript in preparation.
In conclusion, we have identified RXR as an \( \text{IkB}\beta \) interactor and shown that this interaction decreases RXR-driven transcriptional activities in an LPS-dependent manner. This antagonism is in marked contrast to the glucocorticoid-dependent inhibition of NF\( \kappa \)B activity, in which glucocorticoids increase the synthesis of \( \text{IkB}\alpha \) which should then sequester NF\( \kappa \)B in an inactive cytoplasmic form (19). However, it was recently suggested that glucocorticoid-induced \( \text{IkB}\alpha \) synthesis and inhibition of NF\( \kappa \)B activity are two separable biochemical processes (20). Accordingly, glucocorticoid-mediated inhibition of NF\( \kappa \)B activity may involve other mechanisms such as the \( \text{IkB}\beta \)-RXR interactions described here. The antagonistic interaction is consistent with the fact that LPS is one of the best known pro-inflammatory agents (21), whereas retinoids are anti-inflammatory (22–24). Thus, exploration of these interactions may lead to new insights into mechanisms of inflammatory signal transduction pathways and possibly the development of new anti-inflammatory agents.

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