The IκB family of proteins regulates NF-κB-dependent transcription by inhibiting DNA binding and localizing these factors to the cell cytoplasm. IκBa does this by shifting the balance between nuclear import of Rel proteins and their export from the nucleus. Here we show that, unlike IκBo, IκBβ and IκBe appear to sequester p65 or c-Rel in the cytoplasm by inhibiting nuclear import. Furthermore, because IκBβ does not undergo nucleocytoplasmic shuttling, it cannot remove nuclear proteins like IκBo does. We conclude that the mechanism of action differs among IκB family members.

The NF-κB/Rel family of transcription factors plays a central role in immune and inflammatory responses (1). In most cell types these proteins are sequestered in the cell cytoplasm complexed to a family of inhibitory IκB proteins (2, 3). Cellular activation results in IκB degradation, which leaves the DNA-binding protein free to translocate to the nucleus and activate gene expression. Because of the widespread effects of NF-κB activation, its localization in the cytoplasm must be strictly maintained. IκBo-deficient mice are a striking example of the importance of NF-κB sequestration in the cytoplasm; these mice die of a wasting disease that has been attributed to tumor necrosis factor-α production (4, 5). Nuclear factor-κB has also been detected in several diseased tissues, where it has been proposed to contribute to the pathology in part by inhibiting apoptosis (6, 7).

The association of Rel with IκBo has been proposed to hide the NLS of Rel proteins (8, 9), thereby precluding nuclear entry. In addition, we did not detect an association between CRM1 protein and either IκBα or IκBe in yeast two-hybrid assay (15). In this paper we demonstrate that cytoplasmic retention of Rel proteins by IκBβ and IκBe involves sequestration rather than tilting the balance of nuclear import and export as is the case with IκBo. Furthermore, although newly synthesized IκBβ can enter the nucleus, it cannot restore nuclear Rel proteins to the cytoplasm. These observations suggest that IκBβ and IκBe function differently from IκBo.

MATERIALS AND METHODS

Cell Lines and Strains—D5 h3 T hybridoma cells and A20 mature B cells were grown in Dulbecco’s modified Eagle’s medium and RPMI 1640 medium, respectively, with 10% heat-inactivated fetal bovine serum, 50 μM β-mercaptoethanol and antibiotics. COS cells were cultured in Dulbecco’s modified Eagle’s medium with 10% newborn calf serum and antibiotics. Yeast strain W303 and its transformants were generally grown in synthetic medium with the appropriate amino acid and nitrogen base supplement.

Plasmids—pGFP-p65 and pCDNA3-HA-IκBa have been described previously (15). pGFP-cRel contains full-length murine cRel in frame after GFP. pCDNA3-Myc-IκBβ and pCDNA3-Myc-IκBe were made by inserting full-length murine IκBβ and IκBε cDNA, respectively, in frame behind a c-Myc tag (MEQKLISEEDL). Yeast galactose-inducible plasmid encoding GFP-p65 and copper-inducible HA-IκBe (pCuI Be) have been described previously (15). The copper-inducible HA-IκBε was made by replacing the IκBa gene with a murine IκBβ full-length gene in the same vector. All plasmids used in this study were confirmed by sequencing, and expression of proteins was verified by immunoblotting.

Immunostaining—The procedures for immunostaining adherence cells were the same as described previously (15). For staining suspension cells (T and B cells), the procedures were also as described previously (18).

Protease Digestion—The proteases Asp-N and Lys-C were purchased from Roche Molecular Biochemicals. Proteases were used according to the manufacturers’ specifications.

Fluorescence Microscopy—The subcellular localization of GFP and the immunofluorescence signals were observed by fluorescence microscopy (Axiohot II, Zeiss) with a GFP generic filter, fluorescein isothiocyanate, rhodamine, and DAPI filter.

RESULTS

The nuclear export property of IκBo is essential for cytoplasmic location of Rel proteins. However, IκBβ and IκBe, which are not nucleo-cytoplasmic shuttling proteins, can also effectively localize Rel proteins to the cytoplasm. One possibility was that cytoplasmic retention by IκBβ/ε may be mediated by export determinants in the Rel proteins. To test this possibility, we coexpressed green fluorescent protein (GFP)-tagged Rel proteins with IκBβ or IκBe in COS cells and assayed the loca-
Fig. 1. Rel-IκBα complexes shuttle continuously, but Rel-IκBβ complexes do not. A, GFP-p65 was transiently transfected with HA-IκBα, Myc-IκBβ, or Myc-IκBε into COS cells. Half the cells were then treated with LMB (10 ng/ml) for 3 h. Untreated (left panel) or LMB-treated cells (right panel) were fixed for fluorescent visualization. Green fluorescence shows a GFP-p65 signal. The red fluorescence shows an IκB signal from rhodamine-conjugated antibodies against either the HA tag or the Myc tag. Blue fluorescence shows DAPI staining of nuclei. B, A20 B cells were settled on specially treated coverslips (Fisher). Half of these were treated with LMB (100 ng/ml) for 45 min. Cells with or without LMB treatment were fixed and permeabilized for immunostaining. Green fluorescence shows endogenous IκBα (first row) and IκBβ (second row) detected by fluorescein isothiocyanate-conjugated antibodies against anti-IκBα or anti-IκBβ respectively. Representative results are shown from one of three independent experiments.

The shuttling dynamics of endogenous Rel protein complexes were investigated in mammalian cells by transient transfection of Rel proteins by GFP fluorescence. Both p65 and c-Rel (data not shown) were located in the cytoplasm in the presence of IκBα (Fig. 1A, left panel). However, these complexes did not translocate to the nucleus when the cells were treated with LMB, an inhibitor of CRM1-mediated nuclear export (Fig. 1A, right panel). Therefore, CRM1 was not involved in determining the subcellular location of these complexes. Similar results were obtained with IκBβ. As expected, IκBα-associated p65, or c-Rel (data not shown), was predominantly nuclear in LMB-treated cells (Fig. 1A, top row). Thus, cytoplasmic retention by IκBβ and IκBα may involve true sequestration rather than a balance between import and export as is the case with IκBα.

These observations were confirmed in mammalian cells by investigating the shuttling dynamics of endogenous Rel-IκB complexes. Endogenous proteins in mature B (A20) and mature T (D5 h3) cell lines (data not shown) were visualized by staining fixed, permeabilized cells with anti-IκBα, or anti-IκBβ, antibodies in the presence or absence of LMB to block nuclear export. In untreated cells both IκBα were predominantly cytoplasmic (Fig. 1B, left panel). A 1-h LMB treatment induced considerable nuclear translocation of IκBα but not IκBβ (Fig. 1B, right panel). Because most of the cellular IκB is associated with Rel proteins, we concluded that Rel-IκBα complexes shuttled continuously, but Rel-IκBβ complexes did not. Lack of Rel-IκBβ shuttling is consistent with sequestration being the major mechanism of cytoplasmic retention by IκBβ.

We found more direct evidence for differences in interaction between IκBα or IκBβ and p65 through partial proteolysis assays. p65 protein was expressed by transient transfection in BOSC 23 cells in the presence of HA-IκBα or Myc-IκBβ. The p65-IκB complex was immunoprecipitated from whole cell extract with anti-IκBα antibody or anti-IκBβ antibody and digested with different proteases. The p65 fragments were detected using antibodies directed against the N or C terminus of p65 to estimate the cut site from one or the other end of p65. Only two of seven proteases showed significant differences in the pattern of p65 fragments generated in the presence of IκBα or IκBβ.

p65 alone generated one major fragment when treated with Asp-N of ca. 28 kDa when assayed from the C terminus (Fig. 2, lanes 5 and 6); this corresponds to a cut site located 293 amino acids from the N terminus (Fig. 2, top). In the p65-IκBα complex, two bands of approximately equal intensity were seen (Fig. 2, lanes 1 and 2), whereas in the p65-IκBβ complex the
faster mobility (23 kDa) band was enhanced. Therefore, cutting at residue 293 was reduced in the p65-IxBα complex compared with p65 alone, allowing the detection of the cut site at residue 360 (which was not evident with p65 alone). This is presumably because of the protection of the p65 NLS by IxBα, which lies close to residue 293 between residues 301 and 304. Cutting at 293 was further inhibited in the p65-IxBβ complex as shown by a relative increase in the intensity of the 23-kDa compared with the 28-kDa band. These observation suggest that the region around residue 293, including the NLS, is more protected in the p65-IxBβ complex.

p65-IxBα and p65-IxBβ complexes were also probed using the protease Lys-C and p65 antibodies directed against the N terminus. Increased cutting at the residue 425 site was evident in the IxBβ complex compared with the IxBα complex (Fig. 2, lanes 8 and 11). These observations also support the interpretation that IxBα and IxBβ interact differently with p65. We suggest that the p65 NLS is better hidden by IxBβ than by IxBα.

The simplest interpretation of the experiments described above was that Rel-IxBβ complexes did not enter the nucleus because the nuclear localization sequences in both proteins were very effectively hidden in the complex. Therefore, the question of nuclear export did not arise. However, the question remained that if any Rel-IxB complexes formed in the nucleus, would IxBβ be able to bring the complex out to the cytoplasm? Such a situation may occur at the end of cell stimulation when Rel proteins are already nuclear and new IxBs are synthesized to terminate NF-κB-dependent gene expression. We addressed this question in a yeast model.

We have previously shown that export-dependent cytoplasmic localization of p65 by IxBα can be recapitulated in yeast (15). To test the properties of IxBβ, we coexpressed IxBβ and GFP-p65 from galactose-inducible promoters in wild type or Crm1p-deficient (crn1-1) yeast strains. GFP-p65 was located in the cytoplasm under these conditions in both strains (data not shown), correlating closely with the observations in mammalian cells (Fig. 1). In contrast, when GFP-p65 and Ixbα were coexpressed in crn1-1 cells, the complex remained in the nucleus (15). To compare the ability of IxBα and IxBβ to remove nuclear p65, we expressed GFP-p65 using a galactose-inducible promoter, followed by either Ixbα or Ixbβ, from a copper-inducible promoter. A 3-h induction with galactose was followed by growth in glucose to suppress GFP-p65 transcription. In cells that did not contain IxB expression vectors, GFP fluorescence was strictly nuclear. Even when cells contained either Ixbα or Ixbβ expression plasmids, GFP fluorescence was largely restricted to the nucleus, although whole cell expression was observed in ~15% of the cells (Fig. 3, middle and bottom rows, left panel). Cytoplasmic expression under these conditions was most likely due to basal Ixbα, or Ixbβ, expression from the copper-inducible promoter. Induction of Ixbα with copper for 2 h resulted in a significant redistribution of GFP-p65 to the cytoplasm, indicating that the newly synthesized Ixbα exported nuclear GFP-p65 to the cytoplasm (Fig. 3, middle row, right panel). This was mediated by Crm1p because it did not occur in the crn1-1 strain that contains a mutated

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**Fig. 2. Protease digestion of p65-IxBα and p65-IxBβ complexes.** p65 protein was transiently transfected in BOSC 23 cells alone (lane 1, 2, 7, and 8), in the presence of HA-IxBα (lane 3, 4, 9, and 10) or Myc-IxBβ (lane 5, 6, 11, and 12). Anti-IxBα or anti-IxBβ antibodies were used to immunoprecipitate the p65-IxB complex from whole cell extracts. Precipitated materials were digested with Asp-N (left panel, A) and Lys-C (right panel, L). Undigested samples are indicated by a “−” in the figures. Digested and undigested products were fractionated by SDS-polyacrylamide gel electrophoresis and detected using an antibody against the C terminus of p65 (left panel) or antibody against the N terminus of p65 (right panel). Relevant protease sites of p65 were predicted by MacVector version 6.0 (top panel). NLS represents the nuclear localization signal of p65, with critical residues between residues 301 and 304. Arrows (in the lower panels) indicate the relative degree of Lys-C or Asp-N cutting in the IxBβ/p65 and IxBα/p65 complexes.

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**Fig. 3. Sequential induction of GFP-p65 and IxB.** GFP-p65 was cloned into an expression plasmid with a galactose-inducible promoter. HA-tagged IxBα, or IxBβ, was cloned into an expression plasmid containing a copper-inducible promoter. Yeast strain W303 transformed with both GFP-p65 and HA-IxB expression plasmids was treated with galactose for 3 h to induce GFP-p65 expression (left panel). Half of the cells were then treated with glucose to terminate the expression of GFP-p65 followed by 0.75 mM copper sulfate to induce IxB expression (right panel). GFP fluorescence was visualized directly with fluorescence microscopy. Whole cell extracts were made from the cells to confirm the induction of GFP-p65, HA-IxBα, and HA-IxBβ proteins by immunoblotting (data not shown). Results shown are from one of three independent experiments.
CRM1 gene (data not shown). In contrast, there was little redistribution of GFP-p65 after secondary induction of IκBβ (Fig. 3, bottom row, right panel). The small increase in whole cell GFP-p65 expression was probably because of residual GFP-p65 translation during IκBβ induction, which resulted in its cytoplasmic sequestration. These observations indicate that IκBβ cannot remove nuclear Rel proteins to the cytoplasm.

DISCUSSION

We found that p65 or c-Rel associated with IκBβ or IκBe were retained in the cytoplasm, although these IκBs did not shuttle via the CRM1 pathway. We suggest that IκBs, unlike IκBa, sequester rather than shuttle Rel proteins, which implies that there is no available NLS in the Rel-IκBβ (or IκBe) complexes to induce nuclear entry. Conversely, Rel-IκBa complexes must have an available NLS to shuttle. We hypothesize that the Rel and not the IκB component provides the functional NLS of a Rel-IκB complex. Thus, IκBβ or IκBe must hide the Rel NLS more effectively than IκBa. Evidence in favor of this idea was obtained from partial proteolytic studies of p65-IκB complexes.

The sequestration mechanism is based on the lack of an effect of leptomycin B or a mutated CRM1 gene in Rel protein localization by IκBβ. Alternatively, these results could indicate that Rel-IκBβ complexes shuttled by a CRM1-independent pathway. To test this theory, we generated nuclear Rel-IκBβ complexes and determined whether they could reach the cytoplasm by an unidentified pathway. As shown in Fig. 3, IκBβ-mediated GFP-p65 export was inefficient compared with IκBa. We conclude that IκBβ is not an export chaperone like IκBa. Consequently, IκBβ cannot efficiently down-regulate nuclear Rel proteins to restore the resting state of the cell. These results highlight the functional differences between IκBa and IκBβ.

Cheng et al. (19) showed that substituting IκBβ for the IκBa gene compensated for the most obvious defects in IκBa−/− mice. They concluded that IκBa and IκBβ were functionally similar and that regulation of expression accounted for most of the phenotype of IκBa-deficient mice. Our contrasting conclusion regarding the mechanism of IκBa and IκBβ function is not at odds with the biological results. Clearly, if sufficient IκBβ is synthesized in a cell, it can retain Rel proteins in the cytoplasm, albeit by a mechanism different from IκBa. The biological results show that retention of Rel proteins by either mechanism is good enough to rescue lethality. That IκBβ is a less efficient nuclear export chaperone than IκBa may be manifest under conditions that were not directly assayed, such as during an immune response or chronic inflammation. We suggest that control of such situations may require the active export-dependent reduction of NF-κB activity.

REFERENCES

1. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–269
2. May, M. J., and Ghosh, S. (1997) Semin. Cancer Biol. 8, 63–73
3. Whiteside, S. T., and Israel, A. (1997) Semin. Cancer Biol. 8, 75–82
4. Clement, J. F., Rice, N. R., Car, B. D., Abbondanzo, S. J., Powers, G. D., Bhatt, P. H., Chen, C. H., Rosen, C. A., and Stewart, C. L. (1996) Mol. Cell. Biol. 16, 2341–2349
5. Beg, A. A., Sha, W. C., Bronson, R. T., and Baltimore, D. (1995) Genes Dev. 9, 2736–2746
6. Foo, S. Y., and Nolan, G. P. (1999) Trends Genet. 15, 229–235
7. Rayet, B., and Gelinas, C. (1999) Oncogene 18, 6938–6947
8. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) Genes Dev. 6, 1899–1913
9. Zabel, U., Henkel, T., Silva, M. S., and Barhaeuerle, P. A. (1993) EMBO J. 12, 201–211
10. Sachdev, S., Bagchi, S., Zhang, D. D., Mings, A. C., and Hannink, M. (2000) Mol. Cell. Biol. 20, 1571–1582
11. Sachdev, S., Hoffmann, A., and Hannink, M. (1998) Mol. Cell. Biol. 18, 2524–2534
12. Turpin, P., Hay, R. T., and Dargemont, C. (1999) J. Biol. Chem. 274, 6804–6812
13. Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) EMBO J. 18, 6682–6693
14. Huang, T. T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1014–1019
15. Tam, W. F., Lee, L. H., Davis, L., and Sen, R. (2000) Mol. Cell. Biol. 20, 2269–2284
16. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997) Science 278, 141–144
17. Prigent, M., Barlat, I., Langen, H., and Dargemont, C. (2000) J. Biol. Chem. 275, 36441–36449
18. Feske, S., Draeger, R., Peter, H. H., Eichmann, K., and Rao, A. (2000) J. Immunol. 165, 297–305
19. Cheng, J. D., Ryseck, R. P., Attar, R. M., Dambach, D., and Bravo, R. (1998) J. Exp. Med. 188, 1055–1062