p65-NFκB synergizes with Notch to activate transcription by triggering cytoplasmic translocation of the nuclear receptor corepressor N-CoR

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Summary

Notch/RBP-Jκ and nuclear factor-κB (NFκB) complexes are key mediators of the progression of many cellular events through the activation of specific target gene transcription. Independent observations have shown that activation of Notch-dependent transcription generally correlates with inhibition of differentiation. In contrast, activated NFκB complexes are required for progression of differentiation in several systems. Although some interactions between both pathways have been observed, the physiological significance of their connection is unclear. We have now demonstrated that the increase in p65-NFκB protein levels enhances Notch-mediated activation of the Hes1 promoter up to three-fold. This effect does not require NFκB transcriptional activity, and it is independent of the previously described interaction between Notch and p50-NFκB. Furthermore, we show that p65-NFκB can modulate subcellular localization of the transcriptional corepressor N-CoR, abrogating N-CoR mediated repression of the Hes1 promoter. In addition, p65-NFκB is able to upregulate not only the Hes1 but also other promoters containing SRE and AP-1 sites, which are repressed by N-CoR. Thus, we conclude that p65-NFκB can regulate gene expression by a general mechanism that involves cytoplasmic translocation of the transcriptional corepressor protein N-CoR.

Key words: NFκB, N-CoR, Notch, Transcriptional regulation

Introduction

The Notch/lin-12 family of transmembrane receptors plays important roles in regulating embryonic development and tissue homeostasis in the adult (reviewed in Artavanis-Tsakonas et al., 1999; Egan et al., 1998). Multiple observations suggest that Notch might be controlling different cellular events by inhibiting specific signals required for cell-type specification, cell-cycle progression and cell survival. When the Notch receptor binds to its ligand, which is located in neighboring cells, the intracellular domain of the protein (Notch-IC) is released from the cell membrane and translocated to the nucleus. There, active Notch (Notch-IC) interacts with the ubiquitous transcription factor RBPJκ to positively regulate gene transcription (reviewed in Egan et al., 1998). In fact, RBPJκ behaves as a bifunctional factor since, in the presence of Notch, it switches from being transcriptionally repressive to being transcriptionally active (Hsieh et al., 1996). Functional repression by RBPJκ has been described for different genes, including the adenovirus pIX polypeptide (Dou et al., 1994), Hes1 (Kao et al., 1998), IL-6 (Plaisance et al., 1997) or p52/NFκB2 (Oswald et al., 1998). Repression of the Hes1 promoter by RBPJκ has been associated with its interaction with the nuclear corepressors SMRT and N-CoR (Kao et al., 1998).

SMRT and N-CoR are highly homologous proteins, coded by two different genes with different splicing isoforms (Wong and Privalsky, 1998; Park et al., 1999). Although both proteins share many common structural and functional features, inactivation of the N-CoR gene by homologous recombination has shown that their functions are not physiologically redundant (Jepsen et al., 2000).

In recent years, coactivators and corepresors of nuclear receptors, and other transcription factors, have been identified as fundamental components in the regulation of eukaryotic gene expression (reviewed in Xu et al., 1999; Pazin and Kadonaga, 1997). Although transcriptional coactivator complexes possess intrinsic histone acetyl-transferase (HAT) activity, nuclear receptor corepressors achieve their function by recruiting histone deacetylases (HDACs). There is a positive correlation between core histone acetylation and gene transcriptional activity. It is generally accepted that acetylation causes local changes in chromatin structure, thus facilitating the assembly of the transcriptional machinery. Many transcription factors such as RBPJκ (Kao et al., 1998), NFκB, AP-1, SRF (Lee et al., 2000), MyoD (Bailey et al., 1999) and Pbx (Saleh et al., 2000) have been shown to regulate gene transcription by recruiting acetylase/deacetylase activities.

The NFκB/Rel family of transcription factors participates in the regulation of disparate cellular processes such as proliferation, differentiation, immune response and transcription of viral promoters (reviewed in Baldwin, 1996; Ghosh et al., 1998). The Rel family includes p65 (RelA), p105/p50, p100/p52, RelB, c-Rel and the viral oncoprotein v-Rel. These proteins associate as homo or heterodimers to form transcriptional regulatory complexes known as nuclear factor kappa B (NFκB). Transcriptional activity of NFκB is specified

by the composition of the dimers and depends on their subcellular localization and their association with the inhibitory protein IκB (reviewed in Karin, 1999).

There is emerging evidence supporting a functional interplay between Notch and NFκB signaling pathways. For example, Notch can interact with p50-NFκB, thus modulating NFκB-dependent gene transcription (Guan et al., 1996). Conversely, NFκB induces the expression of the Notch ligand Jagged1, triggering Notch activation in adjacent cells (Bash et al., 1999). Interaction between both pathways is specially intriguing since NFκB can either promote or inhibit differentiation in various cell types (Kim et al., 2001; Feng and Porter, 1999; Kaliman et al., 1999; Kaisho et al., 2001) (L.E. and A.B, unpublished), whereas Notch activity is primarily involved in blocking this process (Fortini et al., 1993; Sternberg, 1988; Milner et al., 1996). We have now studied the putative effect of p65-NFκB on modulating the Notch/RBPJκ transcriptional activity. We describe here how the overexpression of p65-NFκB or a mutant that lacks the transcriptional activation (TA) domain facilitates Notch-IC-mediated transcription of the Hes1 promoter by inducing cytoplasmic retention of the nuclear corepressor N-CoR. This effect applies to other promoters repressed by N-CoR, such as those containing SRF or AP-1 sites. Further physiological studies are needed to understand the contribution of this new mechanism of gene regulation in the control of the different cellular events.

Materials and Methods

Plasmids

The Notch1-IC (N1-IC) (AA 1807-2194) and Notch2-IC (N2-IC) (AA 1765-2154) cDNA expression vectors have been described previously (Bigas et al., 1998). Notch3-IC (N3-IC) (AA 1760-2070) was amplified by PCR, sequenced and cloned in the pCS2 vector in frame with the myc tag. Expression vectors for pCMV-flag-p65-NFκB, pCMV-IκBα32-36, pCDNA3-p50-NFκB, pCMV-flag-NcoR, pCMV-p65ΔTA, pCMV-p65ΔNES and reporter plasmids for Hes1-luc, 2×κB-luc, 2×SRE-luc 3×AP-1-luc have been previously described (DiDonato et al., 1996; Harhaj and Sun, 1999; Jarriault et al., 1995; Miralles et al., 1998).

Antibodies

Anti-flag (clone M2) was purchased from Sigma and used at a dilution of 1:1,000 for western blot and 1:750 for immunofluorescence analysis; anti-p65-NFκB (sc-109, Santa Cruz Biotechnology) was used at 1:400. 9E10, anti-myc-tag, was used at 1:1,000 for western blot and 1:200 for immunofluorescence. mN1A antibody (Huppert et al., 2000) was used 1:200 for western blot. Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from DAKO and used 1:2,000 for western blot. Fluorescein-conjugated goat anti-mouse or Cy3-conjugated goat anti-rabbit (Amersham) secondary antibodies were diluted 1:200 and 1:1,000 respectively.

Cell culture and transfections

NIH-3T3 and 293T cells were cultured in Dulbecco's modified Eagle medium and 10% FBS (fetal bovine serum). Cells were plated at subconfluence and transfected by calcium phosphate. Medium was changed after 12 hours, and cells were processed 24 hours later for luciferase assays, immunofluorescence or western blots. Leptomycin B (LMB) was purchased from SIGMA and used at 10-40 ng/ml. Trichostatine A (TSA) was purchased from Calbiochem and used at 600 nM for 12 hours.

Western blot

293T cells were transfected with the different DNA plasmids by calcium phosphate treatment, and 48 hours later were lysed during 30 minutes at 4°C in a buffer containing 20% glycerol, 20 mM Hepes pH 7.6, 350 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.25% NP-40, 5 mM Na Fluoride, 1 mM EGTA, 0.25 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.4 mM Na-ortovanadate and 1 μg/ml Bestatin. Protein extracts were electrophoresed in 6% polyacrylamide gels and transferred to PVDF membranes overnight. Membranes were blocked with 5% non-fat dried milk in TBS and incubated with the appropriate antibody in TBS and 0.5% tween20 (TBS-T) with 5% non-fat dried milk for 90 minutes. Membranes were washed and incubated with a secondary HRP-conjugated antibody for 1 hour. After extensive washing, immunoreactive proteins were detected by using the Enhanced Chemiluminiscent Detection System (ECL, Amersham Pharmacia Biotech) as specified by the manufacturer.

Northern blot analysis

Total RNA was extracted from cells using the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). RNAs were size-fractionated by electrophoresis, transferred onto Hybond-N+ nylon membranes (Amersham) and then hybridized with a radiolabeled Hes1 probe. Radioactivities of each signal were measured by a PhosphorImager using Quantity One software (Bio-rad). Densitometric analysis was performed by using Phoretics software.

Luciferase assays

NIH-3T3 were plated on 12-well plates and transfected with the indicated expression vectors or the empty vector as a control. In the different experiments we used 1 μg of Hes1-luc, 2×κB-luc, 3×AP-1-luc or 2×SRE-luc as reporter plasmids and 0.5 μg RSV-β-gal as internal control. pCS2 vector was added when necessary to keep the amount of DNA constant. A luciferase assay (Luciferase Assay System, Promega) was performed 48 hours after transfection, following the manufacturer's instructions. Luciferase values were normalized for β-galactosidase activity. At least three independent experiments were performed in duplicates.

Immunofluorescence

NIH-3T3 or 293T cells were seeded on slides at 20% confluence and transfected with 10 μg N-CoR, 4 μg p65-NFκB or 4 μg N1-IC. After 48 hours, cells were fixed in 3% paraformaldehyde in PBS for 25 minutes at 4°C, washed in PBS, permeabilized in 0.1% Triton X-100 in PBS, 5% non-fat dry milk for 25 minutes at 4°C. After washing, cells were incubated with the indicated primary antibody for 90 minutes at 4°C and extensively washed in PBS 1% non-fat dry milk. After 90 minutes of incubation with the appropriate secondary antibody, slides were extensively washed and mounted with Vectashield plus DAPI (Vector). Cells were visualized in an Olympus BX-60 microscope with the appropriate filters. Representative cells were photographed and slides were digitalized with Adobe Photoshop.

Results

p65-NFκB synergizes with Notch to transactivate the Hes1 promoter in a p50-NFκB-independent manner

Notch-IC and NFκB are both involved in the control of cellular processes such as proliferation, differentiation and apoptosis. Previous observations showed that Notch-IC can modulate NFκB-regulated promoters both positively, by sequestering RBPJκ (Oswald et al., 1998), or negatively, by

interacting with the p50-NFκB subunit (Guan et al., 1996). To investigate whether NFκB had a reciprocal effect on Notch/RBPJκ-regulated promoters, different Notch-IC and p65-NFκB expression vectors were cotransfected along with the Hes1-luc reporter plasmid into NIH-3T3 cells. Compared with Notch1-IC (N1-IC) alone, we observed that coexpression of full-length p65-NFκB (p65) increased the activity of the reporter gene in a dose-dependent manner (Fig. 1A) without affecting Notch-IC protein levels (Fig. 1A, lower panel). The maximum effect (two- to three-fold activation) was reached when equivalent amounts of Notch-IC and p65-NFκB (p65) were cotransfected. We found a similar increase when p65 was cotransfected with different activated Notch homologs: N1-IC (Fig. 1A), N2-IC (Fig. 1B) or N3-IC (Fig. 1C). In the absence of Notch-IC, no increase in the Hes1 transcriptional activity by p65-NFκB overexpression was observed, suggesting that there was no significant endogenous Notch activity in NIH-3T3 cells.

We next tested whether the previously reported interaction between Notch-IC and p50-NFκB (Guan et al., 1996) was responsible for the above-described effect. We hypothesized that addition of p65-NFκB would liberate Notch-IC from a putative p50 inhibition, resulting in an increase in Notch activity. Cotransfection of p50-NFκB did not affect the Notch activation of the Hes1 promoter nor the observed p65-mediated upregulation (Fig. 1D), although it increased NFκB-dependent transcription (data not shown). Altogether, these results demonstrate that p65-NFκB can synergize with Notch-IC to activate Hes1 transcription. Moreover, this effect is not due to a competition of both transcription factors for binding to the p50-NFκB subunit, indicating that other mechanisms may be involved.

Activation of NFκB-dependent transcription is not required for Notch and p65-NFκB synergism

Since NFκB participates in the transcriptional activation of several genes including the Notch ligand *Jagged1* (Bash et al., 1999), we reasoned that the observed effect on the Hes1 promoter could be mediated by p65-NFκB transcriptional activation of any Notch pathway gene. To check this hypothesis, we repeated the p65/Notch-IC cotransfection experiments in the presence of the constitutive repressor of NFκB, IκBα$_{32\text{-}36}$ (DiDonato et al., 1996). As expected, expression of IκBα$_{32\text{-}36}$ inhibited p65-NFκB-mediated transactivation of the 2×κB-luc reporter in a dose-dependent manner (Fig. 2A) by sequestering NFκB in the cytoplasm even in the presence of Notch-IC (Fig. 2C). In these conditions, inhibition of NFκB transcriptional activity did not abrogate the p65-mediated upregulation of the Hes1 promoter (Fig. 2B). These results indicate that the synergistic effect of p65-NFκB and Notch on the Hes1 promoter requires neither colocalization of Notch and p65-NFκB in the nucleus nor NFκB transcriptional activity. To further demonstrate this observation, we coexpressed N1-IC with a deletion mutant of p65-NFκB (p65ΔTA), which lacks the transactivation domain (AA 451-551) (Harhaj and Sun, 1999). In the presence of N1-IC, expression of p65ΔTA resulted in the increased dose-dependent activation of the Hes1 promoter (Fig. 2D). Moreover, the effect of p65ΔTA was even stronger (up to four-fold) than the maximum effect observed with the p65 wild type (p65 wt).

Hes1 promoter activity is repressed by N-CoR, and this effect is reversed by ectopic expression of p65-NFκB

We have demonstrated that neither transcriptional activation by NFκB nor nuclear localization of p65 subunit are required for upregulating the Notch-dependent Hes1 promoter activation. Thus, we examined whether p65-NFκB was facilitating Notch activity by displacing a Hes1 repressor molecule.

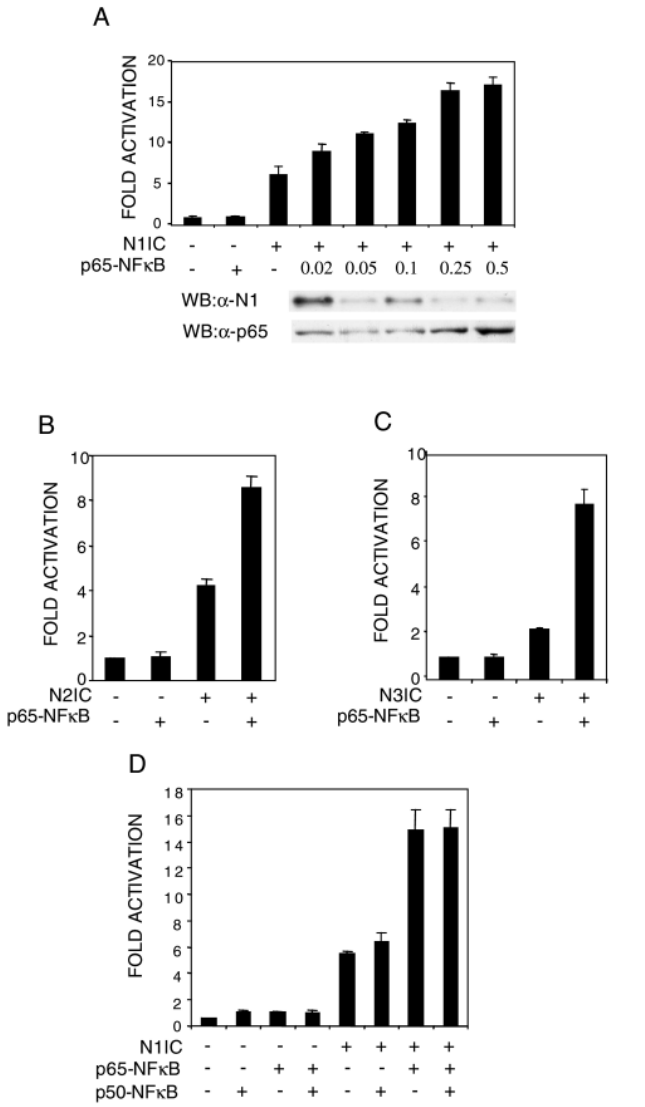


Fig. 1. Synergistic effect of p65-NFκB and Notch-IC in the transactivation of the Hes1 promoter. (A) N1-IC (0.5 μg) was cotransfected along with the Hes1-luc reporter (0.75 μg) in NIH-3T3 cells and increasing amounts of p65 wt. Lower panels show N1-IC and p65 protein levels. Protein loading for each lane was normalized according to β-galactosidase expression. (B,C) p65-NFκB can synergize with different Notch-IC homologs. 0.5 μg of N2-IC (B) or N3-IC (C) were cotransfected with p65 wt (0.5 μg) along with the Hes1-luc reporter plasmid (1μg) in NIH-3T3 cells. (D) Synergistic effect of p65-NFκB and Notch-IC is not modified by ectopic expression of p50-NFκB. NIH-3T3 cells were cotransfected with different combinations of equivalent amounts (0.5 μg) of p50 subunit, N1-IC and p65 wt expression vectors. Luciferase activity is represented as the fold induction relative to the basal level measured in cells transfected with empty vector. The average and standard deviation of duplicates from a representative experiment are presented.

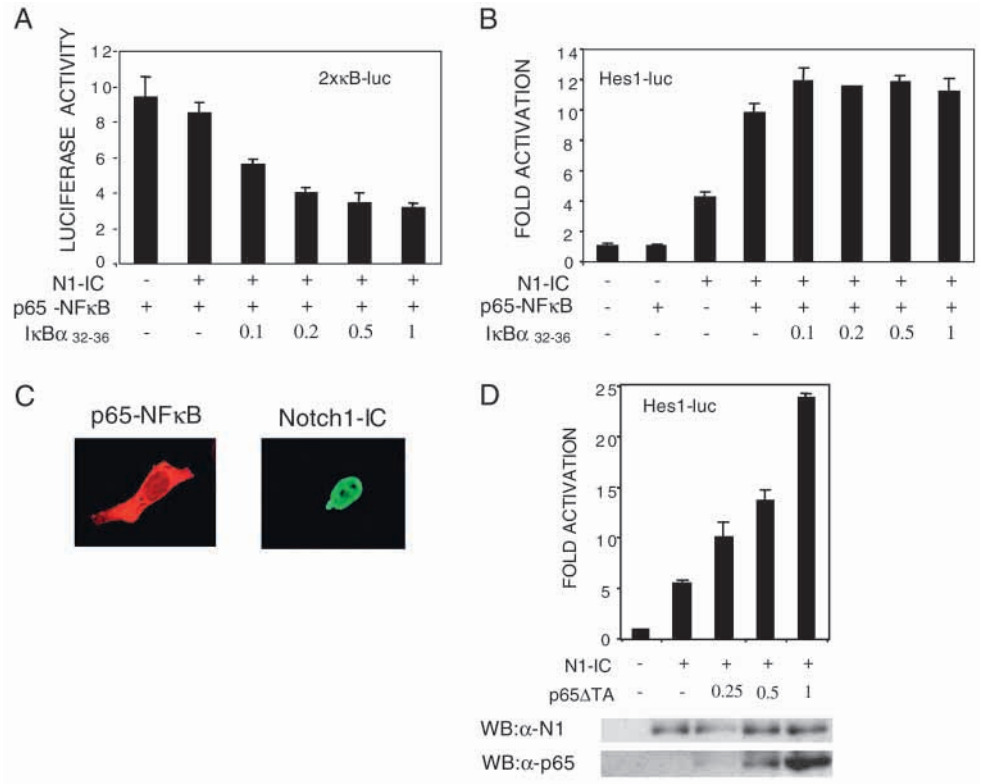


Fig. 2. Transcriptional activation of NFκB-dependent genes is not required for p65-mediated upregulation of the Hes1 promoter. NIH-3T3 cells were transfected with increasing amounts of $I\kappa B\alpha_{32\text{-}36}$ in the presence of N1-IC (0.5 μg) and p65 wt (0.5 μg). The reporter constructs were (A) 2×κB-IL2-luc (1 μg) or (B) Hes1-luc (1 μg). (C) Immunofluorescence assays to determine the subcellular localization of N1-IC and p65wt when coexpressed with $I\kappa B\alpha_{32\text{-}36}$ in NIH-3T3 cells. Cells were cotransfected with N1-IC, p65 wt and $I\kappa B\alpha_{32\text{-}36}$. N1-IC was detected with the 9E10 antibody and a FITC-labeled secondary antibody. p65 was detected with α-p65 and a Cy3-conjugated secondary antibody. (D) Luciferase fold activation obtained by cotransfecting the indicated amounts p65ΔTA with N1-IC (0.5 μg) and the Hes1-luc reporter (1 μg). Luciferase activity is presented as the fold induction relative to the basal level measured in cells transfected with empty vector. The average and standard deviation of duplicates from a representative experiment are presented. Lower panels show N1-IC and p65ΔTA protein levels. Protein loading for each lane was normalized according to β-galactosidase expression.

As previously described for the SMRT corepressor (Kao et al., 1998), we found that N-CoR was able to repress Hes1 promoter activity in a dose-dependent manner (Fig. 3A). To investigate whether p65-NFκB was able to modulate N-CoR-mediated repression, we cotransfected N-CoR and increasing amounts of p65 wt or p65ΔTA along with the Hes1 promoter. Our results demonstrate that ectopic expression of p65wt (data not shown) or p65ΔTA were able to reverse the inhibitory effect of N-CoR in a dose-dependent manner (Fig. 3B). This suggests that the previously observed effect of p65-NFκB on the Hes1 promoter may be mediated through its interaction with N-CoR.

We next asked whether overexpression of p65-NFκB was also modifying the expression of the endogenous *Hes1* gene. We observed a moderate increase (1.5-fold by densitometric analysis) in the Hes1 mRNA from 293T cells transfected with Notch-IC and N-CoR in the presence of p65 wt (Fig. 3C) or p65ΔTA (data not shown).

p65ΔTA expression triggers cytoplasmic translocation of N-CoR

We then examined the subcellular localization and protein levels of N-CoR in the presence or absence of p65ΔTA. In the absence of p65ΔTA, N-CoR was localized in the nucleus, displaying a speckled distribution (upper panels), as previously reported (Horlein et al., 1995). However, in 293T cells

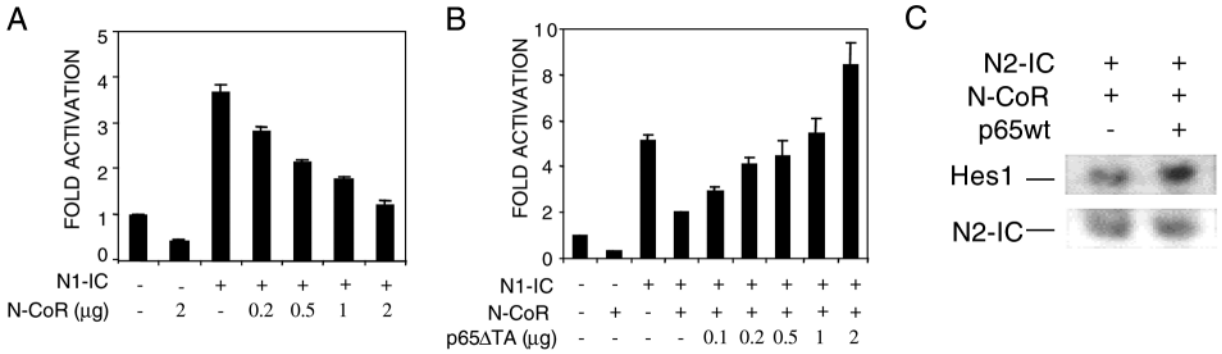


Fig. 3. The Hes1 promoter is repressed by N-CoR, and this effect is reversed by p65-NFκB overexpression. (A) N-CoR represses N1-IC activation of the Hes1 promoter. Indicated amounts of N-CoR were cotransfected with N1-IC expression vector (0.5 μg) into NIH-3T3 cells. (B) p65ΔTA can revert inhibition mediated by N-CoR. Increasing amounts of p65ΔTA were cotransfected with N1-IC (0.5 μg) and N-CoR (2 μg). Luciferase activity is presented as the fold induction relative to the basal level measured in cells transfected with empty vector. The average and standard deviation of duplicates from a representative experiment are presented. (C) Hes1 expression in 293T cells transfected with N2-IC and N-CoR expression vectors or coexpressed with p65wt is shown in the upper panel. Expression of N2-IC plasmid to compare transfection efficiency is shown in the lower panel.

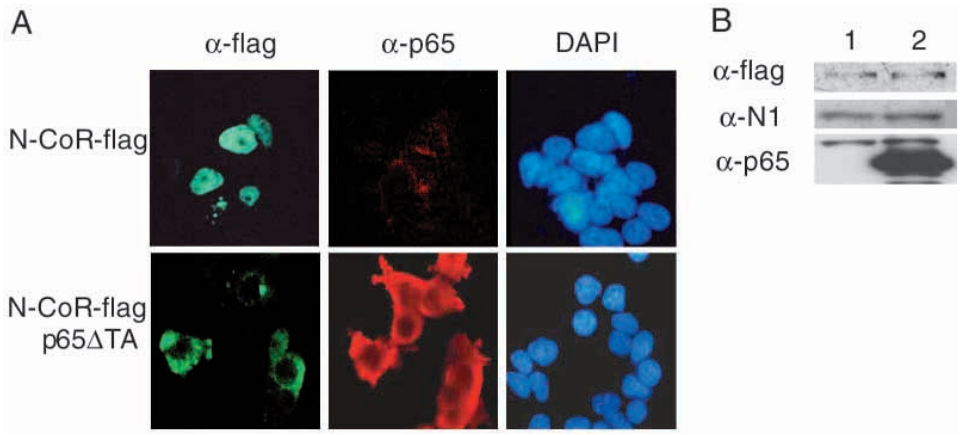
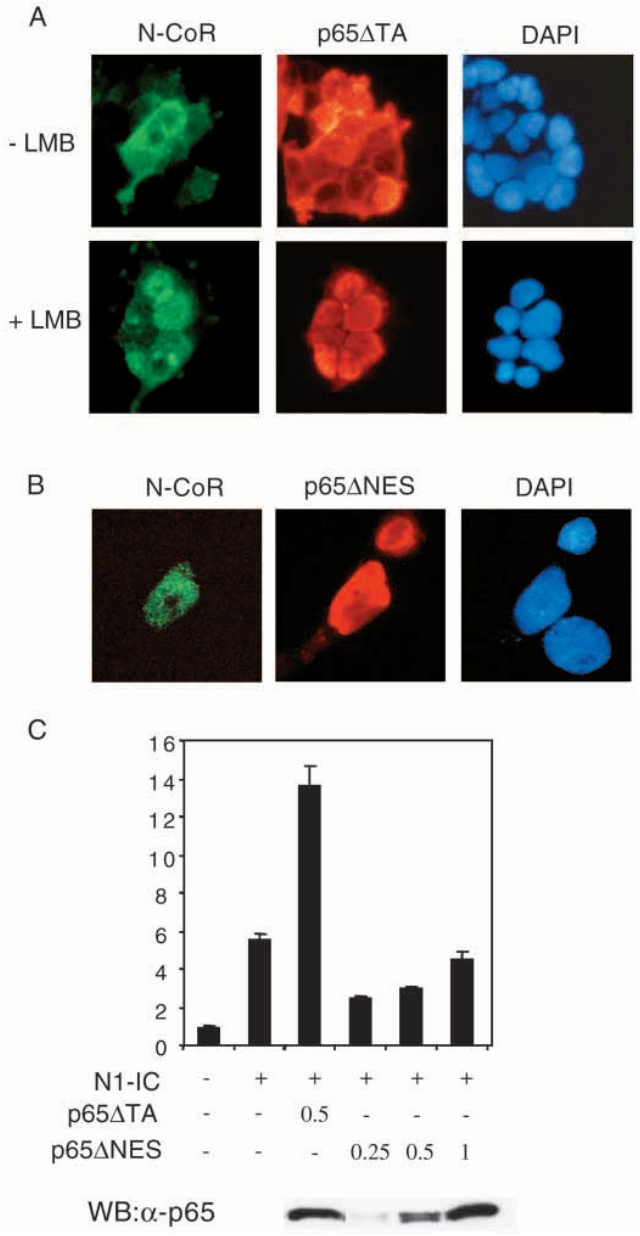


Fig. 4. N-CoR is translocated to the cytoplasm in the presence of p65ΔTA. (A) Immunofluorescence staining of 293T cells transfected with N-CoR in the presence (lower panels) or absence (upper panels) of p65ΔTA. N-CoR-flag was detected by incubating with α-flag antibody and a FITC-conjugated secondary antibody (left panels), α-p65 staining is represented in the middle panels corresponding to Cy3 signal and nuclei were visualized by DAPI staining (right panels). Ectopic expression of p65ΔTA does not affect N-CoR protein levels. (B) Immunoblotting analysis of whole cell extracts isolated from 239T cells transfected with N1-IC and N-CoR-flag (lane 1) and N1-IC, N-CoR-flag and p65ΔTA (lane 2). Cell lysates were separated on a 6% acrylamide gel, and immunoblots were performed with the following antibodies: α-flag (N-CoR), α-N1(N1-IC) and α-p65. Protein loading for each lane was normalized according to β-galactosidase expression and confirmed with α-N1 immunoblot. The western blot for p65 shows endogenous p65 (upper band) and transfected p65ΔTA (lower band).



cotransfected with N-CoR and p65ΔTA, we observed colocalization of both proteins in the cytoplasm, N-CoR being absent from the nucleus (Fig. 4A, lower panels). Identical results were obtained in NIH-3T3 cells (data not shown).

We next quantified N-CoR protein levels in 293T cells cotransfected with N1-IC and N-CoR in the presence or absence of p65ΔTA (Fig. 4B). N-CoR was detected at comparable levels in both conditions, suggesting that protein stability was not affected.

p65-NFκB nuclear export is required for N-CoR cytoplasmic translocation and Hes1 upregulation

There is increasing evidence of continuous nuclear-cytoplasmic shuttling of p65-NFκB within the cell (Carlotti et al., 2000). p65-NFκB subcellular localization is regulated by its NES domain and by its interaction with the p50-NFκB subunit and IκB (Harhaj and Sun, 1999; Huang et al., 2000). It has previously been reported that incubation of cells with the CRM-1-mediated nuclear export inhibitor LMB (Nishi et al., 1994) results in nuclear accumulation of p65-NFκB (Huang et al., 2000). We next investigated whether inhibition of p65 nuclear export by LMB was blocking the cytoplasmic translocation of N-CoR. 293T cells were cotransfected with

Fig. 5. p65-NFκB nuclear export is required for cytoplasmic retention of N-CoR. (A) Immunofluorescence staining of 293T cells transfected with N-CoR-flag and p65ΔTA in the absence (upper panel) or presence (lower panel) of LMB (10 ng/ml). (B) Immunofluorescence staining of 293T cells transfected with N-CoR-flag and p65ΔNES. N-CoR was detected with an α-flag antibody and a FITC-conjugated secondary antibody (left panels), α-p65 staining is represented in the middle panels corresponding to Cy3 signal, and nuclei were visualized by DAPI staining (right panels). (C) Hes1-luc transcriptional activity of NIH-3T3 cells cotransfected with N1-IC, N-CoR or/and p65ΔTA plasmids as indicated. Luciferase activity is represented as the fold induction relative to the basal level measured in cells transfected with empty vector. The average and standard deviation of duplicates from a representative experiment are presented. Lower panels show p65 protein levels. Protein loading for each lane was normalized according to β-galactosidase expression.

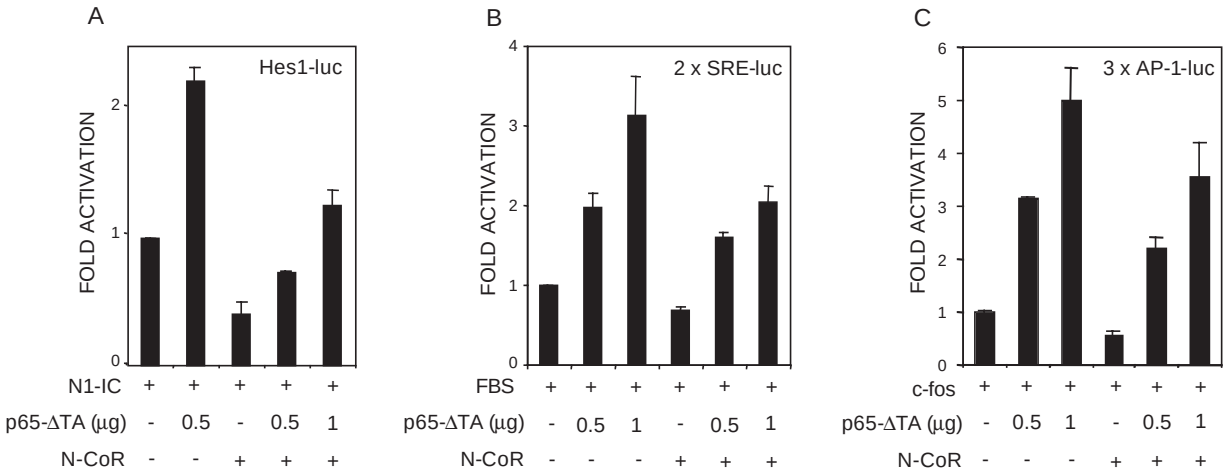


Fig. 6. p65ΔTA increases the activity of promoters repressed by N-CoR. NIH-3T3 cells were cotransfected with the above indicated luciferase reporters (A). N1-IC (0.5 μg) and N-CoR (1 μg) were cotransfected in the presence or absence of p65ΔTA (0.5 μg). Fold activation was calculated relative to cells transfected with N1-IC alone. (B) Cotransfection of p65ΔTA (0.5 μg) with or without N-CoR (1 μg) expression vector. Cells were incubated for 12 hours in the presence of 20% FBS, and fold activation was calculated relative to cells transfected with the reporter alone. (C) Cells were transfected with a c-fos expression vector (0.5 μg), p65ΔTA (0.5 μg) and/or N-CoR (1 μg). Fold activation was calculated relative to cells transfected with c-fos alone.

p65ΔTA and N-CoR and incubated in media alone or in the presence of 10 ng/ml of LMB. Fig. 5A shows that when CRM1-dependent nuclear export was inhibited, both p65ΔTA and N-CoR remained in the nucleus.

To investigate whether p65 nuclear retention but not LMB incubation per se is responsible for inhibiting N-CoR cytoplasmic translocation, we used a p65 mutant (p65ΔNES) lacking the NES domain, which localizes exclusively in the nucleus (Harhaj and Sun, 1999). When coexpressed with p65ΔNES, N-CoR shows an exclusively nuclear localization (Fig. 5B). Consistent with this observation, increasing levels of p65ΔNES with N1-IC did not result in upregulation of Hes1 promoter (Fig. 5C). These results strongly suggest that p65 cytoplasmic translocation is required for N-CoR cytoplasmic retention and that this mechanism is responsible for p65-mediated Hes1 upregulation. Moreover, we have observed that incubation with the HDAC inhibitor trichostatine A (TSA) results in a more nuclear localization of p65-NFκB, as reported by Chen et al. (Chen et al., 2001). Nevertheless, the colocalization of N-CoR with p65-NFκB is not modified in these conditions, suggesting that deacetylase activity is not necessary for interactions between both proteins (data not shown).

p65-NFκB increases the activity of other promoters repressed by N-CoR

Repression of genes dependent on NFκB, Activator Protein-1 (AP-1) or Serum Response Factor (SRF) has been associated with the interaction of these transcription factors with SMRT (Lee et al., 2000). We next speculated that p65-induced N-CoR cytoplasmic translocation should also be affecting the expression of genes other than Hes1. We transfected N-CoR with or without p65ΔTA into NIH-3T3 cells and determined the transcriptional activity of SRF- and AP-1-dependent luciferase reporters. When incubated in 20% FBS, expression of p65ΔTA upregulates SRE reporter activity in a dose-dependent manner, up to a maximum of a three-fold increase (Fig. 6B). When the AP-1-dependent promoter was assayed in the presence of p65ΔTA, luciferase activity was five-fold higher compared with the maximum activity of this reporter in the presence of c-fos. (Fig. 6C). Moreover, and similar to its effect on the Hes1 reporter, we show that N-CoR was able to repress both promoters and that this repression was overridden by coexpression of p65ΔTA (Fig. 6A,B,C). These results clearly demonstrate that p65-NFκB can regulate gene expression not only through binding to promoters containing κB-sites but also by triggering cytoplasmic translocation of the nuclear corepressor N-CoR.

Discussion

Integration of different signal transduction pathways results in activation of specific subsets of genes and is responsible in the last term for specifying cell behavior. Notch and NFκB signaling pathways participate in the regulation of essential cellular events. In this study, we demonstrate that p65-NFκB enhances the transcriptional activity of Notch and that of other transcription factors by regulating the subcellular localization of the nuclear corepressor N-CoR.

First, we show that p65-NFκB cooperates with Notch in activating the Hes1 promoter. Second, we show that this effect is independent of transcriptional activity and colocalization of p65-NFκB and Notch-IC. Next, we demonstrate that ectopic expression of p65-NFκB can modify subcellular localization of the nuclear corepressor N-CoR. Finally, we demonstrate that this new mechanism affects not only Notch-dependent gene expression but also the expression of other genes (such as SRF- and AP-1-responsive genes) that are targets for N-CoR repression.

Notch and NFκB signaling pathways

Several interactions between Notch and NFκB pathways have already been reported; however, the physiological significance

of their interplay is not well understood. For instance, a Notch-IC and p50-NFκB direct interaction leads to the inhibition of NFκB transcriptional activity, supporting the idea that Notch activation promotes downregulation of NFκB-dependent genes (Guan et al., 1996). Conversely, it has been shown that Notch3 homologue is able to upregulate the expression of NFκB-dependent genes, probably by promoting degradation of the NFκB inhibitor IκBα (Bellavia et al., 2000). On the other hand, NFκB participates in the transcriptional regulation of the Notch ligand Jagged1 gene (Bash et al., 1999), thus modifying the balance between receptors and ligands that is critical in Notch pathway regulation (Heitzler and Simpson, 1991).

Notch and NFκB can play different roles in regulating cell proliferation (Baonza and Garcia-Bellido, 2000; Carlesso et al., 1999; Kontgen et al., 1995), cell differentiation (Kaisho et al., 2001; Kaliman et al., 1999; Guttridge et al., 2000; Feng and Porter, 1999; Egan et al., 1998; Milner and Bigas, 1999) and apoptosis (reviewed in Barkett and Gilmore, 1999; Jehn et al., 1999; Shelly et al., 1999; Ohishi et al., 2000). However, it is tempting to speculate that they should exert antagonistic or synergistic effects depending on the cellular context. In myogenic systems, NFκB can both promote or inhibit differentiation depending on the external stimuli. For example, it mediates TNF-α-induced inhibition of differentiation in C2C12 cells (Guttridge et al., 1999), and it is required for the IGFII-induced differentiation of L6E9 myoblasts (Kaliman et al., 1999). In contrast, Notch activation is mainly associated with maintaining the undifferentiated phenotype (Kopan et al., 1994) by upregulating Hes1 (Kuroda et al., 1999), inhibiting MEF2C (Wilson-Rawls et al., 1999) or by combined mechanisms.

In T cell development, constitutive activation of Notch (Robey et al., 1996) and suppression of NFκB activity by expression of $I\kappa B\alpha_{32\text{-}36}$ (Boothby et al., 1997) results in opposite phenotypes for the acquisition of the $CD8^+CD4^-$ T cell fate. In fact, in a previous step, both Notch and NFκB activities are necessary for the survival of double-positive $CD4^+CD8^+$ cells when the appropriate TCR signal is received (Deftos et al., 2000; Hettmann and Leiden, 2000). Synergistic mechanisms of crosstalk between NFκB and Notch pathways (Oswald et al., 1998; Bash et al., 1999; Bellavia et al., 2000) (this paper) may be controlling thymocyte-positive selection by regulating the expression of anti-apoptotic genes. Consistent with this, both Notch and NFκB can activate bcl-2 expression in lymphoid cells (Zong et al., 1999; Deftos et al., 2000).

On the other hand, alterations in the Notch or NFκB pathways have been associated with oncogenic processes. Several cancer cells show constitutive nuclear NFκB activity, including those from lymphoid, breast, ovarian, lung, thyroid and melanoma origin (reviewed in Rayet and Gelinas, 1999). Moreover, there is emerging evidence that alteration of the Notch pathway affects many tumorigenic transformation and tumor progression processes (Capobianco et al., 1997; Leethanakul et al., 2000; Gallahan and Callahan, 1997). The NFκB constitutive activity found in Notch3-IC-transformed T cells is the first evidence that suggests a possible synergistic effect of both pathways in tumorigenesis (Bellavia et al., 2000). Taken together, these results indicate that coordination between Notch and NFκB pathways may be crucial in controlling cellular events.

Transcriptional activation mediated by p65-NFκB

Different combinations of Rel/NFκB subunits bind to specific target genes, directly regulating their transcriptional activity. Although NFκB-mediated transcriptional regulation has been extensively studied (reviewed in Siebenlist et al., 1994), NFκB-mediated effects on promoters lacking κb-sites or possible interactions of single NFκB subunits with other transcriptional complexes need further clarification.

For example, it has been reported that p65-NFκB can upregulate SRE-containing promoters independently of classical NFκB activity (Franzoso et al., 1996). In addition, the transforming ability of v-rel, a mutated version of the avian c-Rel that lacks the transactivation domain (reviewed in Luque and Gelinas, 1997), correlates with high levels of c-jun and c-fos proteins and increased AP-1 transcriptional activity (Kralova et al., 1998). Consistent with this, we have shown that overexpression of p65-NFκB regulates not only the transcriptional activity of Hes1 but also the activity of promoters containing SRE and AP-1 elements that lack NFκB sites. Thus, our results can provide an explanation of the previously published observations, reinforcing the idea that p65-NFκB exerts a positive effect over a broad spectrum of transcription factors, even in the absence of its transcriptional activation (TA) domain. In this sense, our results suggest that the p65ΔTA mutant may be more effective at overriding N-CoR transcriptional repression than the full-length protein, perhaps reflecting the presence of some regulatory elements in the C-terminal half of the protein.

Although more physiological studies should be done, these observations may reflect a new crosstalk between NFκB and other signaling pathways, which may be responsible for regulating transcription of specific sets of genes.

A role for p65-NFκB in regulating chromatin modifying activities

Assembly of multimeric protein complexes at their appropriate target DNA sequences results in transcriptional activation or repression, thus generating specific gene expression patterns (reviewed by Merika and Thanos, 2001). Recruitment of coactivators or corepresors by transcription factors and hormone receptors is crucial to determine the nature and activity of these complexes. Coactivators and corepressors exert their function by increasing or decreasing histone acetylation and thus modifying chromatin structure. N-CoR and SMRT nuclear corepressors are highly homologous, although N-CoR contains a unique N-terminal region that might be involved in unreported specific functions. Although preliminary studies associated both proteins with repression of hormone nuclear receptors (reviewed by Xu et al., 1999), SMRT can also repress c-fos-, NFκB-, SRF- (Lee et al., 2000) and Notch/RBPJκ-dependent genes (Kao et al., 1998). Here we show that most of these promoters are also repressed by N-CoR, and we demonstrate that this repression can be modulated by p65-NFκB.

Physical association of SMRT and HDACs with p65-NFκB has been reported to repress NFκB-regulated genes (Lee et al., 2000; Ashburner et al., 2001; Chen et al., 2001). Our results suggest that p65-NFκB may interact with N-CoR, inducing its translocation to the cytoplasm and acting as a rapid and potent tool for regulating gene expression. Although experiments with TSA suggest that this mechanism does not depend on

deacetylase activity, the role of HDACs or other proteins known to interact with p65 or N-CoR is currently under investigation. It is also worth considering that phosphorylation or acetylation events affecting N-CoR or p65-NFκB may regulate their interaction. In this sense, it has been reported that SMRT can translocate to the cytoplasm in response to phosphorylation events (Hong and Privalsky, 2000; Jang et al., 2001).

Coactivators and corepressors bind to nuclear receptors competing for similar consensus regions (Nagy et al., 1999). Interestingly, p65-NFκB can synergize with IRF1 and ATF2/c-jun to activate transcription of the IFNβ promoter by recruiting transcriptional coactivators through a leucine-rich domain (AA 430-458) similar to that present in nuclear receptors (Merika et al., 1998). In this system, the effect of p65-NFκB is dependent on the binding to DNA of all three transcription factors. By contrast, the mechanism that we describe here does not require the presence of a κB-binding site in the target promoter. Since the NES is included in the p65-NFκB leucine-rich domain, it is tempting to speculate that there is a potential physical interaction between p65-NFκB and N-CoR involving this domain.

Taken together, our results indicate that p65-NFκB can exert an important role in coordinating gene transcription of specific sets of genes by a new mechanism that involves cytoplasmic translocation of N-CoR. We speculate that this new p65-NFκB function may be crucial in the integration of multiple signal transduction pathways.

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