p105-IκBγ and Prototypical IκBs Use a Similar Mechanism to Bind but a Different Mechanism to Regulate the Subcellular Localization of NF-κB*

Anu K. Moorthy and Gourisankar Ghosh‡

From the Department of Chemistry & Biochemistry, University of California at San Diego, La Jolla, California 92093-0359

Received for publication, July 25, 2002, and in revised form, October 22, 2002
Published, JBC Papers in Press, October 23, 2002, DOI 10.1074/jbc.M207515200

p105, also known as NF-κB1, is an atypical IκB molecule with a multi-domain organization distinct from other prototypical IκBs, like IκBo and IκBβ. To understand the mechanism by which p105 binds and inhibits NF-κB, we have used both p105 and its C-terminal inhibitory segment known as IκBγ for our study. We show here that one IκBγ molecule binds to NF-κB dimers wherein at least one NF-κB subunit is p50. We suggest that the obligatory p50 subunit in IκBγ-NF-κB complexes is equivalent to the N-terminal p30 segment in all p105-NF-κB complexes. The nuclear localization signal (NLS) of the obligatory p50 subunit is masked by IκBγ, whereas the NLS of the nonobligatory NF-κB subunit is exposed. Thus, the global binding mode of all IκB-NF-κB complexes seems to be similar where one obligatory (or specific) NF-κB subunit makes intimate contact with IκB and the nonobligatory (or nonspecific) subunit is bound primarily through its ability to dimerize. In the case of IκBα and IκBβ, the specific NF-κB subunit in the complex is p65. In contrast to IκBα-NF-κB complexes, where the exposed NLS of the nonspecific subunit imports the complex to the nucleus, p105-NF-κB and IκBγ-NF-κB complexes are cytoplasmic. We show that the death domain of p105 (also of IκBγ) is essential for the cytoplasmic sequestration of NF-κB by p105 and IκBγ. However, the death domain does not mask the exposed NLS of the complex. We also demonstrate that the death domain alone is not sufficient for cytoplasmic retention and instead functions only in conjunction with other parts in the three-dimensional scaffold formed by the association of the ankyrin repeat domain (ARD) and NF-κB dimer. We speculate that additional cytoplasmic protein(s) may sequester the entire p105-NF-κB complex by binding through the death domain and other segments, including the exposed NLS.

The NF-κB family of transcription factors plays an important role in a large number of cellular processes including immune response and inflammation, cellular development, and differentiation (1–4). This family comprises of five distinct members, p50, p65, p52, Rel-R, and ResB, that exhibit a high degree of sequence homology at their N termini. This region, known as the Rel homology region, is responsible for important functions like DNA binding, dimer formation, nuclear localization, and IκB binding (1–4).

NF-κB dimers are regulated by inhibitor IκB proteins, which include IκBα, IκBβ, IκBε, IκBγ, p105 (NF-κB1), p100 (NF-κB2), Bcl-3, IκBγ, and neural (4–21). In most cells NF-κB remains inactive as a complex with IκB. In response to a variety of extracellular signals, the IκB molecule is phosphorylated by IκB kinases, which leads to the ubiquitination and subsequent degradation of IκB by the proteosome machinery within the cell (22).

It has long been thought that all IκB proteins inhibit NF-κB by masking the nuclear localization signal (NLS) of NF-κB and thereby sequestering them in the cytoplasm (1–4, 22). However, recent studies have indicated that the IκB proteins differ with regard to their regulation of NF-κB subcellular localization. The IκBα-NF-κB complex exhibits dynamic shuttling between the cytoplasm and the nucleus (23–27). However, despite its transient presence in the nucleus, NF-κB remains bound to IκBα, and this prevents DNA binding and activation of transcription. IκBβ, on the other hand, sequesters NF-κB in the cytoplasm of resting cells (26–28). The detailed mechanism of this process is not clear. It has been suggested that other ancillary proteins may be required for this function.2

p105 and p100 are the precursors of NF-κB subunits p50 and p52, respectively, which are located in their N termini. Both p105 and p100 have similar structural organizations (3) (Fig. 1). The central portion of these molecules has a glycine-rich region that has been shown to play a critical role in processing of the precursor (29, 30). The C termini resemble other IκB molecules that possess ankyrin repeats (AR) (14, 15, 31). These two proteins also contain a death domain immediately C-terminal to the ankyrin repeat domain (ARD). The IκB kinase phosphorylation sites are located further downstream within a region called the destruction box (see Fig. 1). A separate gene also encodes the C-terminal part of p105. This gene product, known as IκBγ, has been shown to exist only in certain cell types, like mouse pre-B cells (12) (Fig. 1).

In resting cells, p105 is partially processed, generating p50. The exact mechanism of this limited processing event is not known, although co-translational processing events have been proposed (32, 33). The unprocessed p105 functions as an inhibitor molecule and nonspecifically inhibits almost all NF-κB

---

1. The abbreviations used are: NLS, nuclear localization signal; AR, ankyrin repeat; ARD, ankyrin repeat domain; LMB, leptomycin B; HA, hemagglutinin; PBS, phosphate-buffered saline.

2. S. Malek, Y. Chen, and G. Ghosh, unpublished observation.
subunits, including p50 (34–36). An earlier study has shown that p105 retains itself, as well as other NF-κB molecules, in the cytoplasm (37). The mechanism of inhibition is, however, still unclear. The p105 molecule, like the classical IκB inhibitors, can also undergo complete degradation in response to signals and the sequential action of IκB kinases, ubiquitin ligases, and the 26S proteasome (38–41). Both the death domain and the destruction box have been shown to be important for IκB kinase phosphorylation (41, 42). In contrast to p105, the mechanism of p100 processing is very different. In resting cells, most of p100 remains unprocessed, and in response to appropriate signals all of it gets processed into p52 (43, 44).

In this study, we address the functional properties of p105 by asking the following questions. How does p105 regulate subcellular distribution of NF-κB subunits? How does p105 nonspecifically inhibit other NF-κB proteins? What is the relationship between p105-NF-κB and IκB-NF-κB complexes? We find that although IκBx resembles other IκBs in exhibiting a 1:1 stoichiometry of binding to NF-κB dimers, it has a unique specificity for NF-κB dimers that contain at least one p50 subunit. This obligatory p50 subunit in IκBx-NF-κB complexes is structurally equivalent to the N-terminal p50 segment of p105 in p105-NF-κB complexes. Interestingly, although IκBx fails to mask one NF-κB NLS, it still retains NF-κB in the cytoplasm. We observe that the death domain of p105 is necessary but not sufficient for this cytosolic retention. We suggest that p105 and IκBx inhibitors may require an as yet unknown cellular factor(s) to sequester NF-κB in the cytoplasm.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification from Escherichia coli—The cloning, expression, and purification of the NF-κB subunits has been described previously (45, 46). Full-length and truncated glutathione S-transferase-IκBα were made by cloning into the pGEX-2T vector (Amersham Biosciences). The fusion protein was expressed in E. coli BL21 DE3 and purified by glutathione-agarose column chromatography following the manufacturer’s protocol (Amersham Biosciences).

Native Polyacrylamide Gel Electrophoresis—Proteins and protein complexes were diluted in 10 mM Tris (pH 7.5), 200 mM NaCl, 4% glycerol, and 2 mM β-mercaptoethanol. The reactions were allowed to equilibrate at room temperature for 1 h. Native gel loading dye (50 mM Tris, pH 7.5, 0.1% bromphenol blue, 10% glycerol, and 1.25 mM β-mercaptoethanol) was then added to each sample. 10% native polyacrylamide gels were prepared with 0.25× Tris-borate-EDTA buffer. The samples were loaded on the gel and run in Tris-borate-EDTA buffer for 2 h at a constant current (3 mA). The protein bands were visualized by Coomassie staining.

Fluorescence Polarization Competition Assay—Fluorescence polarization competition assays were done as described previously (47). Briefly, varying concentrations of IκB were mixed with constant amounts of p50 homodimer pre-equilibrated with fluorescein-labeled DNA. The competition assay binding curves were analyzed for IC50 values, defined as the concentration of IκB at 0.5 fractional occupancy.

Plasmids, Cell Culture, and Transfections—DNAs encoding full-length p50 and full-length truncated p105 were cloned into a pDNA vector (Invitrogen) containing either an N-terminal FLAG (p50 constructs) or an N-terminal HA (p105-IκBα constructs) tag. HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics and transfected with plasmid DNA using the Lipofectamine Plus reagent (Invitrogen). Protein expression was checked by Western blot.

Immunofluorescence—HeLa cells were grown on 8-well chamber slides (Lab Tek). The cells were transfected with a total of 0.2 μg of plasmid DNA. After 24 h, the cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were then permeabilized with 0.25% Nonidet P-40 in PBS for 1 min and blocked with 5 mg/ml bovine serum albumin in PBS containing 0.1% Tween 20 at room temperature for 30 min. Fluorescent detection was done by incubating the cells with monoclonal antibody 12CA5 (against HA), M2 (against FLAG), and H-286 (against p65) in PBS supplemented with 0.1% Tween 20 at room temperature for 30 min. The cells were washed three times with buffer containing 0.2% Nonidet P-40 in PBS and incubated with fluorescein-labeled secondary antibody at room temperature for 1 h. Finally, the cells were washed three times with buffer containing 0.1% Tween 20 in PBS, and the slide was mounted with Vectashield (Vector Laboratories).

Immunoprecipitation—For immunoprecipitation from whole cell lysates, HeLa cells were harvested 24 h post-transfection and lysed with buffer containing 20 mM Tris (pH 7.5), 0.2 M NaCl, 1% Triton X-100, 1 mM EDTA, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma). Immuneiprecipitation was carried out using the protein A pull-down method. Briefly, 0.1–0.5 mg equivalent of protein from transfected cell extract was diluted with 300 μl of lysis buffer. The appropriate antibodies were added to the extract and incubated overnight with protein A-Sepharose 4B beads (Sigma) at 4 °C. The beads were pulled down by brief centrifugation and washed three times with the lysis buffer. The immunoprecipitates were then eluted from the beads with 2× Laemmli buffer devoid of β-mercaptoethanol by heating at 95 °C for 5 min. The bound proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The bound proteins were then identified by Western blot. In vitro immuno-
precipitation was carried out with a similar protocol using purified E. coli proteins in place of whole cell extract.

RESULTS

Interactions between NF-κB Dimers and IkBγ—Unprocessed p105 functions as an inhibitor of NF-κB, although its mode of inhibition appears to be quite different from the prototypical IkB proteins such as IkBa and IkBβ. As opposed to IkBa and IkBβ that bind through p65, p105 inhibits all NF-κB members nonspecifically. To understand the biochemical basis of NF-κB inhibition by p105, we wanted to first test how the p105 C terminus (IkBγ) binds to NF-κB dimers. We have characterized the interactions between IkBγ and various NF-κB dimers by protein-protein gel shift assays under native conditions. To simplify the assay we generated a truncated IkBγ containing only the ARD fused to a poly-His peptide. This construct will be referred to as ARD-IkBγΔC. We observe that ARD-IkBγΔC binds strongly to the p50-p50 homodimer and p50-p65 heterodimer but only weakly to the p65-p65 and c-Rel-c-Rel homodimers (Fig. 2A). Although no stable complexes are formed between IkBγ and p65-p65 or c-Rel-c-Rel homodimers, we do not observe free IkBγ in these lanes. It is likely that these complexes are weaker and smear during electrophoresis. Based on these results we suggest that complexes between IkBγ and non-p50 containing NF-κB dimers are highly unstable.

The fact that p105 binds to all NF-κB proteins and IkBγ binds to the p50 dimers (any NF-κB dimer that contains at least one p50 subunit) suggests that the required p50 subunit in IkBγ-NF-κB complexes is likely the N-terminal p50 segment of p105 in p105-NF-κB complexes. Thus, the p105-p50 complex is...
The binding data presented above suggest that IkB prefers p50 containing NF-κB dimers. However, these experiments do not reveal whether one or two molecules of IkB bind to one molecule of NF-κB dimer. This is important considering that Bcl-3, which contains seven AR like IkB domains, has been proposed to bind to p50 homodimers in a 2:1 molar ratio (48–50). To elucidate IkB-NF-κB binding stoichiometry, we have used two different IkBγ constructs, one is the full-length IkBγ expressed as a glutathione S-transferase fusion protein (FL-IkBγ), and the other is the previously described ARD-IkBγΔC. As seen in Fig. 2C (lanes 4 and 5), both of these protein bind p50 p50 homodimer efficiently, and the resulting complex migrates to different positions in the native gel. In a reaction mixture containing both IkBγ and the p50 homodimer, binding of two molecules of IkBγ to the homodimer would result in a distinct, additional complex composed of these IkBγ molecules of different lengths and p50. However, when equivalent amounts of FL-IkBγ, ARD-IkBγΔC, and p50-p50 homodimer were mixed, only two distinct complexes, one corresponding to the FL-IkBγ-p50-p50 complex and the other to the ARD-IkBγΔC-p50-p50 complex, were seen (Fig. 2C, lane 6). Thus, we can conclude that one molecule of IkBγ binds to one p50-p50 homodimer.

The Role of the NF-κB NLS in IkB Binding—The presence of one free NLS is a prime reason for the nucleocytoplasmic shuttling of the IkBα-NF-κB complex. To elucidate the status of the NF-κB NLS(+) in its complex with IkBγ, p50-p50 homodimers of different lengths, both with and without the NLSs, were prepared, and their binding to ARD-IkBγΔC was tested using native gel shift assays. Fig. 3A shows that all three of the p50-p50 dimers were capable of binding to ARD-IkBγΔC, although the dimer with both NLSs deleted (p50-350) seems to bind IkBγ relatively weakly (Fig. 3A, lanes 5–7). This suggests that IkBγ may not use the NLS sequence for p50 binding, or at least this region does not seem to contribute significantly. Similar binding patterns were also observed with FL-IkBγ (data not shown).

Co-immunoprecipitation experiments were performed to further confirm the presence of at least one free NLS in the IkBγ-NF-κB complex. If the p65 NLS in the IkBγ-p50-p50 complex were free, then anti-p65 NLS antibody would interact with the p65 NLS. Here, the p50-p50 heterodimer was used because of the availability of the p65 NLS-specific monoclonal antibody. Free NF-κB p50-p50 heterodimer and the IkBγ-p50-p50 heterodimer complex were incubated with p65 NLS antibody. It was seen that the antibody was able to pull down NF-κB in both the free and IkBγ complexed form (Fig. 3B, top panel). This demonstrates that the p65 NLS in the p50-p50 heterodimer is not protected by IkBγ. As a parallel control, the IkBα-p50-p50 complex was also tested. The p65 NLS antibody was unable to pull down NF-κB in the IkBα-p50-p50 complex (Fig. 3B, bottom panel). This corroborates with earlier results that have shown that the p65 NLS is masked in the
IxBp50-p65 complexes (26, 27, 52, 53). Thus, these experiments show that at least one NF-κB NLS remains unmasked in the IxBp50-NF-κB complex.

**IxBp50-NF-κB and p105-NF-κB Complexes Are Cytoplasmic**—The above observation that at least one NF-κB NLS is free in the IxBp50-NF-κB complex suggests that these complexes should localize to the nucleus. The subcellular distribution of the complex was tested by simultaneously transfecting HeLa cells with IxB, p50, and p65. Immunostaining shows that free p50 and p65, which are most likely to be present as a heterodimer, are nuclear (Fig. 4A, left panel), but co-expression of IxBp50 leads to cytoplasmic retention (Fig. 4A, right panel). Thus, although the IxBp50-p65 has at least one NLS free, the complex does not localize to the nucleus.

The subcellular distribution of p105 and IxB complexes with p50 was also tested. Immunofluorescence studies show both these complexes to be cytoplasmic (Fig. 4B). Free p50, as expected, is localized in the nucleus (data not shown).

To further test the ability of these complexes to be retained in the cytoplasm, a mutant p50 with an additional NLS (p50-NLS) was made. Cells co-transfected with p50-NLS and p105 or IxBp50 reveal that the complexes are retained in the cytoplasm (Fig. 4C, left panels). To test whether the p105-p50-NLS complex shuttles between the nucleus and cytoplasm, HeLa cells were treated with the nuclear export inhibitor leptomycin B (LMB), and the subsequent changes in localization were monitored. LMB treatment does not appear to alter the localization of the p105-p50-NLS or the IxBp50-NLS complexes (Fig. 4C, right panels), implying that these complexes do not shuttle. This result is in contrast to that observed for IxBp50-NF-κB complexes where addition of the inhibitor confines both proteins within the nucleus (26). Thus, the p105-NF-κB complexes seem to be similar to the IxBp50-NF-κB complexes, which are primarily cytoplasmic. Free p50-NLS, as expected, is localized within the nucleus (data not shown).

**The Death Domain Is Necessary for Cytoplasmic Retention of NF-κB**—To assess the possible role of the death domain and the C-terminal tail containing the destruction box in the cytoplasmic retention of NF-κBs, deletion mutants of both p105 and IxBp50 were constructed. HeLa cells were co-transfected with p50 and various truncated forms of p105 or IxBp50. Subcellular localization of these complexes was monitored by immunofluorescence. As mentioned in the previous results section, the p105-p50 complex is cytoplasmic (Fig. 5A, left panel). Removal of the last ~100 residues, which includes the destruction box, p105ΔDB, did not alter the localization of the complex (Fig. 5A, right panel). However, deletion of the C-terminal 171 residues (encompassing the death domain), p105ΔC, localizes the complex exclusively in the nucleus (Fig. 5A, middle panel). These results suggest that the death domain of p105-IxBp50 plays a role in the retention of NF-κB complexes within the cytoplasm.
p50 is again retained in the cytoplasm when co-expressed with a truncated p105, p105ΔN (Fig. 1), where the N-terminal, DNA-binding immunoglobulin domain (residues 1–244) is deleted (Fig. 5B, left panel). However, when both the N- and C-terminal domains (p105ΔNΔC) are deleted, these proteins localize to the nucleus (Fig. 5B, right panel). Similar results were obtained with various deletion mutants of IκBγ. Co-expression of full-length IκBγ and p50 lead to cytoplasmic retention of the complex (Fig. 5C, left panel). Deletion of the C terminus, IκBγΔC, leads to nuclear localization of both proteins (Fig. 5C, right panel). Also, a shorter construct of IκBγ comprising the ARD and all residues downstream, ARD-IκBγ (Fig. 1) and the corresponding C terminally deleted form, ARD-IκBγΔC, showed a similar retention pattern (Fig. 5D).

It is known that NF-κB dimers are nuclear proteins, and it is only in complex with IκB that they are retained in the cytoplasm. As described above, co-expression of p50 and death domain-deleted p105 or IκBγ leads to nuclear localization of both components. To test whether these proteins localize independently or are present as a complex in the nucleus, co-immunoprecipitation experiments were done. FLAG antibody (M2) was used to immunoprecipitate FLAG-tagged p50 from whole cell lysates that had been co-transfected with FLAG-p50 and HA-p105 or HA-IκBγ. Subsequent Western blotting was done using the HA antibody. The results clearly show that
these proteins form a complex because the FLAG antibody was able to pull down both p50 and p105 (or IkBγ) (Fig. 5E). In all, the above experiments suggest that the death domain of p105-IκBγ is necessary for cytoplasmic retention.

The Death Domain Is Not Sufficient for Cytoplasmic Retention—Next, we wanted to test whether the death domain was the exclusive retention signal in these IκB molecules. Full-length IkBγ when expressed in HeLa cells exhibits a nucleocytoplasmic distribution (Fig. 6A, left panel). The death domain-truncated IkBγΔC, as expected, was localized within the nucleus (Fig. 6A, right panel). Both ARD-IκBγ and ARD-IκBγΔC, by themselves, were present in the nucleus (Fig. 6B). Thus, the presence of the death domain is not sufficient for cytoplasmic localization.

To further test the role of the death domain, two more constructs were made. One containing only the death domain, p105 (800–887), and the other with all the segments further downstream, p105 (800–971). Both of these proteins localized within the nucleus (Fig. 6C). This suggests that neither the death domain by itself nor its presence in the IkBγ molecule provides the retention signal.

The Death Domain Does Not Participate in NF-κB Binding—Because the death domain of p105-IκBγ seems to be important for cytoplasmic localization of NF-κB, a possible model of the complex could be one in which the death domain binds and masks the exposed NLS of NF-κB, thereby preventing nuclear import. If this were true, then full-length IkBγ must interact with the p50-p50 homodimer with higher affinity than the death domain-truncated IkBγ. Although the native gel shift assays showed that the death domain was not essential for NF-κB binding, these experiments were of a qualitative nature. Fluorescence polarization competition assays were done to determine the binding affinity of NF-κB for both full-length and death domain-truncated IkBγ. In a solution-based competition assay, fixed amounts of fluorescein-labeled κB DNA bound to the p50-p50 dimer was incubated with increasing amounts of IkBγ. In this experiment, the IkBγ-dependent dissociation of the p50-p50 dimer from DNA is accompanied by a distinct change in fluorescence polarization. The DNA binding inhibition constant at equilibrium was then calculated. As shown in Fig. 7, both IkBγs inhibit NF-κB DNA binding to a similar extent. Thus, the death domain does not seem to play a major role in NF-κB binding to IkBγ.

Limited Processing of p105: a Possible Model of the Cytoplasmic Complex—In addition to testing the localization of p50 complexes with the full-length and truncated forms of p105, we have also expressed just p105 and its truncated forms in HeLa cells and tested their individual subcellular distribution. p105 is present exclusively in the cytoplasm (Fig. 8A, left panel), whereas the C-terminally truncated p105, p105ΔC, was exclusively nuclear (Fig. 8A, right panel). The N-terminally truncated p105, p105ΔN, also localized to the cytoplasm (Fig. 8B, left panel). The corresponding C-terminal deleted p105, p105ΔNC, was nuclear (Fig. 8B, right panel). Localization patterns of p105 and its derivatives are thus identical to that observed previously for the p105-p50 complexes in HeLa cells, expressing both p105 (or its derivatives) and p50.

p105 is known to undergo limited processing to generate p50 molecules. To test whether the other truncated forms of p105 were also capable of similar processing, Western analysis was done using HA monoclonal antibody. We observe that like full-length p105, its truncated forms were also processed in a limited manner within the cell. The appearance of an additional band of lower molecular weight indicates a processed band, derived from the C-terminal processing of the intact protein. The lengths of the processed products vary according to the truncations of p105. Cells transfected with the C-terminally truncated form, p105ΔC, show a processed band corre-
Regulation of Localization of NF-κBs by p105 and IκBγ

Fig. 8. Subcellular localization of full-length and N-terminally truncated p105. A, transfection of full-length p105 leads to cytoplasmic localization of the protein (left panel). Transfection of death domain- and destruction box-truncated p105 (p105ΔC) leads to nuclear accumulation of the protein (right panel). B, transfection of N-terminally truncated p105, p105ΔN, leads to cytoplasmic localization of the protein (left panel). Transfection of death domain and destruction box truncated p105 (p105ΔNΔC) leads to nuclear accumulation of the protein (right panel). C, processing of p105 in the presence and absence of co-expressed p50. Western blot showing the processing of various p105 mutants. HeLa cells were transfected with C-terminally (p105ΔC), N-terminally (p105ΔN), and both C- and N-terminally (p105ΔNΔC) truncated p105 in the presence and absence of p50. As seen, all three mutants undergo processing (lanes 1–3), and this is abrogated in the presence of co-expressed p50 (lanes 4–6). D, N-terminally deleted p50 binding by ARD-IκBγΔC. Native gel mobility shift assay of ARD-IκBγΔC binding to N-terminally deleted p50 dimers. Lane 1, ARD-IκBγΔC; lane 2, p50ΔN-376; lane 3, p50ΔN-363; lane 4, ARD-IκBγΔC+p50ΔN-376; lane 5, ARD-IκBγΔC+p50ΔN-363. p50ΔN-376 and p50ΔN-363 represent p50 (245–376) and p50 (245–363), respectively. The free and complexed proteins are indicated by arrows.

Earlier studies using competition assays have shown that IκBγ preferentially inhibits p50-50 homodimer (18, 31, 51). In the present study, we have performed direct binding assays to demonstrate that IκBγ interacts stably with both p50-65 and p50-50 dimers but not with p65-65 or c-Rel-c-Rel dimers. This suggests that IκBγ may have a preference for p50 containing NF-κB dimers. We and others have previously shown that IκB interacts strongly with p65 containing dimers (23, 24, 26). The x-ray structure of IκBα-p50-p65 heterodimer showed that the p65 subunit makes extensive contacts with IκBα (52, 53). Thus, a central theme seems to govern all IκB-NF-κB binding wherein the stability of the interaction is dictated by the association of an IκB molecule with one specific NF-κB subunit. The second subunit, which we refer to as the nonspecific subunit, associates with the specific subunit through its dimerization domain. This is illustrated in Fig. 9A. The nature of the specific subunit is directly correlated to the nature of the binding IκB molecule. For IκBα, p65 serves as the specific subunit. IκBβ also exhibits similar dimer specificity, requiring p65 as the primary binding partner. On the other hand, in IκBγ, the specific subunit appears to be p50. In p105-NF-κB complexes, by the very nature of the molecule, the docking occurs intramolecularly with the N-terminal p50 arm serving as the specific subunit. It is possible that in IκB-NF-κB complexes the nonspecific subunit can be exchanged with other nonspecific subunits, and this may dictate the cellular pool of active NF-κB.

Sequesteration of NF-κB in the cytoplasm can be considered to be one of the most simplistic ways by which IκB molecules exhibit their inhibitory function. They can do this by blocking
the NLS of NF-κB dimers. However, both IκBα and IκBβ, upon binding to the specific NF-κB dimers, mask only the NLS of the specific NF-κB subunit. As expected therefore, IκBα-NF-κB and IκBβ-NF-κB complexes are not truly cytoplasmic but dynamic nucleocytoplasmic (23–26). We observe that in the IκBγ-p50-p65 complex the p65 NLS is free. Although no structural evidence exists to confirm that the NLS of the specific subunit is completely masked, two lines of evidence indicate this to be most likely. First, we show that when both NLSs are removed the affinity of the p50-p50 homodimer for IκBγ appears to be reduced, and second, we show that the NLS of the nonspecific p65 subunit of the IκBγ-p50-p65 complex is free. Therefore, reduced binding affinity of p50-p50 homodimer for IκBγ is likely due to the removal of the NLS of the p50 subunit that makes specific contacts with IκBγ.

The domain architecture of the C-terminal inhibitory domain of p105 is different from that of IκBα and IκBβ. p105 does not contain a PEST sequence in its C-terminal tail as in IκBα and IκBβ, but it does contain a death domain. The PEST sequence in IκBα and IκBβ is important for NF-κB binding. In case of IκBγ, we observe that the death domain does not play any role in NF-κB binding, suggesting that this domain is not involved in masking the exposed NLS of the nonspecific NF-κB subunit in p105-NF-κB or IκBγ-NF-κB complexes. The death domain,
Regulation of Localization of NF-κB by p105 and IκBγ

however, seems critical for cytoplasmic retention. When this domain is removed from p105-IκBγ, the subcellular localization of the truncated molecules in their free form or their complexes with NF-κB change completely, from cytoplasmic to nuclear. This observation is consistent with earlier studies showing that removal of C-terminal 191 residues alters the localization from the cytoplasm to the nucleus (37). However, although this clearly suggested the role of the C-terminal domain of p105, this large truncation also removed most of the last ARD. Therefore, it was not clear whether removal of part of the ARD contributed to altered localization of p105. Interestingly, the death domain, by itself, does not contain necessarily the exact same mechanism. Thus, a bridging factor, which requires the death domain and the NF-κB scaffold, could be involved in masking the NLS. The death domain has a putative, cytoplasmic protein that leads to its docking. This could then explain the inability of the p105-NF-κB or the IκBγ-NF-κB complex to move to the nucleus even with an additional NLS. We conclude that the IκB family proteins employ diverse mechanisms for cytoplasmic sequestration of NF-κB dimers. Elucidating each of these mechanisms in detail is imperative for understanding their cellular function and participation in unique signal transduction pathways.

Acknowledgments—We thank Prof. M. Yoshida at the University of Tokyo for providing LMB. We acknowledge Amanda Fusco for aid in performing the fluorescence polarization experiment (Fig. 7) and Tom Huxford and Rashmi Talwar for critically reading the manuscript.

REFERENCES

1. Baerle, P. A., and Baltimore, D. (1996) Cell 87, 13–20
2. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
3. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 255–260
4. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) Genes Dev. 9, 2723–2735
5. Whiteside, S. T., and Israel, A. (1997) Semin. Cancer Biol. 8, 75–82
6. Baerle, P. A., and Baltimore, D. (1988) Science 242, 540–546
7. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mundal, K., Ralph, P., and Baldwin, A. S., Jr. (1991) Cell 65, 1291–1298
8. Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995) Cell 80, 573–582
9. Simonidis, S., Liang, S. H., Alyn, L., and Thanos, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14372–14377
10. Whiteside, S. T., Epatin, J. C., Rice, N. R., and Israel, A. (1997) EMBO J. 16, 1413–1426
11. Li, Z., and Nabel, G. J. (1997) Mol. Cell. Biol. 17, 6184–6190
12. Inoue, J., Kerr, L. D., Kaczikaiz, A., and Verma, I. M. (1992) Cell 68, 1109–1120
13. Lipovsky, S., Schmid, R. M., Perkins, N. D., Meltzer, P., Altbehr, M. B., McPherson, J. D., Wasmuth, J. J., and Nabel, G. J. (1992) Genomics 13, 287–292
14. Bourj, V., Burd, P. R., Brown, K., Villalobos, J., Park, S., Byeast, R. P., Bravo, R., Kelly, K., and Siebenlist, U. (1992) Mol. Cell. Biol. 12, 685–695
15. Mercurio, F., Didonato, J., Rosette, C., and Karin, M. (1992) DNA Cell Biol. 11, 523–537
16. Ghosh, S., Gifford, A. M., Liu, A., and Nolan, G. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2489–2493
17. Kieran, M., Blank, V., Logeat, F., Van deckerkrohe, J., Lottspeich, F., Bai, L., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israel, A. (1993) Cell 62, 1007–1018
18. Hatada, E. N., Nieters, A., Wulczyn, F. G., Naumann, M., Meyer, R., Nucifora, G., McKeithan, T. W., and Scheidereit, C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5289–5293
19. Wulczyn, F. G., Naumann, M., and Scheidereit, C. (1992) Nature 358, 597–599
20. Yamasaki, S., Muta, T., and Takegaki, K. (1991) J. Biol. Chem. 266, 26577–26582
21. Kitamura, H., Kanai, K., Otake, K., Morimatsu, M., and Saito, M. (2000) FEMS Lett. 485, 53–56
22. Karam, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
23. Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) EMBO J. 18, 6682–6693
24. Tam, W. F., Lee, L. H., Davis, L., and Sen, R. (2000) Mol. Cell. Biol. 20, 2286–2294
25. Huang, T. T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1014–1019
26. Malek, S., Chen, Y., Huxford, T., and Ghosh, G. (2001) J. Biol. Chem. 276, 42225–42235
27. Ghosh, S., and Karin, M. (2002) Cell 109, suppl. S81–S86
28. Tam, W. F., and Sen, R. (2001) J. Biol. Chem. 276, 7701–7704
29. Lin, L., and Ghosh, S. (1998) Mol. Cell. Biol. 16, 2248–2254
30. Oriain, A., Schwartz, A. L., Israel, A., Whiteside, S., Kahana, C., and Cheah-nover, A. (1999) Mol. Cell. Biol. 19, 3664–3673
31. Liu, H. C., Noland, G. P., Ghosh, S., Fujita, T., and Baltimore, D. (1992) EMBO J. 11, 3093–3099
32. Lin, L., DeMartino, G. N., and Greene, W. C. (1998) Cell 92, 819–828
33. Lin, L., DeMartino, G. N., and Greene, W. C. (2000) EMBO J. 19, 4712–4722
34. Rice, N. R., MacKibben, M. L., and Israel, A. (1992) Cell 71, 245–253
35. Naumann, M., Wulczyn, F. G., and Scheidereit, C. (1993) EMBO J. 12, 213–222
36. Mercurio, F., Didonato, J. A., Rosette, C., and Karin, M. (1993) Genes Dev. 7, 705-718
37. Henkel, T., Zabel, U., van Zee, K., Muller, J. M., Fanning, E., and Baeuerle, P. A. (1992) Cell 68, 1112–1123
38. Heissmeyer, V., Krappmann, D., Hatada, E. N., and Scheidereit, C. (2001) Mol. Cell. Biol. 21, 1024–1035
39. Oriain, A., Gonen, H., Bercovich, B., Fajerman, I., Eytan, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A. L., and Chuehanover, A. (2000) EMBO J. 19, 2580–2591
40. Chechanover, A., Gonen, H., Bercovich, B., Cohen, S., Fajerman, I., Israel, A., Mercurio, F., Kahana, C., Schwartz, A. L., Iwai, K., and Oriain, A. (2001) Biochimie (Paris) 83, 341–349
41. Cohen, S., Oriain, A., and Chechanover, A. (2001) J. Biol. Chem. 276, 26765–26776
42. Beinke, S., Belich, M. P., and Ley, S. C. (2002) J. Biol. Chem. 277, 24162–24168
43. Betta, J. C., and Nabel, G. J. (1996) Mol. Cell. Biol. 16, 6363–6371
Regulation of Localization of NF-κBs by p105 and IκBγ

44. Xiao, G., Harhaj, E. W., and Sun, S. C. (2001) *Mol. Cell.* 7, 401–409
45. Chen, F., Kempiak, S., Huang, D., Phelps, C., and Ghosh, G. (1999) *Protein Eng.* 12, 423–428
46. Chen, F. E., Huang, D. B., Chen, Y. Q., and Ghosh, G. (1998) *Nature* 391, 410–413
47. Phelps, C. B., Sengchanthalangsy, L. L., Huxford, T., and Ghosh, G. (2000) *J. Biol. Chem.* 275, 29840–29846
48. Michel, F., Soler-Lopez, M., Petosa, C., Cramer, P., Siebenlist, U., and Muller, C. W. (2001) *EMBO J.* 20, 6180–6190
49. Wulczyn, F. G., Naumann, M., and Scheidereit, C. (1992) *Nature* 358, 597–599
50. Bundy, D. L., and McKeithan, T. W. (1997) *J. Biol. Chem.* 272, 33132–33139
51. Matthews, J. R., Watson, E., Buckley, S., and Hay, R. T. (1993) *Nucleic Acids Res.* 21, 4516–4523
52. Huxford, T., Huang, D. B., Malek, S., and Ghosh, G. (1998) *Cell* 95, 759–770
53. Jacobs, M. D., and Harrison, S. C. (1998) *Cell* 95, 749–758
54. Weber, C. H., and Vincenz, C. (2001) *Trends Biochem. Sci.* 26, 475–481
