NF-κB inhibits transcription of the H^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPase α\textsubscript{2}-subunit gene: role of histone deacetylases

WENZHENG ZHANG AND BRUCE C. KONE

Departments of Internal Medicine and of Integrative Biology, Pharmacology, and Physiology, The University of Texas Medical School at Houston, Houston, Texas 77030

Received 22 April 2002; accepted in final form 19 June 2002

Zhang, Wenzheng, and Bruce C. Kone. NF-κB inhibits transcription of the H^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPase α\textsubscript{2}-subunit gene: role of histone deacetylases. Am J Physiol Renal Physiol 283: F904–F911, 2002.—The H^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPase α\textsubscript{2}-subunit gene plays a central role in potassium homeostasis, yet little is known about its transcriptional control. We recently demonstrated that the proximal promoter confers basal transcriptional activity in mouse inner medullary collecting duct 3 (IMCD3) cells. We sought to determine whether the κB DNA binding element at −104 to −94 influences basal HK\textsubscript{α2} gene transcription in these cells. Recombinant NF-κB p50 footprinted the region −116/−94 in vitro. Gel shift and supershift analysis revealed NF-κB p50- and p65-containing DNA-protein complexes in nuclear extracts of mouse inner medullary collecting duct 3 cells. A promoter-luciferase construct with a mutated −104/−94 NF-κB element exhibited higher activity than the wild-type promoter in transfection assays. Overexpression of NF-κB p50, p65, or their combination trans-repressed the HK\textsubscript{α2} promoter. The histone deacetylase (HDAC) inhibitor trichostatin A partially reversed NF-κB-mediated trans-repression of the HK\textsubscript{α2} promoter. HDAC6 overexpression inhibited HK\textsubscript{α2} promoter activity, and HDAC6 coimmunoprecipitated with NF-κB p50 and p65. These results suggest that HDAC6 restrains HK\textsubscript{α2} transcription and identify NF-κB p50 and p65 as novel binding partners for HDAC6.

kidney; colon; trichostatin A; promoter

THE H^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPASE \alpha\textsubscript{2}-SUBUNIT (HK\textsubscript{α2}) is a member of the X^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPase multigene family, which includes the gastric H^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPase and the Na^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPase isoforms. The X^+\textsuperscript{+}-K^+\textsuperscript{+}-ATPases share homologous structures, common catalytic mechanisms, and a requirement for heterodimeric (α/β) assembly (22). HK\textsubscript{α2} is principally expressed in distal colon and the renal collecting duct, in which it plays a critical role in potassium and acid-base homeostasis. Mice with targeted ablation of the HK\textsubscript{α2} gene develop fecal K^+ wasting and profound hypokalemia during potassium restriction (27) and sodium restriction (34). HK\textsubscript{α2} also appears to contribute to bicarbonate absorption by the kidney (29) and distal colon (27) and increased ammonium secretion in the inner medullary collecting duct (IMCD) during chronic hypokalemia (37). Recent studies also suggest that HK\textsubscript{α2}, possibly functioning as a Na^+\textsuperscript{+}-K^+\textsuperscript{+} exchanger, participates in the chronic adaptation to altered sodium and aldosterone balance (32).

The HK\textsubscript{α2} gene is differentially expressed in kidney and distal colon under basal conditions and in response to chronic potassium or sodium deprivation. Under basal conditions, the gene is robustly expressed in distal colon, but only weakly expressed in the renal medulla. After chronic K^+ deprivation, however, HK\textsubscript{α2} gene expression is upregulated in the rat and mouse kidney outer medulla but not in distal colon (1, 32). In contrast, chronic sodium deprivation upregulates HK\textsubscript{α2} expression in distal colon but not in the renal collecting duct (32).

We recently isolated and completely sequenced the cDNA and structural gene encoding the mouse HK\textsubscript{α2} gene and localized it to mouse chromosome 14C3 (41). Using deletion analysis of promoter-reporter gene constructs, we demonstrated functional activity of the HK\textsubscript{α2} promoter in cultured renal collecting duct cells and found that the proximal 177 bp of the promoter appear to be essential for collecting duct-selective expression. This proximal promoter region contains several consensus sequences for transcription factors. Among these is an NF-κB site −104 GGGGGCTCCCC −94. The mammalian NF-κB/Rel family comprises five known members: p50, p52, p65, c-Rel, and RelB. NF-κB subunits form homo- or heterodimers through the Rel homology domain, forming transcription factor complexes that exert a broad range of DNA-binding and -activation potentials (4). The protein is bound in the cytoplasm with members of the inhibitor of κB (IKB) family, which prevents phosphorylation of the active unit and its translocation to the nucleus. A variety of stimuli and signaling events leads to activation of NF-κB, and there is evidence that NF-κB can transport between cytoplasm and nucleus even in unstimulated cells. In several tissues, including kidney, NF-κB DNA-binding activity is evident under basal conditions. It has been suggested that NF-κB in these
settings suppresses or activates basal gene expression in these cells (10, 18). In this context, it has been recently shown that NF-κB can suppress target gene transcription by means of interactions of p65 with histone deacetylase (HDAC)1 and HDAC2 corepressor proteins (3). Indeed, interaction with other transcription factors and accessory proteins appears to lend NF-κB versatility and specificity in mediating transcriptional responses.

Histone acetylation is a dynamic process regulated by the activities of two histone-modifying enzymes, histone acetyltransferase(s) and HDACs. Present models indicate that HDACs are recruited to target sequences through protein-protein interactions (23). HDAC activity results in histone hypoacetylation, chromatin condensation, and, generally, transcriptional repression (23). HDACs are typically found as components of large corepressor complexes. They affect transcriptional activity not only by modifying chromatin structure but also by deactivating transcription factors and altering their transcriptional competency. For example, HDAC1 and HDAC2 have been shown to repress transcription through direct interaction with transcription factors. HDAC1 directly interacts with MyoD to silence MyoD-dependent transcription of p21 (25). HDAC2 interacts with the YY1 transcription factors, converting them from activators to repressors (39). Deacetylation of RelA by HDAC3 serves as a molecular switch within the nucleus that controls the NF-κB transcriptional response (11).

In this report, we examined the role of basal NF-κB expression on HKα2 promoter activity in renal medullary collecting duct cells. We demonstrate that NF-κB activation complex through pro-B, and activator protein-2 (AP-2) nuclear extract (rhNF-B) can suppress target gene transcription and identify novel interactions of NF-κB in mediating transcription and, generally, transcriptional repression (23). Deacetylation of RelA by HDAC3 serves as a molecular switch within the nucleus that controls the NF-κB transcriptional response (11).

In vitro DNase I footprinting. DNase I footprinting analyses were performed with the Core Footprinting System (Promega), according to the manufacturer’s instructions. A PCR fragment corresponding to −476 to +82 of the native murine HKα2 5′-flanking region was obtained by using two primers: WZ162 (5′-ATCCAGACGGTATAGATTTCCCCGCACCCTCTATTACAC-3′, sense), MluI site (underlined) and a mutated p65, p50, p52, c-Rel, or HDAC6 antisense). This fragment was used as the DNA template for footprinting, and the unlabeled fragment also was sequenced with oligo 3 (5′-GTCGGGTTCTG-AUGCAGGAGA-3′, antisense). This fragment was used as the DNA template for footprinting, and the unlabeled fragment also was sequenced with oligo 3 as a marker (T7 Sequenase, version 2.0, DNA sequence kit, Amersham Pharmacia Bio tech). The transcription factors examined were NF-κB p50 and, as a control, AP-2. For the NF-κB experiment, the same final binding buffer was used as described (21). Briefly, the labeled template DNA was incubated in a final volume of 50 μl with or without 2 μl of recombinant human NF-κB in the final buffer containing 10 mM HEPES (pH 7.9), 0.2 mM EDTA, 50 mM KCl, 2.5 mM dithiothreitol, 10% glycerol, and 0.05% Nonidet P-40 for 10 min on ice. After addition of 50 μl CaCl2/Mg2+ solution (5 mM CaCl2, 10 mM MgCl2), 3 μl of DNase I was used for digestion for 1 min at room temperature. The reaction was terminated by adding 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, 100 μg/ml yeast RNA). Samples were extracted with phenol/chloroform/isoamyl alcohol, precipitated, resuspended in sequencing loading buffer, and separated on 8% sequencing gels. Bands were visualized by autoradiography.

EMSAs and supershift assays. Nuclear extracts were prepared from mIMCD-3 cells as detailed in earlier work from our laboratory (15). Double-stranded oligonucleotides were generated corresponding to nucleotides −109 to −90 containing the κB element (sense strand: wild-type, 5′-CCCCA-GGGCCGTCCCCAGCTG-3′, κB binding element underlined) and a mutated κB element (5′-CCCCATAGCCGTCCCCAGCTG-3′, mutations double underlined) of the native murine HKα2 promoter region. These were 5′-labeled with [γ-32P]ATP (3,000 Ci/mmol) using T4 polynucleotide kinase. Nuclear extracts proteins (12 μg) were preincubated with or without 4 μg antibodies specific for NF-κB p65, p50, p52, c-Rel, or HDAC6 in the final binding buffer [10 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 4% glycerol, and 1 μg poly (dl-dC)] at 4°C for 1 h or overnight. Binding reactions were performed for 30 min at room temperature by adding 1.75 pmol of duplex DNA probe (~2 × 105 cpm) in the presence or absence of a 10-fold molar excess of nonradiolabeled competitor oligonucleotides. The final reaction volume was adjusted to 20 μl. Aliquots of the reactions were resolved
RESULTS

NF-κB p50 footprints the HKα2 gene in vitro at −116 to −94. The proximal promoter of the HKα2 gene was initially analyzed by TESS software. A putative NF-κB and an overlapping AP-2 binding site were identified at −104 to −93, with the NF-κB site ranging from −104 to −94 and the AP-2 site from −99 to −93. To determine the significance of these sites, DNase I footprinting assay with recombinant NF-κB p50 (Fig. 1) and AP-2 was performed. Protected or hypersensitive sites in the region −116/−94 were detected in the binding reactions that included NF-κB p50, when compared with the reaction in which p50 was omitted. The antisense of this protected region reads as 5′-GGGGACGCC-CCCTGGGTAGGGAAA-3′, with the NF-κB site underlined. In addition, no footprint was observed with recombinant AP-2 under the condition tested (data not shown).

−109/−90 Sequence binds NF-κB p65 and p50. Binding of NF-κB to the −104/−94 element was further investigated by gel shift assays with mIMCD-3 cell nuclear extracts and the radiolabeled −109/−90 sequence containing the κB element as probes. Sequence-specific DNA-protein complexes were detected (Fig. 2A). Supershift assays demonstrated that antibodies to NF-κB p65 and p50 supershifted the DNA-protein complex (Fig. 2B). In contrast, the mobility of the DNA-protein complex in the presence of antibodies to p52, c-Rel, or STAT3 (as a negative control) was not altered. Furthermore, mutation of these sites dramatically decreased, although did not completely prevent,

Fig. 1. DNase I footprinting analysis of H⁺-K⁺-ATPase α2 (HKα2) proximal promoter. A DNA probe (−476 to +82) labeled with 32P at the 5′-end on the noncoding strand was incubated with (+) and without (−) recombinant NF-κB p50 protein. A sequencing ladder was also generated for definition of footprinted or hypersensitive sites. Filled bar, region with hypersensitive sites produced by NF-κB p50 protein; −104, −94, nucleotide positions relative to the transcription start site of the HKα2 gene.
NF-κB binding to this site (data not shown). These data are consistent with the conclusion that NF-κB p50/p65 heterodimers are basally expressed in the nuclei of mIMCD-3 cells.

NF-κB p65 and p50 trans-repress the HKα2 promoter. To determine the functional effect of NF-κB on HKα2 promoter activity, trans-activation/trans-repression assays were performed in mIMCD-3 cells. Overexpression of NF-κB p65 or p50 resulted in ~22 and ~30%, respectively, lower rates of HKα2 promoter activity compared with vector-transfected controls (Fig. 3A). Overexpression of NF-κB p65 and p50 together resulted in a dramatic, synergistic inhibition (~87%) of HKα2 promoter activity (Fig. 3A). In concert with these findings, overexpression of an IkBα mutant designed to limit NF-κB translocation to the nucleus resulted in a 40% increase in HKα2 promoter activity (Fig. 3A). Importantly, overexpression of NF-κB p65 or p50 in these same cells strongly activated activity of the NF-κB reporter plasmid p36B(−)NF-κB3-luc (Fig. 3B), indicating that the classic gene activation by NF-κB signaling under these conditions was intact and that the observed inhibitory effects on HKα2 promoter activity likely reflected the influence of the promoter context and/or coregulatory molecules.

To address the functional importance of the NF-κB binding site at −104/−94 in the NF-κB-mediated re-

---

**Fig. 2.** NF-κB p50 and p65 bind the −104/−94 κB element of HKα2 proximal promoter. A: nuclear proteins extracted from mouse inner medullary collecting duct 3 (mIMCD-3) cells were subjected to EMSA with a 32P-labeled oligomer containing the −104/−94 κB binding element of the HKα2 gene (S*). To demonstrate binding specificity, reactions were also conducted in the presence of a 10-fold molar excess of unlabelled −104/−94 κB binding element oligomer (S) or nonspecific (NS) oligomers (the corresponding, mutated version of −104/−94 κB binding element oligomer). The autoradiogram is representative of 3 independent experiments performed on separate preparations of nuclear extracts. B: polyclonal IgGs specific for NF-κB p50, p52, p65, c-Rel, and STAT3 (as a negative control) were used in supershift experiments with nuclear extracts from mIMCD-3 cells and the 32P-labeled −104/−94 κB binding element oligomer. The autoradiograms are representative of 3 independent experiments performed on separate preparations of nuclear extracts. SS, S, supershifted and shifted complexes, respectively; no Ab, no antibody.

**Fig. 3.** NF-κB p50 and p65 trans-repress the HKα2 promoter. A: mIMCD-3 cells were transfected with the pGL3–0.48MHKα2 reporter construct (Wild-Type) or pGL3–0.48MHKα2mut (Mutant), which harbors a mutation of the −104/−94 κB binding element, and the Renilla luciferase expression plasmid pRL-SV40 in the presence of the expression vector for NF-κB p50 (pRSV-p50), p65 (pRSV-p65), p50+p65 (pRSV-p50+pRSV-p65), an IκBα dominant-negative mutant (pCMV-IκBαΔ1–36), or an insertless mammalian expression vector containing the cytomegalovirus promoter (pCMV-500). Twenty-four hours after transfection, cell lysates were prepared and firefly and Renilla luciferase activities in lysates of the cells were assayed. Firefly luciferase activity was normalized to Renilla luciferase activity. B: identical methods as in A, except that an NF-κB consensus element reporter construct p36B(−)NF-κB3-luc and its parent plasmid p36-luc were used instead of pGL3–0.48MHKα2 plasmid and pCMV500, respectively. Values are means ± SEM of 4 separate experiments. *P < 0.05.
expression of the HK\(\alpha_2\) promoter, we constructed pGL3–0.48MHK\(\alpha_2\)mut, which harbors the same mutation in the NF-\(\kappa\)B binding site as that generated for the mutant probes used in the gel shift assay. When compared with expression of pGL3–0.48MHK\(\alpha_2\), expression of pGL3–0.48MHK\(\alpha_2\)mut was significantly augmented in all cases examined (Fig. 3A). In the control cells transfected with pCMV500 vector, the mutation augmented expression by \(\sim110\%\), suggesting that the binding of NF-\(\kappa\)B to this site is important for the endogenous NF-\(\kappa\)B-mediated repression of the HK\(\alpha_2\) promoter. Similarly, the repression resulting from overexpression of p65, p50, or both was at least partially relieved by the mutation, resulting in higher level expression of the HK\(\alpha_2\) promoter-reporter (Fig. 3A). HK\(\alpha_2\) promoter-reporter activity was greatest when nuclear expression of NF-\(\kappa\)B was limited by overexpressing the I\(\kappa\)B\(\alpha\) mutant and the HK\(\alpha_2\) promoter harboring the mutated \(\kappa\)B element (Fig. 3A). Taken together, these data support the conclusion that NF-\(\kappa\)B binding to \(-104/-94\) results in downregulation of activity of the HK\(\alpha_2\) promoter. It is interesting to note that the HK\(\alpha_2\) promoter harboring the mutated \(\kappa\)B element, while exhibiting higher activities compared with the wild-type promoter when p65, p50, p65/p50, or the I\(\kappa\)B\(\alpha\) mutant was overexpressed, was still partially inhibited by p65 and p65/
p50, but not p50, and augmented by the I\(\kappa\)B\(\alpha\) mutant. This result probably reflects, in part, the fact that the mutation did not completely abolish NF-\(\kappa\)B binding on gel shift assays and the likelihood that NF-\(\kappa\)B may also mediate repression through other mechanisms independent of its binding to \(-104/-94\).

TSA augments and HDAC6 overexpression inhibits HK\(\alpha_2\) promoter activity. NF-\(\kappa\)B is known to interact with HDAC1, HDAC2, and HDAC3 (3, 11, 19) in other cell types and promoter contexts (3), and HDACs are known to act as corepressors in some instances. To determine whether the state of histone acetylation influences the ability of NF-\(\kappa\)B to trans-repress the HK\(\alpha_2\) promoter in mIMCD-3 cells, HK\(\alpha_2\) promoter-luciferase activity was measured in the presence and absence of the potent and specific HDAC inhibitor TSA. TSA treatment promoted a 2.5-fold increase in basal HK\(\alpha_2\) promoter activity (Fig. 4A). As shown in Table 1, TSA treatment resulted in significantly greater activity of the NF-\(\kappa\)B consensus element reporter in the vector-transfected controls c-transfected with either the wild-type HK\(\alpha_2\) promoter (pGL3–0.48HK\(\alpha_2\)) or the HK\(\alpha_2\) promoter harboring the mutation in the NF-\(\kappa\)B site (pGL3–0.48HK\(\alpha_2\)mut). However, p65, p50, and p65+p50 had less ability to inhibit pGL3–0.48HK\(\alpha_2\)mut promoter activity compared with the wild-type pGL3–0.48HK\(\alpha_2\) construct in the presence of TSA, indicating that TSA partially reversed the NF-\(\kappa\)B-dependent trans-repression of the HK\(\alpha_2\) promoter (Table 1). These findings are consistent with the involvement of an HDAC in the process. In accordance with these findings, overexpression of HDAC6 inhibited HK\(\alpha_2\) promoter activity (Fig. 5A). Interestingly, the inhibitory effect appeared to be specific for HDAC6, because overexpression of HDAC1, HDAC3, HDAC4, and HDAC5 had no appreciable effect on HK\(\alpha_2\) promoter activity (Fig. 5A), despite roughly comparable levels of expression (not shown).

TSA treatment also accentuated basal activity of the NF-\(\kappa\)B reporter plasmid p36B(–)\(\kappa\)B\(\beta\)_3-luc (Table 2). However, in contrast to its effects on NF-\(\kappa\)B-dependent trans-repression of the HK\(\alpha_2\) promoter, TSA did not affect the ability of overexpressed p65, p50, or p65+p50 to trans-activate the NF-\(\kappa\)B reporter plasmid p36B(–)\(\kappa\)B\(\beta\)_3-luc (Table 2). This result suggests

Table 1. Effects of trichostatin A on activity of the wild-type \(H^+\)-K\(^+\)-ATPase \(\alpha_2\)-promoter and promoter harboring a mutation in the \(-104/-90\) NF-\(\kappa\)B element

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Wild-Type HK(\alpha_2) (pGL3–0.48HK(\alpha_2))</th>
<th>HK(\alpha_2) ΔNF-(\kappa)B Mutant (pGL3–0.48HK(\alpha_2)mut)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>TSA</td>
</tr>
<tr>
<td>Vector</td>
<td>1.9 ± 0.2 (74%)</td>
<td>15.2 ± 1.4 (74%)</td>
</tr>
<tr>
<td>p65</td>
<td>0.5 ± 0.1 (37%)</td>
<td>6.8 ± 0.5 (56%)</td>
</tr>
<tr>
<td>p50</td>
<td>1.2 ± 0.1 (37%)</td>
<td>8.4 ± 0.5 (45%)</td>
</tr>
<tr>
<td>p50/p65</td>
<td>0.5 ± 0.1 (74%)</td>
<td>6.4 ± 0.1 (58%)</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 3\). Percent change from vector-treated vehicle is in parentheses. mIMCD3 cells were transfected with the pGL3–0.48HK\(\alpha_2\) reporter construct or pGL3–0.48HK\(\alpha_2\)mut, which harbors a mutation of the \(-104/-94\) binding element, together with the expression vector for NF-\(\kappa\)B p50 (R5V-p50), p65, (R5V-p65), p50+p65 (R5V-p50+p50-p65), or an insertless vector (pCMV500) containing the cytomegalovirus (CMV) promoter. The cells were then treated with vehicle or 100 nM trichostatin A (TSA) for 16 h, after which cell lysates were prepared and luciferase activities measured and normalized to protein content. The percent inhibition (−) or stimulation (+) relative to the corresponding vector-transfected cells for each condition is noted in parentheses.
that promoter context is important for the functional effects of HDAC activity on transcription.

HDAC6 and NF-κB interact in mIMCD-3 cells in vivo. EMSA and supershift studies using the −104/−94 κB element as probe, nuclear extracts from mIMCD-3 cells, and anti-HDAC6 antibodies or IgG (as a negative control) demonstrated that anti-HDAC6 partially supershifted the κB-specific DNA-protein complex (Fig. 6). In agreement with these results and those of the HKα2 promoter assays, coimmunoprecipitation experiments demonstrated interaction of HDAC6 and NF-κB proteins (Fig. 7). HDAC6 was overexpressed as a FLAG-tagged construct in mIMCD-3 cells, and the cells were then subjected to coimmunoprecipitation with antibodies to NF-κB p50, NF-κB p65, the FLAG epitope (as a positive control), or IgG (as a negative control), followed by blotting with the anti-FLAG M2 antibody. As seen in Fig. 7, both NF-κB p50 and p65 coprecipitated with HDAC6.

Table 2. Effects of TSA on ability of NF-κB proteins to trans-activate NF-κB consensus element reporter construct p36B(−)NF-κB3-luc

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Vehicle</th>
<th>TSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>0.5 ± 0.1</td>
<td>2.4 ± 0.4*</td>
</tr>
<tr>
<td>p65</td>
<td>3.0 ± 0.8</td>
<td>9.5 ± 0.5*</td>
</tr>
<tr>
<td>p50</td>
<td>1.3 ± 0.1</td>
<td>4.3 ± 0.4*</td>
</tr>
<tr>
<td>p50/p65</td>
<td>5.9 ± 1.6</td>
<td>43.6 ± 7.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3. mIMCD3 cells were transfected with the NF-κB consensus element reporter construct p36B(−)NF-κB3-luc together with expression vector for NF-κB p50 (RSV-p50), p65, (RSV-p65), p50+p65 (RSV-p50+RSV-p65) or an insertless vector (pCMV500) containing the CMV promoter. The cells were then treated with vehicle or 100 nM TSA for 16 h, after which cell lysates were prepared and luciferase activities measured and normalized to protein content. TSA treatment resulted in a ∼5-fold greater activity of the NF-κB consensus element reporter in the vector-transfected controls. TSA treatment did not, however, significantly limit the ability of overexpressed p65, p50, or p50+p50 to stimulate promoter activity. *P < 0.05.

DISCUSSION

In this study, we demonstrated that NF-κB p65 and p50 DNA binding activities are basally expressed in cultured renal collecting duct cells (Fig. 2) and that these transcription factors serve to trans-repress the HKα2 gene by binding to a κB element in the proximal promoter region of this gene (Fig. 3A). The fact that blockade of HDAC activity with TSA relieves NF-κB-mediated trans-repression of the HKα2 gene (Fig. 4) and that HDAC6 overexpression inhibits HKα2 promoter activity (Fig. 5) indicates that the state of histone or NF-κB acetylation strongly influences HKα2 promoter activity. We further demonstrate that NF-κB p50 and p65 interact with HDAC6 in supershift experiments with the −104/−94 κB element (Fig. 6) and in coimmunoprecipitation assays (Fig. 7). These results provide the first data concerning transcriptional regulation of the HKα2 gene, give a novel example of the ability of NF-κB to suppress basal gene expression, and identify for the first time HDAC6 as a binding partner with NF-κB p50 and p65. However, it remains to be defined whether p50 and p65 are subject to acetylation/deacetylation and whether HDAC6 is directly involved in this process.

Fig. 5. Histone deacetylase (HDAC) 6 overexpression inhibits activity of the HKα2 promoter. mIMCD-3 cells were transfected with the pGL3−0.48MKα2 reporter construct and a Renilla expression plasmid together with expression plasmids for HDAC1, HDAC2, HDAC4, HDAC5, or HDAC6 or an insertless vector containing the CMV promoter (pCMV-500). Cell lysates were prepared and luciferase activities measured. Values are means ± SE; n = 4. *P < 0.05.

Fig. 6. HDAC6 contributes to the −104/−94 κB element-protein complex of the HKα2 proximal promoter. Supershift experiments with nuclear extracts from mIMCD-3 cells and the 32P-labeled −104/−94 κB binding element oligomer were performed in the presence and absence of polyclonal antibodies against HDAC6 or with nonimmune IgG. The autoradiograms are representative of 3 independent experiments performed on separate preparations of nuclear extracts.

Fig. 7. HDAC6 interacts with NF-κB p65. HDAC6 was overexpressed as a FLAG-tagged protein in mIMCD-3 cells, cell extracts were prepared and immunoprecipitated (IP) with polyclonal antibodies directed against the FLAG epitope, NF-κB p50, NF-κB p65, or IgG, separated by SDS-PAGE, and immunoblotted with anti-FLAG M2 antibody. Data are representative of 3 independent experiments.
The fact that the −104/−94 region was footprinted in vitro by NF-κB p50 (Fig. 1) and that mutation of the κB element partially relieved repression of HKα2 promoter activity (Fig. 3A) suggests that much of the effect of NF-κB on the promoter was direct and mediated by DNA binding. However, because the effect was only partial, and because overexpression of NF-κB p65 or p55/p50, but not p50 alone, still inhibited the mutant HKα2 promoter (Fig. 3A), other undefined mechanisms are apparently involved. The κB element rests 53-bp upstream of the TATA box and is flanked by an AP-2 site. However, AP-2 did not footprint the region in vitro (Fig. 1). Because the combination of p50 and p65 overexpression synergistically suppressed HKα2 promoter activity (Fig. 3A) and because these NF-κB proteins contributed to the κB-specific DNA-protein complexes in supershift assays (Fig. 2B), we conclude that p50/p65 heterodimers are likely the dominant NF-κB species binding the HKα2 promoter and the most effective in trans-repressing the HKα2 gene.

NF-κB is subject to complex control. The principal mode of regulation has long been considered to be the retention of NF-κB in the cytoplasm by association with IκB proteins and its release for translocation to the nucleus on IκB phosphorylation and degradation (4). This regulatory pathway appears to be operative at least to a degree in our study, because overexpression of an IκBα dominant-negative mutant augmented basal HKα2 promoter activity (Fig. 3A). In addition, NF-κB has been shown to shuttle between cytoplasm and nucleus under basal conditions. Chen et al. (11) showed that deacetylation of RelA by interaction with HDAC3 promotes effective binding to IκB and results in IκBα-dependent nuclear export of the complex. Ashburner et al. (3) established that NF-κB p65 in the nucleus of unstimulated cells interacts with HDAC1 and HDAC2 to suppress basal gene expression. The glucocorticoid receptor has also been shown to recruit HDAC2 to the p65-AMP response element binding protein (CBP) histone acetyltransferase complex to inhibit IL-1β-induced gene expression (19). NF-κB has been found in the nucleus of unstimulated cells, in which it is theorized to repress and/or activate basal gene expression (10). The differential association of NF-κB with coregulatory proteins may regulate this function. CBP and p300 coactivators interact with p65 to enhance its ability to activate transcription (4). The histone acetyltransferase function of the p300/CBP-associated factor coactivator (9) and the steroid receptor coactivator-1 (28) were shown to interact with p50 to potentiate NF-κB-mediated trans-activation.

Histone acetylation and deacetylation play essential roles in modifying chromatin structure and regulating expression of eukaryotic genes. HDACs are part of transcriptional corepressor complexes. HDAC interacts with NcoR (nuclear corepressor) and SMRT (silencing mediator of receptor transcription) to mediate nuclear receptor repression, as well as with the Mad-Max complex to confer transcriptional repression (2, 16, 17). In our study, TSA treatment increased HKα2 promoter activity, consistent with the model that TSA blocks activity of HDAC, resulting in hyperacetylation of histones and, consequently, a higher level of gene expression (40). Consistent with this, overexpression of HDAC6 inhibited HKα2 promoter activity (Fig. 6). We do not know whether TSA promotes dissociation of HDAC from p65 or p50, facilitating the binding of CBP or other coactivators.

Acetylation regulates transcription factors other than NF-κB, including p53 (6), GATA-1 (7), and MyoD (25), and it alters transcription factor function in several ways, including altering protein-protein interactions (5), affecting conformation (33), and altering half-life (26). Direct interaction with HDACs has only been demonstrated for a few transcription factors. Retinoic acid receptor alpha (24) and DNA topoisomerase II (36) interact directly with HDAC1, SP1 (20) and YY1 (35) interact with HDAC2, GATA-2 couples with HDAC3 and HDAC5 (30), and myocyte enhancer factor 2 was shown to interact directly with HDAC4 and HDAC5 (14). Our finding of an interaction between HDAC6 and NF-κB p50 and p65 adds to this list. Why specific HDAC isoforms are active in some cell types but not others likely relates to the specific promoter context and other coregulatory molecules. Nonetheless, this protein-protein interaction could lend cell specificity and versatility to the regulatory response. In addition, the ability of NF-κB to function as both activator and repressor in mIMCD-3 cells indicates that this transcription factor may exist in different complexes with different acetylation/deacetylation patterns, resulting in differential DNA binding specificities and regulation of expression of its target genes.

The authors thank Dr. Bharrat Agarwal, The University of Texas M.D. Anderson Cancer Center, for the gift of the NF-κB and mutant IκB expression plasmids. Dr. Warren S.-L. Liao, The University of Texas M.D. Anderson Cancer Center, for the gift of the NF-κB expression plasmid, Dr. Edward Seto, University of South Florida, for the FLAG-tagged HDAC2 expression plasmid pME18S-FLAG-HDAC2, and Dr. S. L. Schreiber, Harvard University, for the FLAG-tagged expression plasmids pBJ-HDAC1, pBJ-HDAC4, pBJ-HDAC5, and pBJ-HDAC6. The authors also thank Dr. Jun Chen and Sandra Higham for technical assistance.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-47981.

REFERENCES


