NF-κB/Rel Proteins Are Required for Neuronal Differentiation of SH-SY5Y Neuroblastoma Cells*

(Received for publication, July 12, 1999, and in revised form, August 28, 1999)

Zhiwei Feng and Alan G. Porter‡
From the Institute of Molecular and Cell Biology, The National University of Singapore, Singapore 117609, Republic of Singapore

The expression, cellular localization, and activation of the NF-κB/Rel transcription factors are altered during neuronal differentiation, but the significance is unclear. Here we investigate the requirement for NF-κB/Rel proteins in neuronal differentiation. SH-SY5Y neuroblastoma cells were induced to differentiate with retinoic acid (RA) or 12-O-tetradecanoylphorbol 13-acetate (TPA), and differentiation was demonstrated by morphological criteria and the enhanced expression of Bcl-2. NF-κB was transiently activated after the addition of the differentiation inducers before the morphological signs of differentiation and the enhanced Bcl-2 synthesis. The onset of NF-κB activation coincided with a significant reduction in the amount of only one of four NF-κB-inhibitory proteins examined (I-κBα). In contrast, NF-κB activation and the reduction in I-κBα failed to occur in SH-SY5Y cells transformed with I-κBαM, a dominant-negative inhibitor of NF-κB/Rel proteins. These I-κBαM-expressing cells failed to differentiate into neuronal cell types when treated with RA or TPA, and the increased Bcl-2 synthesis was blocked. Therefore, NF-κB/Rel proteins are required for neuronal differentiation of SH-SY5Y neuroblastoma cells.

The human SH-SY5Y neuroblastoma cell line is a well-established system for studying neuronal differentiation (1, 2). SH-SY5Y cells can be morphologically differentiated into neuronal cells, the phenotype of which varies depending on the inducing agent (1). For example, retinoic acid (RA)1 drives SH-SY5Y cells to differentiate along a sympathetic chromaffin lineage, whereas 12-O-tetradecanoylphorbol 13-acetate (TPA) induces differentiation along a sympathetic lineage (1). However, little is known of the genes or patterns of gene expression that are essential for the process of neuronal differentiation.

The ubiquitously expressed mammalian transcription factor NF-κB is one of a family comprising at least five members (NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel) that bind the consensus DNA motif 5′-GGGPuNNPpyPyCC-3′ as homo- or heterodimers upon activation (3, 4). NF-κB dimers are normally sequestered in the cytoplasm by one of several protein inhibitors (e.g. I-κBα, I-κBβ, I-κBγ, or I-κBε) that mask the nuclear localization signal of NF-κB (3). Receptor-generated signals lead to the phosphorylation and release of I-κB proteins, and the subsequent nuclear translocation and functional activation of NF-κB occurs as the result of the unmasking of its nuclear localization signal (3, 4). NF-κB not only participates in the induction of the expression of many genes, including those encoding proteins that fulfill important roles in the processes of immunity and inflammation (5), but also contributes to the regulation of apoptosis (6, 7).

Studies on B cells from p50/NF-κB knockout mice and on B cells and thymocytes transformed with I-κBαM, a strong dominant-negative inhibitor of the activation of different NF-κB/Rel complexes that bind I-κB proteins, have indicated that NF-κB is required for or participates in immune cell differentiation and development (8, 9).

NF-κB/Rel proteins may have unique roles in the central nervous system, contributing to synaptic transmission and neuronal plasticity as well as neuronal development and differentiation (10). During neurogenesis, NF-κB becomes activated in neurons in certain regions of the brain, showing marked changes in both constitutive and inducible activity postnatally (10). Several in vitro models have suggested that these profound changes in vivo may reflect an involvement of NF-κB in neuronal differentiation (10–12), but this notion is far from being established. Here we show that, in a neuroblastoma cell line, NF-κB is activated prior to neuronal differentiation induced by two different agents and that both its activation and the differentiation into neurons are blocked by a dominant-negative inhibitor of NF-κB.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human SH-SY5Y neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. LipofectAMINE was used for transfections according to the Life Technologies, Inc. manual, and stably transfected clones were selected and maintained with 500 μg/ml G418 (Life Technologies, Inc.). All-trans-retinoic acid and TPA were from Sigma. For differentiation, 1 × 10⁶ cell/ml were grown in 10-cm cell1 plates (Sarsted, Inc.) for 24 h, and then RA (10 μM) or TPA (16 nM) was added (1). The medium was changed every 2 days, and the morphology was observed and photographed at day 4 for both RA and TPA treatments. The plasmids encoding the I-κBαM dominant-negative inhibitor of NF-κB and vector control plasmid were both provided by D. R. Green (13). Polyclonal antibodies to I-κBα and I-κBβ were from Santa Cruz Biotechnology, and the monoclonal antibody to Bcl-2 was from Transduction Laboratories.

Preparation of Cytoplasmic and Nuclear Proteins—Cells stably transformed with the vector control or I-κBαM cDNA (13) were harvested and washed in ice-cold phosphate-buffered saline. For cytoplasmic proteins, the cell pellets were suspended in ice-cold lysis buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of aprotinin, leupeptin, and antipain) and incubated on ice for 30 min. The samples were centrifuged, and supernatants were collected as cytoplasmic proteins. For isolating nuclear proteins, cells were homogenized in a Dounce homogenizer in 20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml of protease inhibitors and incubated at 4 °C for 30 min. The cell pellets were resuspended in 20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 420 mM KCl, 1 mM EDTA, 25% glycerol, 1 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml of protease inhibitors and incubated at 4 °C for 30 min.

* This research was funded by the Institute of Molecular and Cell Biology, Singapore. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Institute of Molecular and Cell Biology, The National University of Singapore, 30 Medical Dr., Singapore 117609, Republic of Singapore. Tel.: 65-874-3761; Fax: 65-779-1117; E-mail: mcbag@mcb.nus.edu.sg.
1 The abbreviations used are: RA, all-trans-retinoic acid; TPA, 12-O-tetradecanoylphorbol 13-acetate.
results
I-κB Synthesis in Stable SH-SY5Y Cell Lines Expressing I-κBαM—It is known that the DNA binding activity of NF-κB is activated by a variety of differentiation-inducing agents in some cells at early stages of neuronal differentiation (2, 10, 11). To clarify the roles of NF-κB members in neuronal differentiation, we generated stable transformed SH-SY5Y neuroblastoma cells expressing I-κBαM, which is a derivative of I-κBα, a binding partner and protein inhibitor of NF-κB (3–5, 8, 13). I-κBαM is an effective dominant-negative inhibitor of NF-κB/Rel complexes, because two key phosphorylation sites in I-κBαM are mutated, thus preventing its phosphorylation, release from NF-κB, and degradation (8, 13). SH-SY5Y cells expressing I-κBαM (or transformed with a vector control plasmid) were induced to differentiate with either RA or TPA (1, 2), and the synthesis of I-κBαM and endogenous I-κBα was followed over 24 h. In the vector control cells, I-κBα protein levels showed a small but insignificant decrease around 1–2 h after treatment with RA or TPA, followed by a marked increase from 8 to 24 h (Fig. 1A, upper panels). In one of several I-κBαM-expressing SH-SY5Y clones, similar amounts of endogenous I-κBα and the faster migrating I-κBαM protein were initially synthesized, but only I-κBαM steadily accumulated in the 24 h following RA or TPA treatments (Fig. 1A, lower panels). The accumulation of I-κBαM is not surprising, as it is a mutant form of I-κBα that is resistant to degradation (13). In the presence of I-κBαM, the enhanced expression of endogenous I-κBα was blocked, and the endogenous I-κBα levels declined dramatically at ∼8–24 h after addition of the differentiation inducers (Fig. 1A, lower panels). This finding indicates that NF-κB function is inhibited by I-κBαM, because NF-κB normally up-regulates I-κBα at the level of transcription (15).

I-κBαM Is an Effective Inhibitor of NF-κB Activation in Neuroblastoma Cells—Previously, NF-κB activation was found to be associated with neuronal differentiation of SH-SY5Y cells, but the relationship of NF-κB activation to differentiation was not further investigated (2). DNA band-shift analyses were performed using an oligonucleotide containing a NF-κB consensus sequence to confirm and extend these findings and to determine whether I-κBαM prevented the activation of NF-κB that occurs in cells treated with different inducers of differentiation (2). At 1–2 h after the addition of RA or TPA, the DNA-binding activity of NF-κB was clearly activated in the vector-transfected SH-SY5Y cells but not in the cells transformed with I-κBαM (Fig. 2). NF-κB activation was transient in the vector control cells (Fig. 2), and no further NF-κB complexes were formed at any times between 8 and 24 h after addition of the differentiation agents in either the vector-transformed or the I-κBαM-expressing cells. Under these conditions (Fig. 2), the DNA complex contains the RelA (p65) subunit of NF-κB (2).

The transient NF-κB activation at 1–2 h following the addition of RA or TPA to the vector-transfected cells (Fig. 2) was accompanied by a significant reduction in the level of the NF-κB-inhibitory protein I-κBβ (3) that began at 1 h and returned to unstimulated levels by 8 h after RA or TPA addition (Fig. 1B, upper panels). In contrast, I-κBβ protein levels remained constant for 8 h after RA or TPA addition in cells transformed with I-κBαM in which NF-κB activation is abolished (Fig. 1B, lower panels). Altogether, these results show that I-κBαM is effective at inhibiting NF-κB activation in SH-SY5Y neuroblastoma cells, which may proceed through the degradation of I-κBβ rather than I-κBα. Complete degradation of I-κBβ was not observed, perhaps because of the transient nature of NF-κB activation and the fact that destruction of I-κB proteins is not the only mechanism capable of activating NF-κB (16).

Absence of Neuronal Differentiation in I-κBαM-expressing SH-SY5Y Cell Lines—SH-SY5Y cells rapidly undergo neuronal differentiation, the phenotype of which depends on the inducing agent (1). Four days after RA or TPA treatment, extensive morphological differentiation was observed in I-κBαM-expressing SH-SY5Y cell cultures transformed with the empty vector, as judged by the shrinkage of the cell body and the extension of neurites, which made cell-to-cell connections (Fig. 3, left panels). As expected (1), the neurites in RA-treated cells were on average much shorter than neurites in RA-treated cells (Fig. 3, left panels). These morphological signs of differentiation were first evident
NF-κB activation is an early event required for neuronal differentiation. Various proteins are known to be activated or synthesized and several genes are up-regulated in neuronal differentiation (1, 10), but it is difficult to distinguish between functions that are essential for differentiation and those that are merely markers of the differentiated state. Bcl-2 and NF-κB Are Two Genes That Have Been Directly Implicated in Neuronal Development and Differentiation (8, 10, 17, 19–22). We and others have shown that Bcl-2 is strongly up-regulated in parallel with neuronal differentiation (17–19). Moreover, Bcl-2 appears to be required for neuronal differentiation of Paju, a neural crest-derived cell (21), and a Bcl-2 antisense oligonucleotide suppressed nerve growth factor-induced neuronal differentiation of PC12 pheochromocytoma cells (19). Bcl-2 also influences the axonal growth of embryonic sensory neurons (22). We found that the characteristic RA- or TPA-induced Bcl-2 up-regulation fails to occur when NF-κB activation and neuronal differentiation are abrogated in I-κBαM-expressing SH-SY5Y cells, further illustrating the close relationship between Bcl-2 and differentiation.

Diverse stimuli activate NF-κB in the brain and in neuronal cells, leading to the expression of genes associated with immune-related functions, growth regulation, inflammation, and cell adhesion (10). Activation of NF-κB/Rel proteins has been observed during neuronal differentiation in the brain (10), in P19 embryonic carcinoma cells (12), and in SH-SY5Y, GOTO, and IMR32 neuroblastoma cells (2, 11), but the timing, requirement, and role of NF-κB in neuronal differentiation have not been established until now. Here we showed that NF-κB is activated in SH-SY5Y cells prior to neuronal differentiation induced by two different agents, and that when this activation is blocked, neuronal differentiation is completely prevented by both morphological and biochemical criteria (i.e. absence of Bcl-2 up-regulation). Thus, NF-κB/Rel proteins are required for neuronal differentiation of SH-SY5Y neuroblastoma cells. The fact that a transient, and not sustained, NF-κB activation is observed well before the morphological signs of differentiation appear suggests that NF-κB activation is an early event required for neuronal differentiation. An earlier study utilizing DNA band-shift analysis suggesting that NF-κB activation accompanies TPA-, but not RA-induced neuronal differentiation of SH-SY5Y cells is puzzling (2), as we found that both RA and TPA activate NF-κB by DNA band-shift analysis. Moreover, our finding that I-κBαM completely prevents neurite out-
NF-κB/Rel Proteins in Neuronal Differentiation

growth, shrinkage of the cell body, and Bcl-2 up-regulation is by itself an indication that one or more NF-κB/Rel proteins are activated and contribute to RA- and TPA-induced neuronal differentiation of SH-SY5Y cells. Our conclusion that NF-κB/Rel proteins are required for neuronal differentiation of SH-SY5Y cells induced by two different types of stimuli is also in accord with recent evidence that this family of transcription factors is involved in or essential for the development or differentiation of hemopoietic cells, including CD4^+CD8^- thymocytes and T and B cells (8, 9, 23–25).

With both differentiation inducers, the onset of NF-κB activation in vector-transformed cells coincided with a reduction in the amount of the NF-κB-regulatory protein IκBα, whereas IκBα levels were essentially unchanged in these cells. Protein levels of two other NF-κB-inhibitory proteins (IκBγ and IκBε) also did not change during RA- or TPA-induced NF-κB activation and neuronal differentiation. These findings suggest that the destruction of IκBα might be a trigger for NF-κB activation, which is unusual and interesting because almost all known NF-κB inducers cause the degradation of IκBα, not IκBβ (3).

If NF-κB is required for neuronal differentiation, why does neuronal development appear normal in mice in which individual NF-κB or Rel proteins are absent (4)? The dominant-negative inhibitor IκBαM inhibits the different NF-κB/Rel complexes that bind various IκBκB complexes and IκBβ). Our results therefore raise the intriguing possibility that more than one class of heterodimeric NF-κB-related transcription factors (comprising NF-κB1 or NF-κB2 complexed with RelA, RelB, or c-Rel) may act together in neuronal differentiation, which is consistent with the failure to obtain viable mice lacking more than one type of NF-κB or Rel subunit (4).

Finally, as NF-κB activation and Bcl-2 up-regulation are abrogated in the IκBαM-expressing cell lines, and increased Bcl-2 synthesis has previously been associated with NF-κB activation in other systems (26, 27), it is worth asking whether bcl-2 is a NF-κB-regulated gene. The long lag between transient NF-κB activation and the sustained increase in Bcl-2 levels strongly argues against bcl-2 being a direct target gene of NF-κB in neuronal differentiation of SH-SY5Y cells, but it still leaves open the possibility that NF-κB indirectly contributes to Bcl-2 up-regulation.

Acknowledgments—We are very grateful to D. R. Green (La Jolla Institute for Allergy and Immunology) for the IκBαM-expressing plasmid and E. Feldman (University of Michigan) for the SH-SY5Y cell line. We thank V. Yu and M. Choi for critically reviewing the manuscript.

REFERENCES

1. Påhlman, S., Hoehner, J. C., Nänberg, K., Hedborg, F., Fagerström, S., Gestblom, C., Johansson, L., Larsson, U., Lavenius, E., Ortoft, E., and Söderholm, H. (1995) Eur. J. Cancer 31A, 453–458
2. Körner, M., Tarantino, N., Fleksoff, O., Lee, L. M., and Debre, P. (1995) J. Neurochem. 62, 1716–1726
3. May, M. J., and Ghosh, S. (1998) Immunol. Today 19, 80–88
4. Baeuerle, P. A., and Baltimore, D. (1996) Cell 87, 13–20
5. Baeuerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
6. Baltch, V. R., and Baeuerle, P. A. (1997) Curr. Biol. 7, R84–R96
7. Lipton, S. A. (1997) Nat. Med. 3, 20–22
8. Siebenlist, U. (1997) Biochim. Biophys. Acts 1332, R7–R13
9. Snapper, C. M., Zelazowski, P., Rosas, F. R., Kehry, M. R., Tian, M., Baltimore, D., and Shu, W. C. (1996) J. Immunol. 156, 183–191
10. O’Neill, L. A. L., and Kaltschmidt, C. (1997) Trends Neurosci. 20, 252–258
11. Kurata, S., Wakabayashi, T., Ito, Y., Miwa, N., Ueno, R., Marunouchi, T., and Kurata, N. (1999) FEBS Lett. 421, 201–204
12. Sheppard, A. M., McQuillan, J. J., Iademarco, M. F., and Dean, D. C. (1995) J. Biol. Chem. 270, 3710–3719
13. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) Science 274, 787–789
14. Feng, Z., Mati, A., Jehn, B., Altermatt, H. J., Chicaiza, G., and Jaggi, R. (1995) J. Cell Biol. 121, 1095–1103
15. Sun, S. C., Guinchi, P. A., Ballard, D. W., and Greene, W. C. (1993) Science 259, 1912–1915
16. Belich, M. P., Salmeron, A., Johnston, L. H., and Ley, S. C. (1999) Nature 397, 363–368
17. Lasorella, A., Iavarone, A., and Israel, M. A. (1995) Cancer Res. 55, 4711–4716
18. Hanada, M., Krajewski, S., Tanaka, S., Cazals-Hatem, D., Spengler, B. A., Ross, R. A., Hiedler, J. J., and Reed, J. C. (1993) Cancer Res. 53, 4978–4986
19. Katoh, S., Misumi, Y., Kitani, K., and Suzuki, T. (1996) Biochem. Biophys. Res. Commun. 229, 653–657
20. Bernier, P. J., and Parent, A. (1998) J. Neurosci. 18, 2486–2497
21. Zhang, K.Z., Westberg, J. A., Höltta, E., and Andersson, L. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4504–4508
22. Hilton, M., Middleton, G., and Davies, A. M. (1997) Curr. Biol. 7, 798–800
23. Ochiai-Garay, J., Kaye, J., and Coligan, J. E. (1997) J. Immunol. 160, 3835–3843
24. Boothby, M. R., Mora, A. L., Scherer, D. C., Brockman, J. A., and Ballard, D. W. (1997) J. Exp. Med. 185, 1897–1907
25. Esslinger, C. W., Wilson, A., Sordat, B., Beermann, F., and Jongeneel, C. V. (1997) J. Immunol. 158, 5075–5078
26. Rowe, M., Peng-Pilon, M., Huen, D. S., Hardy, R., Croom-Carter, D., Lundgren, K., and Richardson, A. B. (1994) J. Virol. 68, 5602–5612
27. DeLuca, C., Kwon, H., Pelletier, N., Wainberg, M. A., and Hiscott, J. (1998) Virology 244, 27–38

Z. Feng and A. G. Porter, unpublished observations.