IkB-\(\alpha\) Enhances Transactivation by the HOXB7 Homeodomain-containing Protein*

(Received for publication, October 7, 1998, and in revised form, November 23, 1998)

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Combinatorial interactions between distinct transcription factors generate specificity in the controlled expression of target genes. In this report, we demonstrated that the HOXB7 homeodomain-containing protein, which plays a key role in development and differentiation, physically interacted in vitro with IkB-\(\alpha\), an inhibitor of NF-\(\kappa\)B activity. This interaction was mediated by the IkB-\(\alpha\) ankyrin repeats and C-terminal domain as well as by the HOXB7 N-terminal domain. In transient transfection experiments, IkB-\(\alpha\) markedly increased HOXB7-dependent transcription from a reporter plasmid containing a homeodomain consensus-binding sequence. This report therefore showed a novel function for IkB-\(\alpha\), namely a positive regulation of transcriptional activity by homeodomain-containing proteins.

Multiple transcription factors establish combinatorial interactions to achieve their in vivo specificity. These protein-protein interactions modulate the activating or repressing abilities of the complexes. The identification of all the partners interacting with a transcription factor is thus essential for the understanding of its biological functions.

Homeodomain-containing proteins are transcription factors that play a crucial role in the development of many species, including humans (1–4). They share a highly conserved 60-amino acid DNA-binding domain, the homeodomain, and control the expression of many target genes, most of which remain unknown (5). These proteins are encoded by 39 HOX genes, which are organized in four clusters (loci A, B, C, and D) located on chromosomes 7, 17, 12, and 2, respectively (6). Interestingly, their pattern of expression along the anteroposterior axis of the developing embryo is closely related to their chromosomal position on the cluster (7), defining a "spatial colinearity." Although homologous recombination experiments have clearly demonstrated their in vivo specificity, all the HOX gene products bind to very similar sequences in vitro (8–10). Their specificity may thus be achieved not only through DNA-protein interactions but also through protein-protein interactions with other transcription factors whose identities remain largely unknown. Among these partners, the extradenticle/Pbx homeodomain-containing proteins were the first to be identified as co-factors for HOX proteins (11). Interaction with the PBX protein requires the pentapeptide, a conserved domain located upstream of the DNA-binding domain of most HOX gene products and required for the interactions of HOX proteins with other peptides (11), as well as the HOX cooperativity motif, a sequence C-terminal to the Pbx homeodomain (12). Because AbdB-like HOX proteins do not harbor any pentapeptide-like sequence, they cannot interact with Pbx proteins (13), thus suggesting that other partners might be involved. Indeed, a recent report has illustrated the existence of heterodimeric complexes between HOX and Meis1 proteins (13). Moreover, it is likely that other proteins yet to be identified also interact with HOX proteins and contribute to their biological function.

HOXB7 cDNA was initially isolated from an SV40-transformed human fibroblast cDNA library (14). The HOX7 protein is involved in a variety of development processes, including hematopoietic differentiation and lymphoid development (15–17). Because of its expression in lymphoid and nonlymphoid cells, the HOXB7 protein might be involved in the regulation of a common transcriptional event rather than in lineage-specific gene expression (18). However, despite the demonstration of HOXB7 protein binding to DNA (19), little is known about its transcriptional properties and interacting partners in vivo. We first demonstrated that the HOXB7 protein as well as a naturally occurring mutant harboring a truncated C-terminal tail both transactivate from a HOX-binding consensus sequence in breast cancer cells (20) and physically interact with the coactivator CREB-binding protein.

The NF-\(\kappa\)B proteins form a family of transcription factors that play a central role in the cellular responses to stress, cytokines, and pathogens (22–24). Indeed, these transcription factors are activated in response to a variety of extracellular signals such as phorbol esters, tumor necrosis factor-\(\alpha\), interleukin-1, lipopolysaccharide, UV irradiation, viral infection, and growth factors (24, 25) and regulate a wide spectrum of immune and inflammatory responses (26). In unstimulated cells, NF-\(\kappa\)B activity is inhibited by another class of proteins that includes IkB-\(\alpha\) (27, 28), IkB-\(\beta\) (29), IkB-\(\epsilon\) (30), p105, and p100. These inhibitory proteins all share ankyrin repeats, sequester the NF-\(\kappa\)B complexes in the cytoplasm, and block their binding to \(\kappa\)B DNA sequences. Initially described as a cytoplasmic protein (28), IkB-\(\alpha\) has since been detected in the nucleus of transfected Vero cells (31) as well as after serum stimulation.

* This work was supported by grants from the National Fund for Scientific Research, Telévie (Belgium) and the Centre Anti Cancéroux (University of Liège, Belgium). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 A. Chariot, C. van Lint, M. Chapelier, J. Gielen, M.-P. Merville, and V. Bours, submitted for publication.

The Journal of Biological Chemistry Vol. 274, No. 9, Issue of February 26, pp. 5318–5325, 1999
Printed in U.S.A.
proteins deleted of 12, 34, 80, and 97 C-terminal amino acids, respectively. These constructs include pGEX-2TK vector (Amersham Pharmacia Biotech) to create GST fusion constructs. pcDNA3 (Invitrogen, San Diego, CA) and pMT 2T. The constructs were subcloned by PCR into the expression vector pcDNA3. The PMT 2T expression vector for IκB-α was previously described (37, 38). The IκB-α expression vectors for p50, RelA, and IκB-α were previously described (37, 38). The IκB-α coding sequence was also subcloned by PCR into the expression vector pcDNA3. The PMT 2T expression vectors for IκB-αΔN and IκB-αΔC lacking the first 53 codons and the last 42 codons of IκB-α, respectively (39), are schematically illustrated in Fig. 1A. The PMT 2T expression vector for IκB-α N-C GST codes for a protein where the ankyrin repeats of IκB-α have been replaced by the GST peptide (Fig. 1A) as described (39).

Both the pT109 and pTCBS reporter plasmids were provided by Dr. Zappavigna (Laboratory of Gene Expression, Department of Biology and Technology, Instituto Scientifico H. S. Raffaele, Milan, Italy). The pTCBS plasmid contains an 8-fold multimerized form of a homeodomainminimal promoter and the luciferase gene (40).

For GST interaction experiments, various functional domains of IκB-α were subcloned by PCR into the BamHI/EcoRI polylinker of the pGEX-2TK vector (Amersham Pharmacia Biotech) to create GST fusion proteins. These constructs include pGEX IκB-α ΔC, pGEX IκB-α ΔN, pGEX ankyrins, pGEX N129, and pGEX C80, and are schematically illustrated in Fig. 1B. The sequence of primer 1 is 5′-TATAGGATC-CTATGTTCCAGGCCTGCCC-3′; primer 2, 5′-TATAGGATCCATCAGCGGCGATCC-3′; primer 3, 5′-TATAGGATCCAGCGGCGATCC-3′; primer 4, 5′-TATAGGATCCAGCGGCGATCC-3′; and primer 5, 5′-TATAGGATCCAGCGGCGATCC-3′.

In Vitro Translation—In vitro transcription and translation were performed using the Wheat Germ TNT kit provided by Promega (Madison, WI) with 1 μg of various DNA templates and [35S]methionine, according to the protocol provided by the manufacturer.

In Vitro Protein-Protein Interactions—GST fusion proteins were produced in the Escherichia coli BL21 bacterial strain. Bacteria were grown in 500 ml of Luria broth to an A600nm of 0.6, induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h and harvested. Bacterial pellets were washed once with phosphate-buffered saline, resuspended in 10 ml of NENT buffer (250 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 1 mM DTT) of at least three independent experiments after normalization to the protein concentration of the extracts.

Nonidet P-40 (1.5%) and sonicated three times for 15 s at 4°C. Insoluble materials were removed by centrifugation. GST fusion proteins were purified after incubation of 1 ml of the supernatant with 10 μl of glutathione-Sepharose beads for 1 h at 4°C (Amersham Pharmacia Biotech). The beads were then washed twice with 1 ml of NENTM buffer (NENT + 0.5% milk) and once with 1 ml of TBW buffer (20 mM Tris, pH 8.0).

2 The abbreviations used are: PCR, polymerase chain reaction; CBS, consensus-binding sequence; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—The MDA-MB231 cell line was obtained from the American Type Tissue Collection (Rockville, MD). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics.

**Plasmids**—Coding sequences for the HOXB7 protein and for a naturally occurring protein lacking 2 amino acids in its C-terminal sequence (B7+ 20) were subcloned by PCR into the expression vectors pcDNAs (Invitrogen, San Diego, CA) and pMT2T. The constructs were sequenced to confirm the integrity of the amplified regions. pcDNA3 expression vectors coding for HOXB7 proteins deleted either in the N- or the C-terminal domain were constructed by PCR amplification. The constructs B7-ΔN18, ΔN54, ΔN86, and ΔN129 generate HOXB7 proteins lacking 18, 54, 86, and 129 N-terminal amino acids, respectively. The constructs B7-ΔC12, B7-ΔC34, B7-ΔC80, and B7-ΔC97 encode HOXB7 proteins deleted of 12, 34, 80, and 97 C-terminal amino acids, respectively.

The mammalian PMT2T expression vectors for p50, RelA, and IκB-α were previously described (32). The nuclear localization of IκB-α is mediated by its second ankyrin repeat, which acts as a nuclear import sequence (33).

In this report, we demonstrated that IκB-α is able to physically interact with the HOXB7 homeodomain-containing protein and to enhance HOXB7 transcriptional activity. We further identified HOXB7 and IκB-α domains involved in this interaction. Our results thus demonstrate a novel function of IκB-α.

**FIG. 1. Schematic illustration of the IκB-α expression vectors (A) and the GST-IκB-α constructs (B).** The ankyrin repeats are illustrated by dark rectangles. The primers designed for PCR amplification are numbered from 1 to 6 and represented by arrows. Primers 4–6 are derived from the complementary strand.
Hepes, pH 7.9, 60 mM NaCl, 1 mM dithiothreitol, 6 mM MgCl₂, 8.2% glycerin, 0.1 mM EDTA). In each case, the expected fusion proteins were visualized on a 12% polyacrylamide gel stained by Coomassie Blue.

Protein-protein interactions were performed by incubating an aliquot of the GST-IkBα fusion protein bound to the glutathione-Sepharose beads with 10 mlof in vitro translated protein in 200 μl of TBW buffer for 1 h at 4°C. Beads were then washed six times with 1 ml of NENTM buffer, resuspended into migrating buffer, and loaded on an SDS-polyacrylamide gel before autoradiography.

**Fig. 3.** HOXB7 does not modify NF-κB transcriptional activity. MDA-MB231 cells were transfected with 1 μg of p50, RelA, and/or IκB-α expression vectors together with various amounts of HOXB7 expression vector (0.5, 1, or 2 μg) and 1 μg of the κB-ICAM-1 reporter plasmid, as indicated in the figure. The figure shows the relative CAT activity over the basal activity observed with 1 μg of the κB-ICAM-1 reporter plasmid alone. Each value represents the mean (± S.D) of at least three independent experiments after normalization to the protein concentration of the extracts.

**Fig. 4.** In vitro protein-protein interaction between HOXB7 and IκB-α requires the HOXB7 N-terminal domain. The HOXB7 expression vectors are schematically represented. B7ΔN18, ΔN54, ΔN86, and B7ΔN129 products are deleted in their N-terminal domain. The homeodomain is illustrated by a large shaded rectangle, whereas the pentapeptide is represented by a small shaded box upstream of the homeodomain, and the acidic C-terminal tail is shown as a cross-hatched box. The expected molecular mass of the resulting proteins is mentioned on the right. 35S-Labeled in vitro translated wild-type and deleted HOXB7 proteins were incubated with a GST-IκB-α fusion protein attached to glutathione-Sepharose beads (lanes 2, 5, 8, 11, and 14), precipitated and run on an SDS-polyacrylamide gel. Beads carrying the GST protein alone were used as negative controls (lanes 3, 6, 9, 12, and 15). In vitro translated proteins (10% of the amounts used in the precipitation experiments) were run on lanes 1, 4, 7, 10, and 13.
RESULTS

p50, RelA, and IkB-α Enhance Transactivation by the HOXB7 Protein—To investigate whether the HOXB7 protein can interact with transcription factors from other families, we transiently transfected MDA-MB231 cells with a HOXB7 expression vector and a variety of constructs coding for different members of the NF-κB/IkB families. Both the pTCBS and pT109 constructs were used as reporter plasmids: the pTCBS plasmid contains a luciferase reporter gene driven by a multimerized HOX CBS that is recognized by most HOX proteins, whereas the pT109 vector does not harbor any HOX-binding sequence and was used as a negative control (42). A 3.6-fold induction over basal luciferase activity was measured when the HOXB7 expression vector was transfected with pTCBS (Fig. 2, column 3), as described previously (20, 41). This effect was mediated by the binding of the HOXB7 protein to the CBS sequence, because no significant effect was observed with the pT109 reporter plasmid (Fig. 2, column 4).

When p50 and RelA expression vectors were transfected with the pTCBS or pT109 reporter plasmids, weak inductions of luciferase activity were observed (Fig. 2, columns 5 and 6).

Moreover, a very weak increase in luciferase activity was observed when the plasmid encoding IkB-α was co-transfected with either the pTCBS or pT109 reporter constructs (Fig. 2, columns 7 and 8), indicating that, as expected, IkB-α did not transactivate through these promoters in MDA-MB231 cells. When the HOXB7 expression construct was co-transfected with p50, RelA, and pTCBS, a 4.9-fold induction over basal luciferase activity was observed (Fig. 2, column 9), indicating that NF-κB members enhanced HOXB7 transcriptional activity. To determine whether IkB-α could inhibit the transactivation observed with HOXB7 and p50-RelA, we co-transfected an IkB-α expression vector with the plasmids generating the HOXB7, p50, and RelA proteins as well as with the pTCBS construct. Surprisingly, a further increase in luciferase activity (7.2-fold induction over basal luciferase activity) was measured (Fig. 2, columns 11). Moreover, the luciferase activity was even more elevated (13.7-fold induction over basal luciferase activity) when we co-transfected only the HOXB7 and IkB-α expression vectors with the pTCBS reporter plasmid (Fig. 2, column 13).

To further characterize the transcriptional properties of the HOXB7 protein, additional transient expression experiments were performed using the b-ICAM-1 reporter plasmid harboring three b-like binding sites upstream of a CAT gene. As expected, transfection of the p50 and RelA expression vectors induced CAT activity (Fig. 3, column 2), and this effect was inhibited by simultaneous expression of IkB-α (Fig. 3, column 3).
Results suggest that the HOXB7 protein does not significantly activate by HOXB7/I

in vitro demonstrating the existence of an lane 3

HOXB7 did not interact with the GST protein (Fig. 4, lane 2). A positive signal was detected (Fig. 4, lane 2). No significant induction of CAT activity was measured when HOXB7 and IκB-α expressing vectors were co-transfected (Fig. 3, columns 14–16). These results suggest that the HOXB7 protein does not significantly modulate the transcriptional abilities of NF-κB members.

**IkB-α Physically Interacts in Vitro with the N-terminal Domain of HOXB7**—To determine whether IκB-α physically interacted with the HOXB7 protein, purified GST-IκB-α fusion protein bound to glutathione-Sepharose beads was incubated with in vitro translated HOXB7. After precipitation of the beads, a positive signal was detected (Fig. 4, lane 2). HOXB7 did not interact with the GST protein (lane 3), thus demonstrating the existence of an in vitro interaction between HOXB7 and IκB-α. To map the HOXB7 domain involved in this interaction, additional constructs including a naturally occurring domain of HOXB7 were designed generating HOXB7 constructs providing HOXB7 and IκB-α fusion proteins attached to glutathione-Sepharose beads, precipitated, and run on an SDS-polyacrylamide gel. Beads carrying the GST protein alone were used as positive controls (lane 3).

**The Ankyrin Repeats and the C-terminal Domain of IκB-α**

for B7-ΔC97 (Fig. 5, lanes 5, 8, 11, 14, and 17). These results indicate that a HOXB7/IκB-α physical interaction can occur independently of the homeodomain sequence and depends exclusively on an intact HOXB7 N-terminal sequence.

**The N-terminal Domain of the HOXB7 Protein Is Required for the Interaction with IκB-α in Vivo**—We previously demonstrated that both the N-terminal domain and the acidic C-terminal tail of the HOXB7 protein mediated its transcriptional properties. Because the N-terminal domain of HOXB7 was required for the interaction with IκB-α in vitro, we transfected MDA-MB231 cells with the B7ΔN129 expression vector and the pTCBS or pT109 reporter plasmid. The B7ΔN129 product, alone or co-expressed with IκB-α, did not induce any luciferase activity (Fig. 6). Moreover, the B7-ΔC12 protein, which lacks the acidic C-terminal domain but still interacts with IκB-α in vivo (Fig. 5), did not behave as a transcriptional activator (Fig. 6). Interestingly, when both the B7-ΔC12 and IκB-α expression vectors were transfected simultaneously with the pTCBS reporter plasmid, an induction of the luciferase activity similar to that measured with both HOXB7 wild-type and IκB-α proteins was observed (Fig. 6). These results suggest that the inhibitor IκB-α potentiates HOXB7 transactivating activities through a physical interaction with the HOXB7 N-terminal domain.

**The Ankyrin Repeats and the C-terminal Domain of IκB-α**
**FIG. 8.** The IκB-α ankyrin and C-terminal domains are required for transactivation by HOXB7. MDA-MB231 cells were transfected with expression vectors coding for HOXB7 (1 μg) and for wild-type or deleted IκB-α (1 μg) as well as with the reporter plasmid (1 μg), as indicated in the figure. The figure shows the relative luciferase activity over the basal activity observed with 1 μg of the pTCBS or the pT109 reporter plasmid alone. Each value represents the mean (± S.D) of at least three independent experiments after normalization as described above.

**Luciferase activity (fold induction)**

| Sample       | Fold Induction |
|--------------|---------------|
| pTCBS        | 1             |
| pT109        | 1             |
| IκB-α        | 1             |
| IκB-α ΔC     | 1             |
| IκB-α ΔN     | 1             |
| IκB-α C      | 1             |

**DISCUSSION**

This report has demonstrated a physical interaction between the HOXB7 homeodomain-containing protein and IκB-α, resulting in an enhanced transactivation by this HOX gene product. Moreover, we identified the HOXB7 and IκB-α functional domains mediating this interaction. These results provide new insights into the transcription properties of the homeodomain-containing proteins and reveal a novel function of the inhibitor IκB-α.

All the homeodomain-containing proteins encoded by the 39 HOX genes share a highly conserved 60-amino acid DNA-binding domain, the homeodomain, and bind to very similar sequences in vitro (8–10). Their in vivo specificity may thus...
involve protein-protein interactions with other transcription factors. In this context, the homeodomain proteins derived from the extradenticle/Pbx genes act as co-factors for HOX gene products that contain a pentapeptide sequence (11), whereas the AbdB-like HOX proteins, which do not harbor a pentapeptide, interact with Meis1 (13). We have provided here evidence that the NF-κB proteins, including the p50-p65 heterodimer, can enhance the transcription potential of the HOXB7 protein in transient expression experiments. This effect is presumably mediated by physical interactions between the p50-p65 complex and HOXB7. Preliminary in vitro experiments have indeed confirmed this hypothesis (data not shown). It is tempting to speculate that these proteins, including the p50-p65 heterodimer, can enhance the transcription potential of the HOXB7 protein in transient expression experiments. This effect is presumably mediated by physical interactions between the p50-p65 complex and HOXB7. Preliminary in vitro experiments have indeed confirmed this hypothesis (data not shown).

We have shown that the inhibitor IκB-α can enhance the HOXB7 transactivating effect. This is the first demonstration that IκB-α interacts with proteins from other families of transcription factors. Previous results had demonstrated that IκB-α can translocate to the nucleus, using its second ankyrin repeat as a nuclear import sequence (33) and subsequently remove the NF-κB complex from its binding site (34). Moreover, IκB-β can repress the 9-cis-retinoic acid-induced transcriptional activity of retinoid X receptor in lipopolysaccharide-treated cells (43). Thus, in both cases, IκB proteins localized in the nucleus negatively regulate the transcriptional activity of their interacting partners. Surprisingly, our study demonstrates that IκB-α can also positively regulate the transcriptional properties of a homeodomain-containing protein. A similar phenomenon was described previously for Bcl3, another member of the IκB protein family. Indeed, Bcl3 can transactivate through κB sites when physically associated with p52 and p50 (37, 44), and it has been demonstrated that the N- and C-terminal domains of Bcl3 are transcriptional activation domains (37). Moreover, Bcl3, but not IκB-β, can also act as a coactivator of the retinoid X receptor (45). These results and
the present report strongly suggest that distinct IκB proteins can modulate, positively as well as negatively, the transcriptional properties of their interacting partners, including transcription factors that do not belong to the NF-κB family.

Interestingly, we demonstrated that both the IκBα ankyrin and C-terminal domains mediate interaction with HOXB7. The same families are also required for the regulation of c-Rel by IκB-α in the nucleus (46). Taken together, these results suggest a critical role for the ankyrin repeats and C-terminal domains in the function of IκB-α in the nucleus.

Several models, which are not mutually exclusive, can explain how IκB-α enhances HOXB7 transcriptional activity (Fig. 9). The first model does not imply a direct interaction between HOXB7 and IκB-α but rather an indirect mechanism mediated by NF-κB. Indeed, we can postulate that NF-κB activates the expression of HOX genes encoding repressors. Therefore, NF-κB inhibition by IκB-α would lead to a decreased expression of these HOX genes and to increased luciferase activity in transient expression experiments (Fig. 9A). This first model is the only one that does not require IκB-α nuclear localization.

The second model is based on a report demonstrating that ankyrin repeats stabilize the DNA binding of other transcription factors (47). The enhanced HOXB7 transcriptional activity would then be mediated by a stronger HOXB7 DNA binding affinity for its target sequence in the presence of IκB-α (Fig. 9B). This hypothesis is supported by the observation that the physical interaction between HOXB7 and IκB-α requires the ankyrin repeats. In the third model, a HOXB7-IκB-α complex is bound to the CBS sequence through the HOXB7 homodimer and transactivates through IκB-α. This hypothesis is supported by previous studies demonstrating that IκB-α can transactivate when fused to a GAL4 DNA-binding domain (35, 36). Moreover, Bcl3, another member of the IκB-α family, also harbors transcriptional activities (37). The fourth model implies a HOXB7-RelA-IκB-α complex activating the luciferase gene through both the HOXB7 and RelA transcription domains. The last two hypotheses are supported by the induction of luciferase activity observed with IκB-α and B7AC12 expression vectors, whereas the B7AC12 protein is not able to transactivate by itself but can physically interact with IκB-α. However, the last model cannot account for the fact that the transcriptional activity was higher after transfection of the HOXB7 and IκB-α expression vectors than in the presence of the same vectors plus RelA (Fig. 2). Further experiments are required to determine which of these models is correct. Unfortunately, the available HOXB7 antibodies did not allow us to study more precisely the HOXB7 multimeric complexes in transfected or unmodified cells.

The functional link between NF-κB-IκB-α and homeodomain proteins was unexpected because of the distinct physiological processes they control. However, a first link between these two families during the outgrowth of the vertebrate limb has recently been described (48, 49). Indeed, NF-κB gene expression has been detected during limb morphogenesis and the alteration of NF-κB activity causes an arrest of the outgrowth (48, 49). Moreover, IκB-α is the human homologue of cactus, a protein that plays a crucial role in the dorsoventral patterning of the Drosophila embryo (21). Because HOX genes are clearly required to establish the anteroposterior axis of the developing embryo, it is tempting to speculate that the interaction between IκB-α and HOX proteins might determine the anteroposterior and dorsoventral polarities of the embryo.

Acknowledgments—We thank Dr. P. T. van der Saag for providing the αβ2-ICAM-1 reporter plasmid and are grateful to Dr. Zappavigna for the pTCBS and pT109 plasmids.