Cytoplasmic IκBα Increases NF-κB-independent Transcription through Binding to Histone Deacetylase (HDAC) 1 and HDAC3*

Patrick Viatour‡, Sylvie Legrand-Poels§, Carine van Lint¶‡‡, Michael Warnier‡, Marie-Paule Merville‡∥, Jacques Gielen‡, Jacques Piette§‡‡, Vincent Bours‡, and Alain Chariot‡∥**

From the ‡Laboratory of Medical Chemistry and Human Genetics and the §Laboratory of Virology and Immunology, Center for Cellular and Molecular Therapy, University of Liège, Sart-Tilman, 4000 Liège, Belgium, and the ¶Laboratory of Molecular Virology, Department of Biological Chemistry, Institute of Biological and Molecular Medicine, Free University of Brussels, 6041 Gosselies, Belgium

IκBα is an inhibitory molecule that sequesters NF-κB dimers in the cytoplasm of unstimulated cells. Upon stimulation, NF-κB moves to the nucleus and induces the expression of a variety of genes including IκBα. This newly synthesized IκBα also translocates to the nucleus, removes activated NF-κB from its target genes, and brings it back to the cytoplasm to terminate the phase of NF-κB activation. We show here that IκBα enhances the transactivation potential of several homeodomain-containing proteins such as HOXB7 and Pit-1 through a NF-κB-independent association with histone deacetylase (HDAC) 1 and HDAC3 but not with HDAC2, -4, -5, and -6. IκBα bound both HDAC proteins through its ankyrin repeats, and this interaction was disrupted by p65. Immunofluorescence experiments demonstrated further that IκBα acts by partially redirecting HDAC3 to the cytoplasm. At the same time, an IκBα mutant, which lacked a functional nuclear localization sequence, interacted very efficiently with HDAC1 and -3 and intensively enhanced the transactivation potential of Pit-1. Our results support the hypothesis that the NF-κB inhibitor IκBα regulates the transcriptional activity of homeodomain-containing proteins positively through cytoplasmic sequestration of HDAC1 and HDAC3, a mechanism that would assign a new and unexpected role to IκBα.

The paradigm of NF-κB activation is based on its cytoplasmic sequestration by IκBα through the masking of its nuclear localization sequence (NLS)[1] in unstimulated cells (1). Recent studies have challenged this hypothesis suggesting that IκBα constantly shuttles in and out of the nucleus via nuclear import (2) and export sequences (3, 4). Because IκBα nuclear export is more efficient than its nuclear import process, the NF-κB·IκBα complex remains mainly in the cytoplasm of unstimulated cells. Upon stimulation by proinflammatory cytokines, viral infection, or bacterial pathogens, IκBα is phosphorylated by the IκB kinase complex and degraded through the proteasome pathway (5). Then NF-κB enters into the nucleus and activates the expression of multiple genes including IκBα. This newly synthesized IκBα protein moves to the nucleus, removes NF-κB from its target genes, and brings it back to the cytoplasm to terminate the phase of NF-κB activation. IκBα is part of a family of ankyrin-containing proteins that also includes IκBβ and IκBε (6) as well as p100, p105, and BCL-3 (7). In contrast to the IκB proteins, BCL-3 is mainly nuclear and harbors some transactivation potential through heterodimer formation with the NF-κB proteins p50 or p52 (8) and recruitment of co-activators such as JAB1 and BARD1 (9).

We previously demonstrated that IκBα enhances the transactivation potential of HOXB7, a homeodomain-containing protein (10). In this study, we elucidated further the underlying mechanism and showed that cytoplasmic IκBα enhances the transactivation potential of other homeodomain-containing proteins such as Pit-1 and PAX8 through binding to some but not all HDAC proteins, a family of proteins sharing the ability to deacetylate their substrates and to repress transcription (11). Taken together, our results therefore suggest that IκBα may have new and NF-κB-independent roles.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Reagents—293, HeLa, HCT116, and MDA-MB231 cells were maintained in Dulbecco's modified Eagle's, McCoy, and RPMI 1640 media, respectively, and supplemented with 10% fetal calf serum (Invitrogen) and antibiotics. Polyclonal anti-HA, -HDAC1, -HDAC3, -p50, -p65, and -IκBα antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The polyclonal anti-HDAC3 antibody used for the indirect immunofluorescence experiments was purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-IκBα antibody was provided by R. Hay (School of Biological and Medical Sciences, University of St. Andrews, Fife, Scotland, UK). Anti-FLAG antibodies and beads were purchased from Sigma.

The HDAC3, FLAG-HDAC1, -2, -3, -4, -5, and -6 constructs were provided by S. Emiliani (Institut Cochin de Génétique Moléculaire, INSERM U529, Paris, France). The HOXB7-expressing vector (10), the pMT₂T expression vectors for IκBα (12), IκBαΔN, N+C GST, and IκBαΔC were described previously (13) as were the pMT₂T expression vectors for p50 and p65 (14). The Pit-1-expressing vector was constructed by subcloning Pit-1 coding sequence into pcDNA3. The IκBα NLS-MT construct harbors three point mutations within the nuclear import sequence (2) and was generated by subcloning the IκBα 110A3 coding sequence, a gift from M. Hannink (University of Missouri, Columbia, MO), into the pMT₂T expression vector. The PAX8-expressing vector was a gift from M. Zannini (Dipartimento Biologia e Patologia Cellulare e Molecolare, Universita' di Napoli Federico II,

[1] The abbreviations used are: NLS, nuclear localization sequence; CBS, consensus-binding sequence; GST, glutathione *S*-transferase; HA, hemagglutinin; HDAC, histone deacetylase; PRL, prolactin; GH, growth hormone; TSHβ, thyroid-stimulating hormone β.

A

B
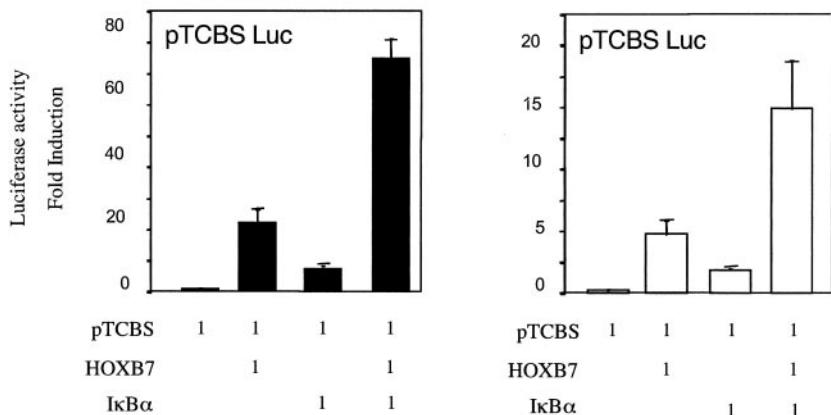
FIG. 1. **IκBα enhances the transactivating activity of HOXB7 (*A*) and PAX8 (*B*).** The effect of IκBα on the HOXB7 (*A*) or PAX8 (*B*) transactivating activity as measured by luciferase assays is shown. *A*, 293 cells or MDA-MB 231 cells (*black* and *white histograms*, respectively) were transfected with the pTCBS reporter plasmid alone or with the indicated expression plasmids as mentioned, and luciferase activities were measured. *B*, 293 or HCT116 cells (*black* and *white histograms*, respectively) were transfected with the *bcl-2* reporter plasmid alone or with various expression plasmids as mentioned, and luciferase activities were measured. The data of three independent experiments performed in triplicate, after normalization with protein concentration of the extracts, are shown (mean values ± S.D.). The amounts of transfected plasmids are mentioned. *Luc*, luciferase.
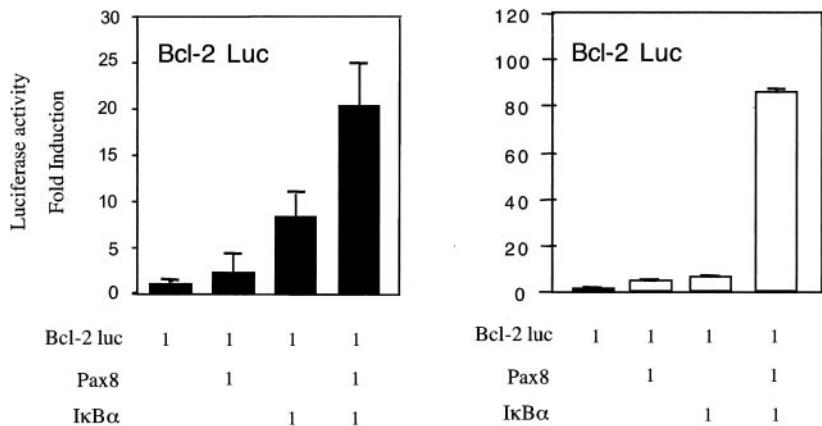
Naples, Italy). The pTCBS plasmid contains an eightfold multimerized form of a homeodomain consensus-binding sequence (CBS) cloned upstream of an HSV-TK promoter and of a luciferase reporter gene, whereas the pT109 construct does not contain the CBS and was used as a negative control (15). Both these reporter plasmids were provided by V. Zappavigna (Transcriptional Regulation in Development, Proteomics Laboratory, Department of Biological and Technological Research, H. San Raffaele, Milan, Italy). The *bcl-2* reporter plasmid was provided by L. Boxer (Department of Medicine, Stanford University School of Medicine, Stanford, CA). The prolactin (*PRL*) and growth hormone (*GH*) luciferase reporters were gifts from M. Muller (Laboratoire de Biologie Moléculaire et de Génie Génétique, Université de Liège, Liège, Belgium) (16) as was the 164-bp *PRL* reporter construct (17). The *TSHβ* luciferase reporter plasmid was described previously (18).

Immunoprecipitation—For immunoprecipitations of overexpressed proteins, 293 cells (3×10^6) were transfected with FuGENE (Roche Applied Science) and expression vectors as indicated. Subsequent lysis and immunoprecipitations were performed as described previously (19) as were the immunoprecipitations of endogenous proteins.

For identification of ternary complexes by immunoprecipitation experiments, 293 cells (10^7) were transfected with FLAG-IκBα as described above. 24 h after transfection, ectopically expressed IκBα was immunoprecipitated with an anti-FLAG antibody for 2 h at 4 °C. Anti-HA immunoprecipitations were performed in parallel as negative controls. The immunoprecipitates were washed five times with the lysis buffer and incubated overnight at 4 °C with the FLAG peptide (Sigma) according to the protocol provided by the manufacturer. The supernatants were subsequently incubated for 2 h with anti-HA (negative control), anti-p50, or anti-HDAC3 antibodies and overnight with protein A-agarose conjugate. The resulting immunoprecipitates were

washed with the lysis buffer and subjected to SDS-PAGE for anti-p65 Western blot analysis.

Indirect Immunofluorescence—293 cells were seeded in 6-well plates and subsequently transfected with the indicated expression plasmids. 24 h later, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde, and permeabilized with ethanol for 6 min at −20 °C. Fixed cells were incubated with monoclonal anti-IκBα antibodies overnight at 4 °C followed by a 60-min incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG (Dako). Coverslips were then mounted with Fluoprep (Biomerieux, Marcy l'Etoile, France), and cells were observed by fluorescent microscopy (Nikon).

Reporter Assays—HCT116, MDA-MB231, or 293 cells (4×10^5) were transfected in 6-well plates with various amounts of expression vectors as indicated. Total amounts of transfected DNAs were kept constant throughout by the addition of appropriate amounts of pcDNA3 empty vector. Cells were harvested 24 h after transfection, and luciferase activities were measured and normalized as described previously (10).

RESULTS

IκBα Enhances Transactivation by Multiple Homeodomain-containing Proteins—We previously established that IκBα enhances the transactivation potential of HOXB7, a homeodomain-containing protein (Fig. 1*A* and Ref. 10). Therefore, we investigated whether this effect also occurred in another cell line and performed identical luciferase assays in 293 cells. Again, IκBα enhanced HOXB7 transactivation potential in this cell line. To determine whether IκBα regulates the transcriptional activity of other homeodomain-containing proteins, we
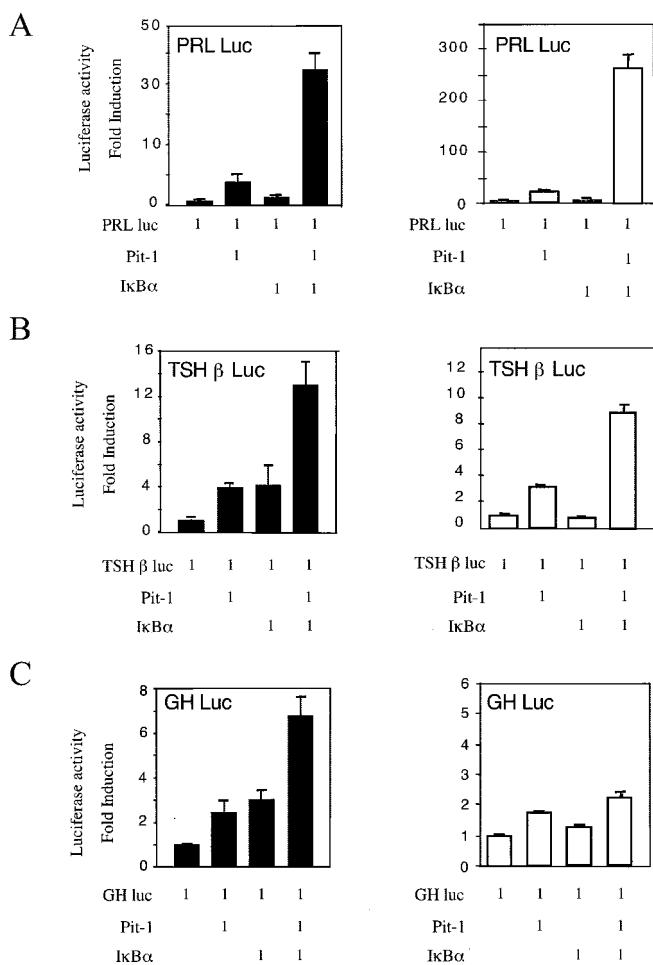
FIG. 2. **IκBα enhances the transactivating activity of Pit-1 on distinct promoters.** *A–C*, 293 cells or HCT116 cells (*black* and *white histograms*, respectively) were transfected with the indicated reporter plasmid alone or with the indicated expression plasmids as mentioned, and luciferase activities were measured. The data of three independent experiments performed in triplicate, after normalization with protein concentration of the extracts, are shown (mean values ± S.D.). The amounts of transfected plasmids are mentioned. *Luc*, luciferase.

selected PAX8, a member of the Paired domain (PAX) family known to transactivate the *bcl-2* promoter (20) and used a small fragment of this promoter as reporter plasmid. IκBα weakly transactivated this promoter in 293 or HCT116 cells but strongly enhanced PAX8-mediated transactivation in both cell lines (Fig. 1*B*). The homeodomain-containing protein Pit-1 is known to regulate the expression of GH, TSHβ, and PRL. We therefore performed similar luciferase assays by transfecting 293 or HCT116 cells with corresponding reporter plasmids either alone or with Pit-1 and/or IκBα. We again detected a strong synergistic effect on the *PRL* promoter in both cell lines (Fig. 2*A*) and to a lesser extent on the *TSHβ* promoter, especially in HCT116 cells (Fig. 2*B*, *right panel*). A weaker effect was observed on the *GH* promoter in 293 cells, whereas no significant synergistic effect was detected in HCT116 cells when the *GH* promoter was used (Fig. 2*C*, *left* and *right panel*, respectively). Therefore, our results indicate that IκBα enhances transactivation by several homeodomain-containing proteins on multiple but not all promoters. Moreover this ability of IκBα to enhance the transactivation potential of the homeodomain-containing proteins does not appear to be cell-specific. Therefore, it is likely that a general mechanism is involved.

Cytoplasmic IκBα Binds HDAC1 and HDAC3 through the

Ankyrin Repeats—Because transcription is also enhanced through cytoplasmic sequestration of HDACs (21), we examined whether the IκBα-mediated gene activation could be mediated through IκBα physical interactions with HDAC proteins. 293 cells were transfected with expression vectors coding for IκBα and for various FLAG-tagged HDAC members. Cell extracts were immunoprecipitated by either anti-IκBα or -HA (negative control) antibodies, and the immunoprecipitates were subjected to an anti-FLAG Western blot (Fig. 3*A*). Ectopically expressed IκBα was associated with FLAG-tagged HDAC1 and HDAC3 (Fig. 3*A*, *upper panel*, *lanes 2* and *6*, respectively) but not with HDAC2, -4, and -5 (Fig. 3*A*, *upper panel*, *lanes 4*, *8*, and *10*, respectively) or with HDAC6 (Fig. 3*B*, *upper panel*, *lane 3*). Similar experiments performed with extracts from untransfected cells showed that this complex occurs physiologically since endogenous HDAC1 or HDAC3 proteins interacted with endogenous IκBα (Fig. 3*C*, *upper panels*, *lanes 2*). Therefore, our results suggest that IκBα associates with some, but not all, histone deacetylase complexes *in vivo*.

To identify the IκBα domain mediating the interaction with the HDAC proteins, co-immunoprecipitation experiments were performed to test the ability of IκBα mutants (Fig. 4*A*) to interact with HDAC3. 293 cells were transfected with FLAG-HDAC3 as well as either IκBα or IκBαΔN, and anti-FLAG immunoprecipitation experiments followed by anti-IκBα Western blots were carried out on the cell extracts. As expected, FLAG-HDAC3 was found to be associated with IκBα (Fig. 4*B*, *upper panel*, *lane 2*) and also with IκBαΔN (Fig. 4*B*, *upper panel*, *lane 4*). Moreover, when IκBα or IκBαΔC were transfected with HDAC3, both proteins were detected in the anti-HDAC3 immunoprecipitates (Fig. 4*C*, *upper panel*, *lanes 2* and *4*, respectively) but not in the anti-HA immunoprecipitates (Fig. 4*C*, *upper panel*, *lanes 1* and *4*). Finally, an IκBα mutant in which the ankyrin repeats were replaced by a GST cassette and named "N+C GST" failed to interact with FLAG-HDAC3 in 293 cells (Fig. 4*D*, *upper panel*, *lane 4*), whereas the wild type IκBα protein was again found in the anti-FLAG immunoprecipitates despite a lower level of expression compared with the N+C GST mutant (Fig. 4*D*, *upper panel*, compare *lanes 2* and *4*, respectively). Taken together, these results strongly suggest that the ankyrin repeats of IκBα are critical in mediating the interaction with HDAC3. This conclusion is supported by the ability of a GST-ankyrin fusion protein to interact with *in vitro* translated HDAC3 (data not shown).

p65 Disrupts the Interaction between IκBα and HDAC3—We next determined the ability of IκBα to bind HDAC3 in the presence of increasing amounts of p65 by immunoprecipitating IκBα from transfected 293 cells and by performing anti-HDAC3 Western blots on the immunoprecipitates (Fig. 5*A*). Increasing amounts of p65 interfered with the ability of FLAG-HDAC3 to bind IκBα (Fig. 5*A*, *upper panel*, *lanes 2–4*). These results suggest that p65 and HDAC3 compete to bind a common domain of IκBα, most likely its ankyrin repeats. We then performed double immunoprecipitation experiments to investigate whether p65, HDAC3, and IκBα are part of a common complex. 293 cells were transfected with FLAG-IκBα, and total cell extracts were subsequently subjected to anti-FLAG or anti-HA immunoprecipitations. The immunoprecipitates were then released from the beads by incubation with the FLAG peptide, and the released material was subsequently immunoprecipitated with either anti-HA or with antibodies to endogenous HDAC3 or p50 proteins. Anti-p65 immunoblots were carried out on the resulting immunoprecipitates. As expected, the p50·p65·IκBα ternary complex was detected (Fig. 5*B*, *upper panel*, *lane 3*). On the other hand, no ternary complex including p65, HDAC3, and IκBα was detectable (Fig. 5*B*, *upper panel*,

Association of Cytoplasmic IκBα with HDAC1 and HDAC3
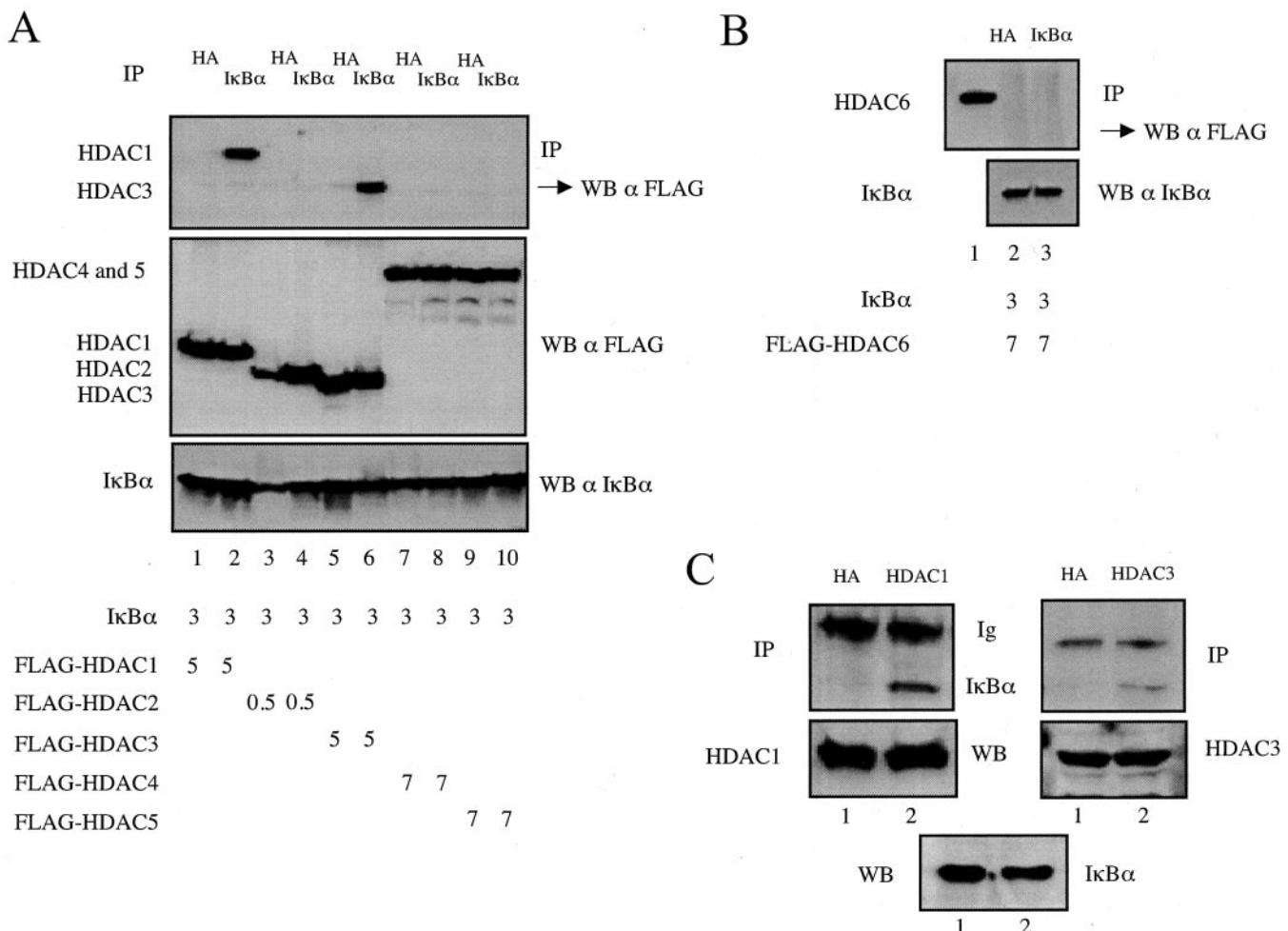
Fig. 3. **IκBα associates with HDAC1 and HDAC3 *in vivo*.** *A*, ectopically expressed IκBα physically interacts with transfected HDAC1 and HDAC3 but not with HDAC2, -4, -5, and -6. 293 cells were transfected with IκBα and either FLAG-HDAC1 (*lanes 1* and *2*), FLAG-HDAC2 (*lanes 3* and *4*), FLAG-HDAC3 (*lanes 5* and *6*), FLAG-HDAC4 (*lanes 7* and *8*), or FLAG-HDAC5 (*lanes 9* and *10*). Anti-IκBα or -HA (negative control) immunoprecipitations were performed on total cell extracts followed by anti-FLAG Western blots as indicated (*upper panel*; *IP*, immunoprecipitation; *WB*, Western blot). The expression of FLAG-HDACs and IκBα proteins in the extracts is demonstrated with Western analyses in the *middle* and *lower* panels, respectively. The amounts of transfected plasmids are mentioned *below* the panels. *B*, IκBα does not interact with HDAC6. Total cell extracts of 293 cells transfected with IκBα and FLAG-HDAC6 were subjected to anti-IκBα and -HA immunoprecipitations. The resulting immunoprecipitates were subjected to an anti-FLAG Western blot. The expression of FLAG-HDAC6 and IκBα in the extracts is illustrated in the *upper panel* (*lane 1*) and in the *lower panel* (*lanes 2* and *3*), respectively. The amounts of transfected plasmids are mentioned *below* the panels. *C*, endogenous HDAC3 and HDAC1 interact with IκBα *in vivo*. 293 cells were lysed, and extracts were subjected to immunoprecipitations with anti-HDAC1, -HDAC3 (*left* and *right panels*, respectively, *lanes 2*), or -HA (*lanes 1*) antibodies followed by anti-IκBα Western blots. The expression of HDAC1, HDAC3, and IκBα is illustrated in the *middle* and *lower panels*, respectively.

lane 6). These results suggest that IκBα binds HDAC3 independently of p65.

HDAC3, but Not HDAC2, Attenuates IκBα-mediated Positive Effect on Pit-1 and HOXB7 Transactivation Potential—We next examined the biological relevance of the interaction of IκBα with some HDAC proteins and noticed that FLAG-HDAC3 strongly decreased IκBα-mediated enhancement of Pit-1-dependent transcription on the *PRL* 164-bp reporter plasmid, whereas similar amounts of FLAG-HDAC2, which does not interact with IκBα (see Fig. 3*A*), had a much weaker effect (Fig. 6*A*). An anti-FLAG Western blot was carried out on the cell extracts to make sure that similar amounts of both FLAG-tagged HDAC proteins were expressed (Fig. 6*A*). We next investigated whether NF-κB is somehow involved in the IκBα-mediated positive effect on the homeodomain-containing protein transactivation potential by using a reporter plasmid that does not harbor any κB binding site and is therefore not induced by p50/p65. Because the *PRL* 164-bp luciferase reporter plasmid turned out to harbor a functional κB site (data

not shown), we selected the pTCBS reporter plasmid, which contains an eightfold multimerized form of a homeodomain CBS and whose activity is not induced by p50/p65 (10), and tested the effect of FLAG-HDAC3 on such reporter plasmid. A similar dose-dependent decrease of the luciferase activity was detected when the pTCBS reporter plasmid was cotransfected with HOXB7, IκBα, and increasing amounts of FLAG-HDAC3 as compared with the luciferase activity measured in the absence of FLAG-HDAC3 (Fig. 6*B*). These results suggest that the HDAC proteins differentially modulate IκBα-mediated enhancement of gene transcription, and this is correlated with their ability to physically interact with IκBα. This newly described IκBα function is NF-κB-independent since it also occurs on promoters lacking any κB binding site.

IκBα Sequesters a Pool of HDAC3 Proteins in the Cytoplasm—The possibility that IκBα causes HDAC3 cytoplasmic sequestration was investigated by studying the subcellular localization of ectopically expressed HDAC3 in HeLa cells either transfected or not with an IκBα expression vector. Transfected
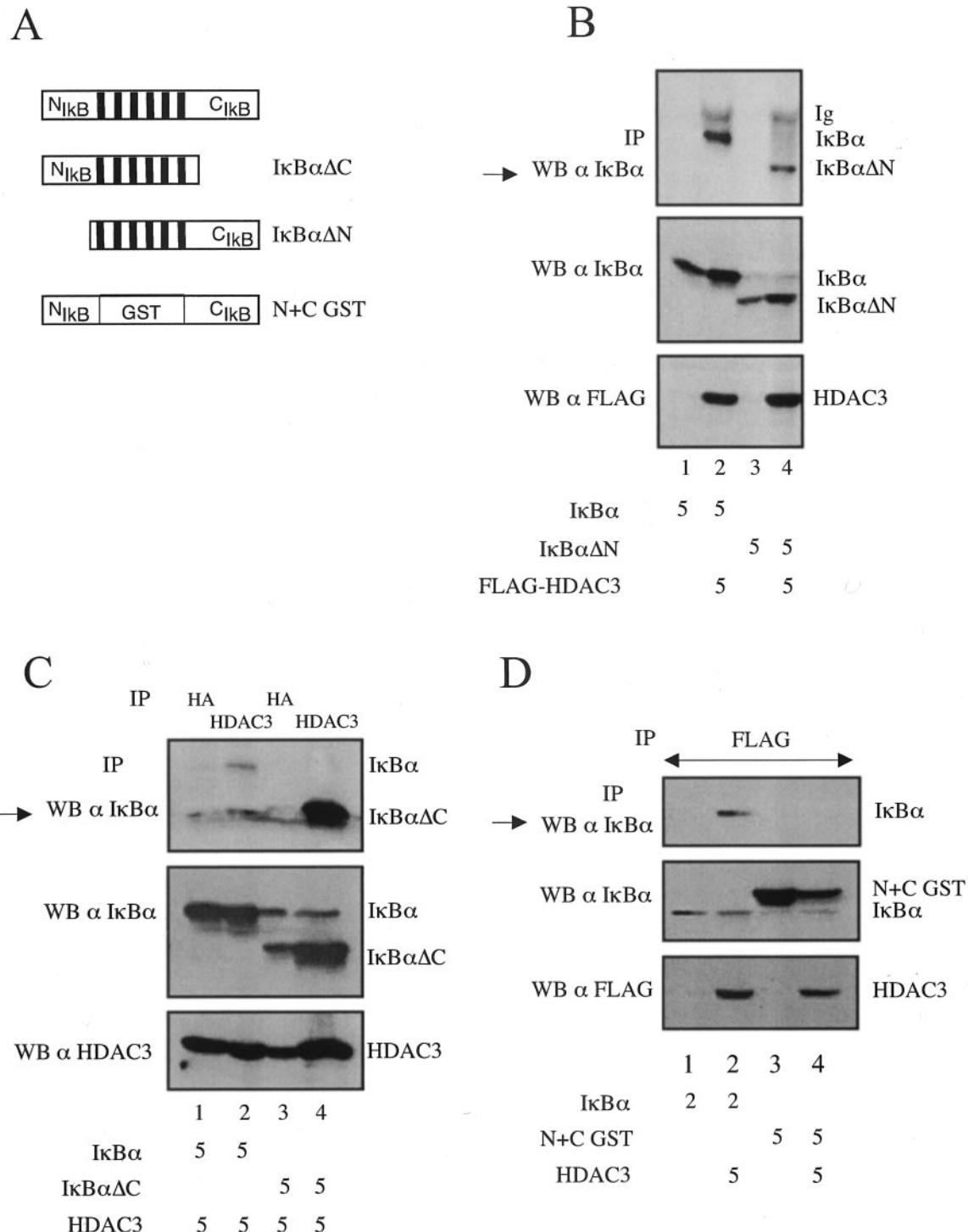
FIG. 4. **IκBα interacts with HDAC3 through the ankyrin-repeats.** *A*, schematic representation of the wild type and mutant IκBα proteins. *B*, both IκBα and IκBαΔN interact with FLAG-HDAC3. 293 cells were transfected with the indicated expression vectors, and anti-FLAG immunoprecipitations followed by anti-IκBα immunoblots were carried out (*upper panel*). The presence of the IκBα wild type protein (*lanes 1* and *2*) and mutant (*lanes 3* and *4*) as well as FLAG-HDAC3 (*lanes 2* and *4*) in the extracts is shown in the *middle* and *lower panels*, respectively. *C*, IκBαΔC interacts with HDAC3. IκBα or IκBαΔC were co-transfected with HDAC3 and subjected to an anti-HA (negative control, *lanes 1* and *3*) or anti-HDAC3 immunoprecipitation (*lanes 2* and *4*) followed by an anti-IκBα Western blot (*upper panel*). Anti-IκBα and -HDAC3 Western analysis was also performed on the cell extracts (*middle* and *lower panels*, respectively). The amounts of transfected plasmids are mentioned *below* the panels. *D*, IκBα but not N+C GST interact with HDAC3. 293 cells were transfected with IκBα (*lanes 1* and *2*) or N+C GST (*lanes 3* and *4*) with (*lanes 2* and *4*) or without FLAG-HDAC3 (*lanes 1* and *3*), and anti-FLAG immunoprecipitation followed by anti-IκBα Western blots was performed (*upper panel*). The presence of FLAG-HDAC3 as well as wild type IκBα or the N+C GST mutant in the cell extracts is illustrated in the *bottom panels*. *IP*, immunoprecipitation; *WB*, Western blot.

HDAC3 is found in the nucleus in most cells, although some cytoplasmic staining was observed in some cases, whereas ectopically expressed IκBα can be found in both cell compartments. In the absence of ectopically expressed IκBα, HDAC3

was nuclear in 47% of the counted cells, and only 13% of the cells showed a predominant cytoplasmic localization. Interestingly, a greater amount of cells (47%) showed a cytoplasmic staining of HDAC3 when both HDAC3 and IκBα were co-
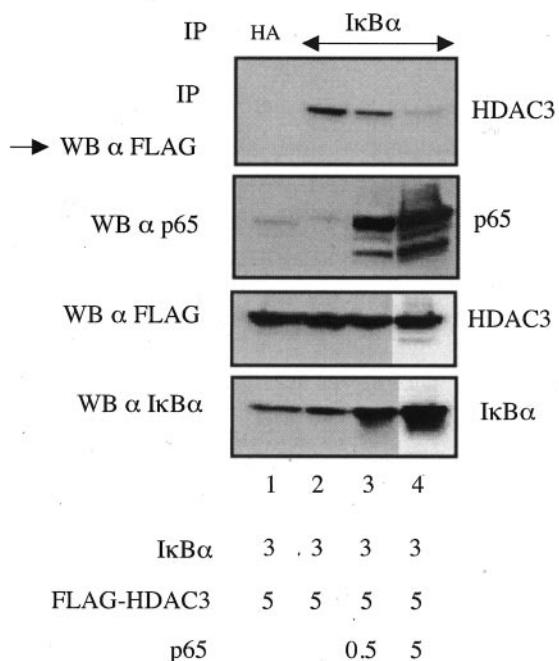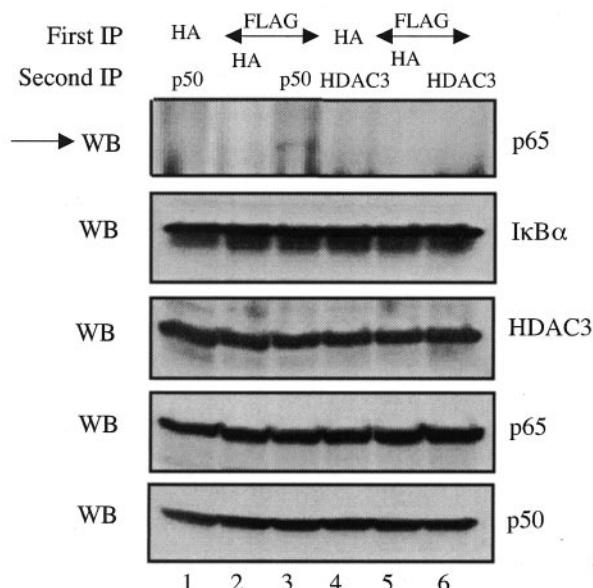
FIG. 5. **IκBα binds HDAC3 independently of p65.** *A*, p65 and HDAC3 compete for binding to IκBα. Cells were transfected with IκBα, FLAG-HDAC3, and increasing amounts of p65 (0.5 or 5 μg, *lanes 3* and *4*, respectively). Extracts were subjected to anti-HA (*lane 1*) or anti-IκBα (*lanes 2–4*) immunoprecipitations, and the presence of FLAG-HDAC3 in the immunoprecipitates was detected by anti-FLAG Western blot (*upper panel*). The presence of transfected IκBα, FLAG-HDAC3, and p65 in the cell extracts is shown in the *three lower panels*. The amounts of transfected plasmids are mentioned *below* the panels. *B*, evidence for a ternary complex associating FLAG-IκBα with endogenous p50 and p65 but not with endogenous HDAC3 and p65. 293 cells were transfected with 3 μg of FLAG-IκBα, (*lanes 1–6*). Cells were harvested 24 h later, and extracts were subjected to an anti-HA (negative control, *lanes 1* and *4*) or anti-FLAG immunoprecipitation (*lanes 2*, *3*, *5*, and *6*). After extensive washes, the FLAG immunoprecipitates were released from the beads by incubating them with a FLAG peptide overnight. Then a second immunoprecipitation was performed with anti-HA, -p50, or -HDAC3 antibodies followed by an anti-p65 Western analysis (*top panel*). The *bottom four panels* show Western analyses for FLAG-IκBα, endogenous HDAC3, p65, and p50 proteins. *IP*, immunoprecipitation; *WB*, Western blot.

expressed (data not shown). Therefore, these results demonstrate that ectopic expression of IκBα increases the cytoplasmic localization of HDAC3. Recent studies have demonstrated that IκBα constantly shuttles between the cytoplasm and the nucleus. To explore whether this shuttling affects the ability of IκBα to interact with HDAC3, we examined by co-immunoprecipitation experiments whether an IκBα mutant (IκBα NLS-MT) lacking a functional NLS (2) could still interact with HDAC3. As expected, this mutant was mainly localized in the cytoplasm when transfected into 293 cells, whereas the wild type IκBα protein was also found in the nucleus (Fig. 7*A*). Interestingly, despite low expression levels as compared with wild type IκBα (Fig. 7*B*, *lower panel*, compare *lanes 3* and *4* with *lanes 1* and *2*), IκBα NLS-MT strongly interacted with HDAC3 or HDAC1 as judged by immunoprecipitation experiments of cells transfected with IκBα (*lanes 1* and *2*) or IκBα NLS-MT (*lanes 3* and *4*) and with either FLAG-HDAC1 or FLAG-HDAC3 (Fig. 7*B*, *upper panels* on the *left* and *right*, compare *lanes 2* and *4*). Moreover IκBα NLS-MT efficiently enhanced Pit-1-mediated transcription through the *PRL* promoter (Fig. 7*C*). Taken together, these results therefore suggest that IκBα enhances Pit-1 transactivating activity through cytoplasmic sequestration of transcriptional repressors such as HDAC1 and -3.

DISCUSSION

The IκBα protein is ubiquitously expressed as an NF-κB cytoplasmic inhibitor. In response to various stimuli, IκBα is rapidly degraded to allow NF-κB nuclear translocation and NF-κB target gene transcription. As IκBα itself is the product of an NF-κB-regulated gene, it is rapidly resynthesized and can then migrate to the nucleus to terminate the first phase of NF-κB activation. Our present data demonstrate that this IκBα inhibitor can also associate with HDAC proteins and sequester them in the cytoplasm. Although we do not have any direct evidence that newly synthesized IκBα binds to HDACs in response to NF-κB-activating stimuli and brings them to the cytoplasm, it is possible that such activity participates in NF-κB target gene transcription. Indeed, upon stimulation, two waves of NF-κB recruitment to its target genes occur (22). The first wave involves immediately accessible genes and appears to be terminated through IκBα-mediated removal of NF-κB from its binding sequences (23), whereas the second wave involves genes whose promoter accessibility requires stimulus-dependent modification of chromatin structure (22). This second wave appears to rely on IκBβ and IκBε, which stabilize NF-κB responses during longer stimulations (23) and, as suggested by the present data, could possibly be favored by IκBα-mediated cytoplasmic sequestration of HDACs.

We previously demonstrated that IκBα physically interacts with HOXB7, and this interaction was required to mediate IκBα positive effect on HOXB7-driven transcription (10). This mechanism presumably relies on a nuclear localization of IκBα, which has been clearly established and would imply that IκBα is capable of recruiting some coactivators in the vicinity of HOXB7 as does the transactivating protein BCL-3, another ankyrin-containing protein of the same family, with the coactivators BARD1 and JAB1 (9). We indeed detected physical

A

B
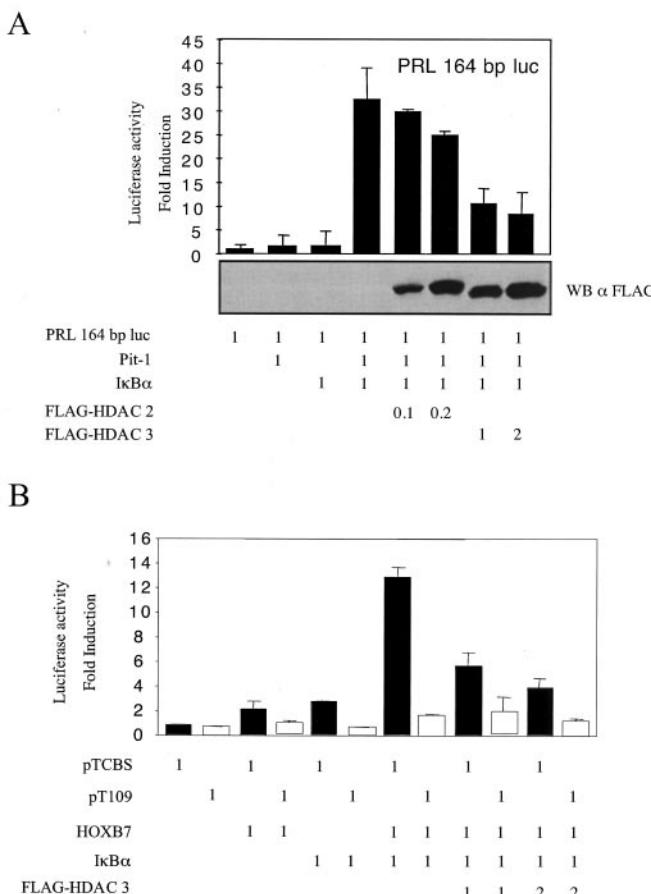
FIG. 6. **HDAC3 decreases IκBα-mediated enhancement of Pit-1 and HOXB7 transactivation potentials on their respective promoter.** *A*, luciferase assays were performed on extracts from 293 cells transfected with 1 μg of the *PRL* 164-bp reporter plasmid alone or with the indicated expression plasmids. The data of three independent experiment performed in duplicate, after normalization with protein concentration of the extracts, are shown (mean values ± S.D.). An anti-FLAG Western blot for the detection of FLAG-HDAC2 and -3 is shown at the *bottom* of the histogram. *B*, identical experiments performed using either the pTCBS or the pT109 reporter plasmid (*black* and *white columns*, respectively) in the MDA-MB231 cell line with the indicated expression plasmids. The amounts of transfected plasmids are mentioned *below* the histograms. *WB*, Western blot; *luc*, luciferase.

interactions of IκBα with BARD1 and JAB1, supporting this hypothesis (data not shown). However, our present report demonstrates that a cytoplasmic IκBα mutant could still strongly induce Pit-1 transactivation potential, therefore suggesting that sequestration of HDACs is the predominant mechanism that accounts for the observed effects. Such a statement, however, does not rule out the ability of IκBα to harbor some transactivating potential in the nucleus through physical interactions with some transcription factors and recruitment of co-activators.

Evidently HDAC sequestration indicates that IκBα also exerts NF-κB-independent activities. Indeed the positive effect of IκBα occurs on promoters limited to the CBS, which lacks any κB binding site, and we previously showed that the heterodimer p50/p65 does not enhance the IκBα effect on the pTCBS reporter but rather attenuates it (10). Moreover, we have no evidence for a ternary complex that includes p65, HDAC3, and IκBα occurring. Rather IκBα binds HDAC3 independently of p65, supporting the hypothesis that IκBα harbors NF-κB-independent functions. Most NF-κB-activating stimuli (proinflammatory cytokines, viruses, DNA damage, etc.) also activate other transcription factors. Our data therefore suggest that IκBα could favor the transcription of genes controlled by

A

WT   NLS-MT

B

C
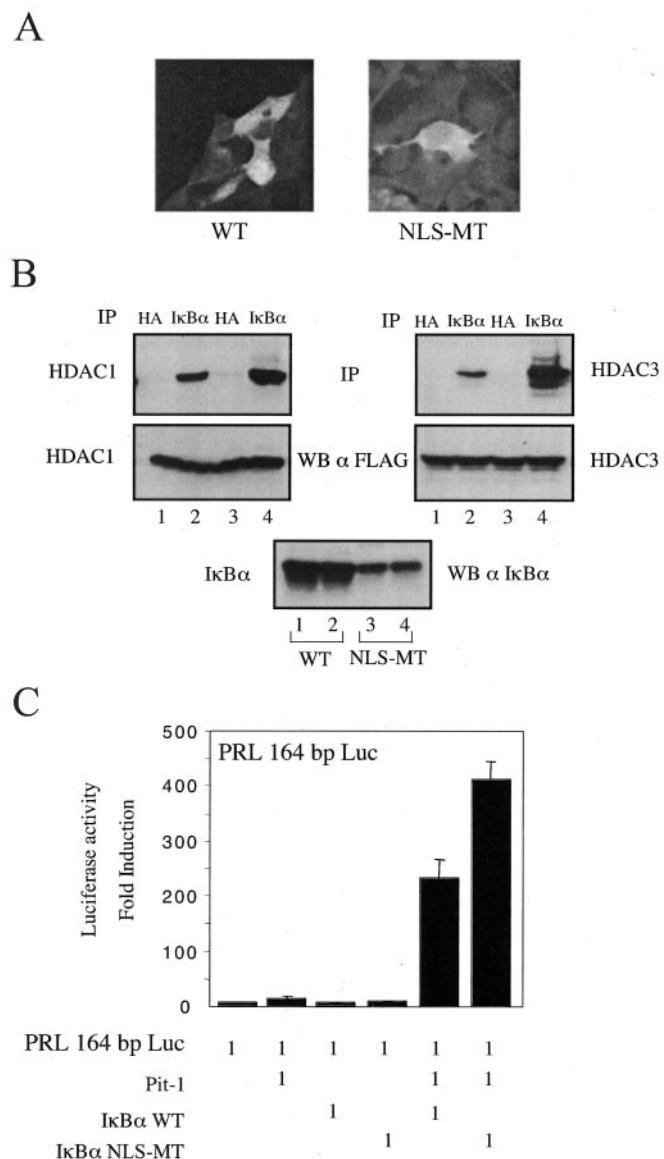
FIG. 7. **An IκBα lacking a functional NLS strongly interacts with HDAC1 and -3 and enhances Pit-1 transactivating activity on the *PRL* promoter.** *A*, indirect immunofluorescence of 293 cells transfected either with IκBα wild type (*WT*, *left panel*) or a mutant harboring mutations within its nuclear import sequence (*NLS-MT*, *right panel*). *B*, the IκBα NLS-MT mutant strongly interacts with HDAC1 and -3. 293 cells were transfected with 7 μg of IκBα wild type (*lanes 1* and *2*) or 7 μg of IκBα NLS-MT (*lanes 3* and *4*) along with 7 μg of FLAG-HDAC1 (*left panels*) or 7 μg of FLAG-HDAC3 (*right panels*), and cell extracts were subjected to anti-HA or -IκBα immunoprecipitations followed by anti-FLAG Western blots (*top panels*) on the immunoprecipitates. Anti-FLAG (*second panels from the top*) and anti-IκBα (*bottom panel*) Western analysis were carried out on the cell extracts. *C*, the IκBα NLS-MT mutant enhances Pit-1 transactivating activity. Luciferase assays were performed on extracts from 293 cells transfected with 1 μg of the 164-bp *PRL* reporter plasmid alone or with the expression plasmids as indicated. Shown are the results of a representative experiment performed in triplicate after normalization with protein concentration of the extracts (mean values ± S.D.). Similar results were obtained in two additional independent experiments. The amounts of the transfected plasmids are mentioned *below* the histograms. *Luc*, luciferase; *IP*, immunoprecipitation; *WB*, Western blot.

these regulating complexes through the maintenance of histone acetylation and promoter accessibility. In other words, once the first round of NF-κB-dependent transcription is over, HDAC/ IκBα interactions would favor the activity of other induced transcription factors.

However, our data demonstrated that endogenous IκBα and

HDACs physically interact in the absence of any stimulus. Therefore, the ubiquitous IκBα cytoplasmic pool could sequester some HDACs and thus favor either basal cellular transcription or transcription in various conditions where NF-κB is not activated. It is currently unclear whether IκBα brings HDAC1 and -3 back to the cytoplasm along with NF-κB proteins after stimulation or whether a pool of these HDACs is sequestered in the cytoplasm through binding to molecules such as IκBα prior to their shuttling to the nucleus. Whether or not nuclear IκBα brings some HDAC proteins along with p65 back in the cytoplasm remains to be investigated.

HDAC molecules regulate histone deacetylation and can thus influence the activity of many transcription factors. However, IκBα interacts specifically with HDAC1 and -3 and leaves the other HDACs unaffected. Among the candidate transcription factors whose activity could be influenced by HDAC1 and -3 sequestration, we demonstrated that transcriptional activity of HOX proteins is enhanced by IκBα expression. Several experiments suggest putative functional interactions between NF-κB and HOX proteins. IκBα knock-out mice are smaller than their wild type littermates, but it remains unclear whether this size difference is due to hormonal defect (GH for instance) or to inflammatory reactions. Moreover, NF-κB and several HOX proteins are simultaneously expressed at various developmental stages, and local NF-κB inhibition following expression of a mutated IκBα has been demonstrated to interfere with limb development (24).

As the present report demonstrates a novel NF-κB-independent function for IκBα, it will be most important to identify the genes whose expression is regulated by IκBα and by the interaction of IκBα with HDACs. These genes are probably highly cell-specific according to the expressed transcription factors and to promoter accessibility. The identification of the IκBα-regulated transcription factors and genes will open a new field of investigation related to a novel and unexpected IκBα activity.

REFERENCES

1. Li, Q., and Verma, I. (2002) *Nat. Immunol.* **2,** 725–734
2. Sachdev, S., Hoffmann, A., and Hannink, M. (1998) *Mol. Cell. Biol.* **18,** 2524–2534
3. Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) *EMBO J.* **18,** 6682–6693
4. Huang, T. T., and Miyamoto, S. (2001) *Mol. Cell. Biol.* **21,** 4737–4747
5. Ghosh, S., and Karin, M. (2002) *Cell* **109,** S81–S96
6. Whiteside, S. T., and Israël, A. (1997) *Semin. Cancer Biol.* **8,** 75–82
7. Wulczyn, F. G., Naumann, M., and Scheidereit, C. (1992) *Nature* **358,** 597–599
8. Bours, V., Franzoso, G., Azarenko, V., Park, S., Kanno, T., Bron, K., and Siebenlist, U. (1993) *Cell* **72,** 729–739
9. Dechend, R., Hirano, F., Lehmann, K., Ansieau, S., Wulczyn, F. G., Scheidereit, C., and Leutz, A. (1999) *Oncogene* **18,** 3316–3323
10. Chariot, A., Princen, F., Gielen, J., Merville, M.-P., Franzoso, G., Brown, K., Siebenlist, U., and Bours, V. (1999) *J. Biol. Chem.* **274,** 5318–5325
11. Khochbin, S., Verdel, A., Lemercier, C., and Seigneurin-Berny, D. (2001) *Curr. Opin. Genet. Dev.* **11,** 162–166
12. Brown, K., Park, S., Kanno, T., Franzoso, G., and Siebenlist, U. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2532–2536
13. Brown, K., Franzoso, G., Baldi, L., Carlson, L., Mills, L., Lin, Y.-C., Gerstberger, S., and Siebenlist, U. (1997) *Mol. Cell. Biol.* **17,** 3021–3027
14. Bours, V., Burd, P. R., Brown, K., Villalobos, J., Park, S., Ryseck, R.-P., Bravo, R., Kelly, K., and Siebenlist, U. (1992) *Mol. Cell. Biol.* **12,** 685–695
15. Zappavigna, V., Sartori, D., and Mavilio, F. (1994) *Genes Dev.* **8,** 732–744
16. Peers, B., Voz, M. L., Monget, P., Mathy-Hartert, M., Berwaer, M., Belayew, A., and Martial, J. (1990) *Mol. Cell. Biol.* **10,** 4690–4700
17. Caccavelli, L., Manfroid, I., Martial, J., and Muller, M. (1998) *Mol. Endocrinol.* **12,** 1215–1227
18. Tagami, T., Madison, L. D., Nagaya, T., and Jameson, J. L. (1997) *Mol. Cell. Biol.* **17,** 2642–2648
19. Chariot, A., Leonardi, A., Müller, J., Bonif, M., Brown, K., and Siebenlist, U. (2002) *J. Biol. Chem.* **277,** 37029–37036
20. Hewitt, S. M., Hamada, S., Monarres, A., Kottical, L. V., Saunders, G. F., and McDonnell, T. J. (1997) *Anticancer Res.* **17,** 3211–3216
21. Baek, S. H., Ohgi, K. A., Rose, D. W., Koo, E. H., Glass, C. K., and Rosenfeld, M. G. (2002) *Cell* **110,** 55–67
22. Saccani, S., Pantano, S., and Natoli, G. (2001) *J. Exp. Med.* **193,** 1351–1359
23. Hoffmann, A., Levchenko, A., Scott, M. L., and Baltimore, D. (2002) *Science* **298,** 1241–1245
24. Bushdid, P. B., Brantley, D. M., Yull, F. E., Blaeuer, G. L., Hooman, L. H., Niswander, L., and Kerr, L. D. (1998) *Nature* **392,** 615–618