REVIEW ARTICLE

The inducible transcription factor NF-κB: structure–function relationship of its protein subunits

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INTRODUCTION

At the level of DNA, gene regulation is governed by *cis* regulatory elements (for a recent review, see Roeder, 1991). Most important for the control of transcription initiation are promoter elements that serve to provide an oriented entry site for DNA-dependent RNA polymerases. In contrast to prokaryotes, eukaryotic polymerases require several additional polypeptides binding in close proximity to the promoter in order to allow transcription initiation. Polymerase II (Pol II), which transcribes most genes in eukaryotes, needs promoter-bound TATA (TF-IID) or initiator binding proteins. A multitude of accessory factors, including TF-IID-associated factors (TAFs), TF-IIA, TF-IIB, TF-IIE and TF-IIF, is required for assembly of a functional transcription initiation complex containing Pol II. Finally, additional proteins binding upstream from TATA and initiator elements can improve the efficacy of a given promoter. Intensive research is going on in order to characterize functionally and structurally the transcription factors required in eukaryotic cells for the initiation of basic transcription.

Despite their complexity, core promoter elements usually provide very little specific regulatory information. Specific regulatory programs are conferred to genes by additional *cis*-regulatory elements, called enhancers. These are frequently found upstream from promoter elements but also in introns or downstream of genes. Enhancers can dramatically enhance the activity of promoters. The relative positional flexibility of enhancers with respect to the invariable position of a promoter might have its basis in the flexibility of DNA segments looping out between physically interacting enhancer- and promoter-binding proteins.

A great diversity of specific DNA-binding proteins are responsible for the specific regulatory potential of upstream promoter and enhancer elements. They can be grouped into transcription factors with activating or repressing potential. Some factors can display both activities. In addition, there are transcription factors that seem to serve an accessory role in sustaining and thereby controlling the effects of activators and repressors. The gene regulatory potential is further augmented by combination of multiple factor binding sites within enhancers and upstream promoters.

Gene regulatory programs are governed by the activity of transcription regulatory proteins. Among the various strategies that have evolved to control transcription factor activity, a common one is the *de novo* synthesis of a transcription factor. But this strategy calls for yet another factor(s) to turn on the gene. This leads into gene networks and hierarchies of transcription factors, a theme frequently exploited during differentiation and determination processes. For rapid gene induction in response to environmental signals, many post-translational modes of transcription factor activation have evolved. The control of DNA binding via association of transcription factors with small diffusible ligands is a common theme in prokaryotes and, in eukaryotes, is the basis of how the many members of the steroid hormone receptor superfamily are activated. Another widespread mechanism is covalent modification of factors, for instance, by addition or removal of phosphoryl groups (reviewed by Hunter and Karin, 1992). Recently, the role of accessory proteins in gene regulation has received great attention (reviewed by Shaw, 1990). A paradigm is serum response factor (SRF), a transcription regulatory protein conferring to genes responsiveness to serum stimulation of cells. SRF is dependent in this property on a second polypeptide, called ternary complex factor, that associates with SRF and a DNA sequence adjacent to the SRF-binding motif. While this is a nuclear event, other transcription factors can comprise the cytoplasmic compartment for their process of activation (reviewed in Schmitz et al., 1991). This allows the factor to participate actively in cytoplasmic/nuclear signalling. Examples are the glucocorticoid receptor (Muller and Renkawitz, 1991), IGSF-3 (Levy et al., 1989) and NF-AT (Crabtree, 1989). A particularly well-studied system in which the activation of a specific transcription factor requires derepression of DNA binding and inducible nuclear uptake is NF-κB. The activity of this factor is controlled by at least three functionally distinct protein subunits. Previous reviews have covered the physiology of NF-κB and its relationship to structurally homologous proteins (Baeuerle and Baltimore, 1991; Baeuerle, 1991; Nolan and Baltimore, 1992; Blank et al., 1992; Grilli et al., 1992). A particular focus of this Review are functional and structural aspects of NF-κB subunits.

NF-κB is a transcription factor that is activated in many different cell types following a challenge with primary (viruses, bacteria, stress factors) or secondary pathogenic stimuli (inflammatory cytokines). The active factor then leads to a rapid induction of genes encoding defence and signalling proteins, suggesting that NF-κB has specialized during evolution as an immediate early mediator of immune and inflammatory responses. There is now increasing evidence that NF-κB and related proteins are also involved in growth control (Gilmore, 1991; Ohno et al., 1991; Neri et al., 1991; Narayanan et al., 1992).

THE DNA-BINDING SUBUNITS: FUNCTION AND STRUCTURE

DNA binding and dimerization

NF-κB was first described as an activity specifically retarding in electrophoretic mobility shift assays (EMSAs) DNA fragments containing the decameric DNA sequence motif 5′-GGGACTTTCC-3′ (Sen and Baltimore, 1986a). This NF-κB binding site, called the B motif, was identified as a B-cell-specific

Abbreviations used: NF, nuclear factor; TF, transcription factor; TAF, TF-IID-associated factor; SRF, serum response factor; EMSA, electrophoretic mobility shift assay; NRD, NF-κB/Rel/dorsal domain; NLS, nuclear location signal.

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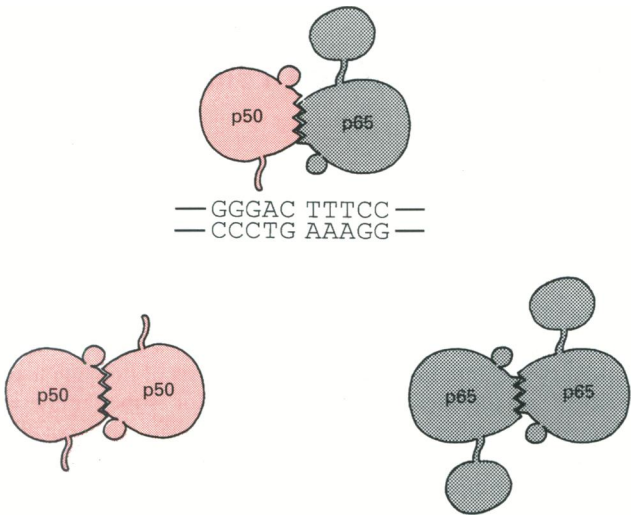

Figure 1 Homo- and hetero-dimeric NF-κB complexes

The p50 subunit is shown with an appendix corresponding to a glycine-rich linker sequence (see Figure 3), a blob corresponding to a nuclear location signal, and a zig-zag line corresponding to a dimerization motif. The additional blob on p65 represents the *trans*-activating C-terminal sequences. The DNA sequence under the p50–p65 heterodimer is found in the enhancers of the immunoglobulin κ light chain gene and in two copies in the HIV-1 LTR. The p50–p65 heterodimer is shown in its preferred orientation over its binding site.

element in the intronic κ light chain enhancer (Lenardo et al., 1988). Soon, it became evident that the element is also functional in pre-B and other cell types, however, not as constitutive but as phorbol ester- and lipopolysaccharide-inducible enhancer element (Sen and Baltimore, 1986b; Nabel and Baltimore, 1987; Pierce et al., 1988). NF-κB is now recognized as ubiquitous factor that occurs, with the exception of a few cell types, in an inducible form requiring certain stimuli in order to appear in nuclei in a DNA-binding form. The activation of NF-κB is independent of protein synthesis (Sen and Baltimore, 1986b). A treatment of cytoplasmic fractions with detergents resulted in a cell-free activation of NF-κB (Baeuerle and Baltimore, 1988a). These findings indicated that the activation of NF-κB involves post-translational mobilization of a sequestered cytoplasmic form.

DNA affinity purification of NF-κB from human cell lines (Kawakami et al., 1988; Baeuerle and Baltimore, 1989), human placenta (Zabel et al., 1991) or rabbit lung (Ghosh et al., 1990) using double-stranded multimers of the recognition sequence 5′-GGGACTTTCC-3′ yielded in each case two polypeptides with apparent molecular sizes of 50 and 65 kDa, referred to as p50 and p65. Reconstitution experiments showed that NF-κB forms a multisubunit complex containing p50 as well as p65 (Baeuerle and Baltimore, 1989). Initial u.v.-crosslinking studies and renaturation experiments using SDS-gel-purified subunits indicated that only p50 has κB-specific DNA binding activity (Kawakami et al., 1988; Baeuerle and Baltimore, 1989). An improved renaturation method and the use of DNA probes for u.v.-crosslinking, in which only one half-site of the decameric motif was photoreactive and radioactively labelled, allowed direct demonstration that the purified p65 subunit has κB-specific DNA binding activity on its own, and that, in NF-κB, both subunits contact the DNA (Urban et al., 1991). Also the p65 protein produced by *in vitro* translation (Ruben et al., 1991) or by baculovirus-infected insect cells (Fujita et al., 1992) could bind to DNA. In another study, DNA binding activity of p65 was only detected after C-terminal truncation of the protein (Nolan et al., 1991).

U.v.-crosslinking experiments showed that in NF-κB p50 and p65 contact DNA as a heterodimer, and glycerol gradient centrifugation analysis suggested that p50 and p65 form a heterodimer in solution (Urban et al., 1991) (Figure 1). A heterotypic dimerization of subunits is also observed with many other transcription factors binding to palindromic sequence motifs (reviewed in Lamb and McKnight, 1992). In the κB sequence 5′-GGGAAATTCC-3′ from the β-interferon enhancer, the p50 subunit in NF-κB preferred binding to the first half-site containing the three GC pairs (Urban et al., 1991). p65, on the other hand, showed a preference for the second half-site, which is usually more degenerate when κB motifs from known target genes are accordingly aligned and compared (Zabel et al., 1991; Baeuerle, 1991). The differential half-site recognition by p50 and p65 was also evident from DNA-binding assays using duplicated half-sites as competitor oligonucleotides (Urban et al., 1991). Upon gel filtration, NF-κB eluted with a size larger than that of immunoglobulin G (Baeuerle and Baltimore, 1989). It is therefore possible that NF-κB can form higher order complexes (for instance, a tetramer), but this awaits further analysis.

p50 and p65 subunits of NF-κB can also form homodimers (Figure 1). This is evident from the following observations. (i) The sedimentation coefficient of p50–p65 NF-κB was intermediate to that of isolated p50 and p65 (Urban et al., 1991). (ii) The mobility of a p50–p65–DNA complex in EMSAs was intermediate to that of a faster-migrating complex containing only p50 and a slower-migrating complex containing only p65. (iii) Isolated p50 and p65 subunits were u.v.-crosslinked equally well to both half-sites of the motif 5′-GGGAAATTCC-3′.

p50 homodimers can occur as constitutive factor in nuclei of certain cell types (Kieran et al., 1990; Kang et al., 1992). The DNA-binding activity of p50 was discovered in parallel to that of NF-κB and is referred to in the literature as KBF-1 (Israel et al., 1987; Kieran et al., 1990), EBP1 (Clark et al., 1990) or H2TF1 (Baldwin and Sharp, 1988). H2TF1 is now recognized to be a distinct factor (A. Baldwin, personal communication). It was noted that the p50 homodimer has a higher affinity for a palindromic 11-bp motif from the enhancer of MHC class I gene (5′-GGGGATTCCCC-3′) than it has for the less symmetric decameric κB motif 5′-GGGACTTTCC-3′ (Kieran et al., 1990; Urban and Baeuerle, 1991). This finding supports the notion that the slight asymmetry of most κB motifs might have evolved to bind preferentially p50–p65 heterodimers. Isolation of ideal binding motifs from a pool of random oligonucleotides using recombinant p50 homodimer showed that p50 prefers binding to highly symmetric GC-rich 11-bp motifs with the consensus 5′-GGGGPuNT/GPyCCC-3′ (Kunsch et al., 1992).

Oligonucleotides selected by p65 homodimers showed a consensus sequence markedly different from that of p50: 5′-(G)GGPuNTTTCC-3′ (Kunsch et al., 1992). There is no apparent requirement for an eleventh base pair and even a decreased requirement for a conserved tenth base pair. The GC content is much lower and the half-site sequence 5′-TTTCC-3′ extremely conserved. NF-κB is a rare example of a dimeric transcription factor in which the DNA-binding subunits have distinguishable DNA-binding specificity. This allows the evolution of *cis*-acting elements preferentially recognized by heterodimers but not by the respective homodimers. It is not yet clear to what extent homodimer-specific binding sites are used for gene regulation.

p65 homodimers were not yet identified in nuclear extracts, even when artificial motifs were used in EMSAs that preferentially bind to purified p65 (Urban and Baeuerle, 1990). When equal amounts of p65 and p50 homodimers are mixed, it takes less than

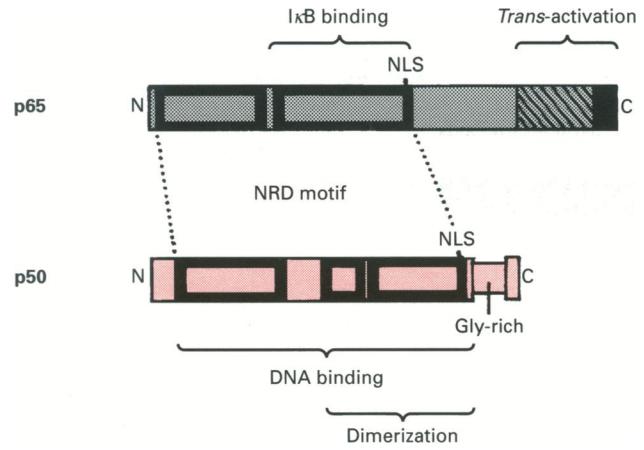

Figure 2 Structure and function of p65 and p50 NF-κB subunits

Bold boxes indicate the highly homologous parts of the NRD motif which are differently interrupted by unique sequences in p65 and p50. Dotted lines connect the N- and C-terminal boundaries of the NRD motif. The filled box in the *trans*-activating C-terminus of p65 indicates TA_1. TA_2 is contained within the shaded area. Both TA regions are separately active. N, N-terminus; C, C-terminus.

10 min at 37 °C for subunit exchange and complete conversion of the homodimers into the heterodimer (M. Urban and P. Baeuerle, unpublished work). This shows that p50 and p65 have a much higher tendency to form heterodimers than homodimers, and that the half life of homodimeric complexes is below 10 min. It is thus unlikely that appreciable amounts of p50 and p65 homodimers can coexist within the cell, unless they are stabilized by additional proteins or DNA.

The various subunit combinations of NF-κB bind to DNA with an extremely high affinity. Dissociation constants (K_D) of 0.4×10^{-12} and 0.9×10^{-12} M, respectively, were determined for p50–p65 and p50–p50 complexes formed with SDS-gel-purified NF-κB subunits from human placenta (Urban and Baeuerle, 1991). Using the same κB motif (5′-GGGACTTTCC-3′), proteins expressed in insect cells by the baculovirus system yielded dissociation constants of 5.7×10^{-12}(p50–p65) and 6.7×10^{-12} M (p50–p50) (Fujita et al., 1992). Depending on the position of the C-terminus (see below), bacterially expressed p50–p50 gave K_D values between 2.6×10^{-12} (443 amino acids) and 8.3×10^{-12} M (503 amino acids) (Kretzschmar et al., 1992). With p50–p65 NF-κB purified from the human cell line HeLa, the same workers found a K_D of 1.3×10^{-12}M. Given the systemic differences and potential sources for errors, the various numbers are in reasonable agreement and document an extremely high affinity of NF-κB for its cognate motif. In one case, a dissociation constant was determined for a p65 homodimer and amounted to 32.2×10^{-12} M (Fujita et al., 1992). This comparatively low affinity might explain why much less protein–DNA complex is usually obtained in EMSAs with p65 compared to similar amounts of p50 or NF-κB (see Urban et al., 1991; Schmitz and Baeuerle, 1991; Nolan et al., 1991). Very similar affinity constants for p50, p65 and NF-κB as above were obtained when three other κB motifs were tested (Fujita et al., 1992). The very low abundance of NF-κB in cells (Lenardo et al., 1989; Henkel et al., 1992) might be one reason why NF-κB requires such an extreme affinity for its cognate motifs.

It was noted that the position of the binding site 5′-GGGACTTTCC-3′ within circularly permutated DNA fragments strongly influenced the mobility of NF-κB–DNA complexes while the mobility of the uncomplexed DNA was not affected (Schreck et al., 1990). Such mobility changes are indicative for an induced alteration of the DNA structure at the site of protein binding. The relative temperature-independence of the mobility effect suggested that NF-κB caused DNA bending rather than an increased flexibility of the DNA. The estimated bend angles of DNA were more than 100 ° for complexes containing p50–65 and p65–p65, and about 57 ° for p50–p50 DNA complexes. Polycations or a site-specific cleavage of the DNA backbone close to the κB motif strongly facilitated DNA binding of NF-κB. Presumably, this is due to a lowered energy requirement for bending which, otherwise, needs to be overcome upon binding of the protein.

The primary structures of p50 and p65

The similar DNA-binding specificity and the homo- as well as hetero-dimerization properties of p50 and p65 have their molecular basis in a 300-amino-acid-long region of sequence similarity (Kieran et al., 1990; Ghosh et al., 1990; Ruben et al., 1991; Nolan et al., 1991; Meyer et al., 1992) (Figure 2). The homology region was found much earlier to be shared by the v-*rel* oncogene product from the avian retrovirus REV-T and the morphogenic protein dorsal from the fruit fly *Drosophila melanogaster* (reviewed in Govind and Steward, 1991). However, the function of this region in the viral and fly proteins remained unknown until it was discovered and functionally analysed in the NF-κB subunits. Now, this region of sequence similarity, which we refer to as NF-κB/Rel/dorsal (NRD) domain, is recognized as the minimal requirement for DNA binding and dimerization of the proteins and defines a novel family of transcription factors (reviewed in Blank et al., 1992; Nolan and Baltimore, 1992). The other members of this family and their relationship to NF-κB will be briefly discussed below.

Compared to dimerization and DNA-binding motifs of other transcription factor families, sharing, for instance, basic leucine zipper, helix–loop–helix or homeo domains, the NRD domain is unusual in that it requires a fairly long and intact stretch of approximately 300 amino acids. As demonstrated by coimmunoprecipitation experiments with deleted forms of p50, the C-terminal half of the NRD domain in p50 is sufficient for dimerization (Logeat et al., 1991) (Figure 2). This points to a role of the N-terminal half in contacting the DNA. Preliminary results indeed suggest that the fine differences of DNA-binding specificity between p50 and p65 can be exchanged between the subunits by swopping less than 30 N-terminal amino acids of the NRD domains (S. Grimm and P. Baeuerle, unpublished work). Within this sequence, a highly conserved cysteine residue (position 62) was shown to interfere with DNA binding of p50 upon mutation or oxidation *in vitro* (Matthews et al., 1992). This might explain the earlier observation by Toledano and Leonard (1991) that the DNA binding of NF-κB can be reversibly controlled *in vitro* by oxidation/reduction. Similar observations were made with the homologous cysteine residue of the related c-Rel protein (Kumar et al., 1992). The physiological significance of these observations is not known.

Both p50 and p65 have at the C-terminal end of their NRD domains a cluster of positively charged amino acid residues: Arg-Lys-Arg-Gln-Lys and Lys-Arg-Lys-Arg, respectively. Similar sequences in nuclear proteins were reported to serve as signals for receptor-mediated nuclear uptake and are referred to as nuclear location signals (NLS) (for review see Garcia-Bustos et al., 1990) (Figure 2). Upon separate overexpression of p50 and p65, both proteins were detected in nuclei (Blank et al., 1991; Henkel et al., 1992; Beg et al., 1992; Zabel et al., 1993). While p50 was exclusively nuclear (Blank et al., 1991; Henkel et al.,

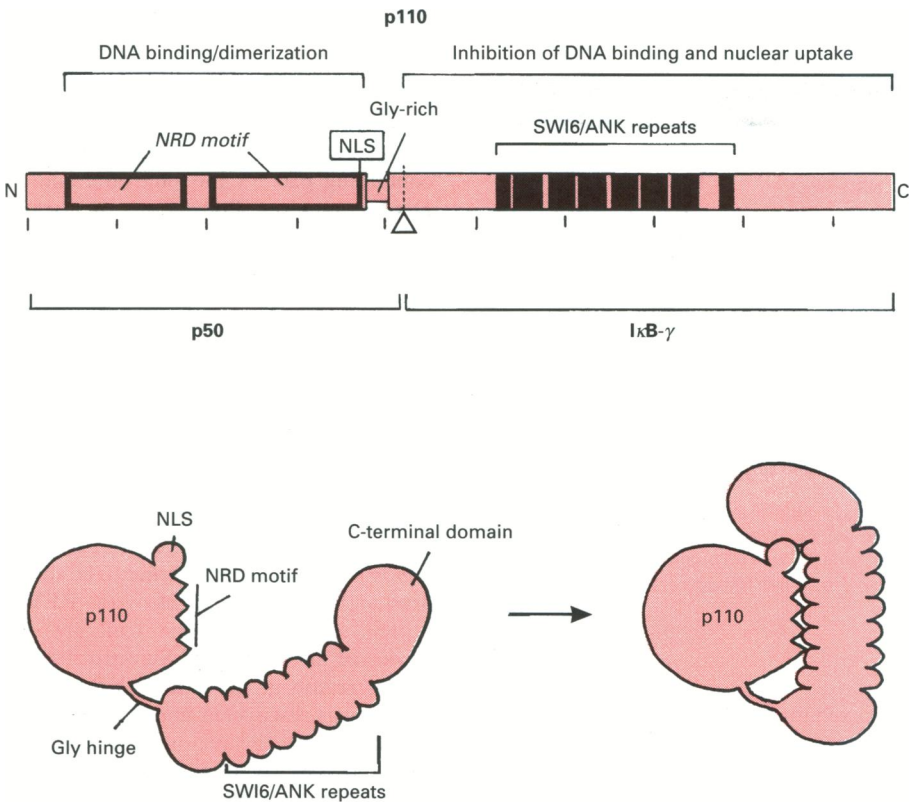

Figure 3 Structure and function of p110, the precursor for p50

The upper part of the panel shows the primary structure of p110. The bars indicate distances of 100 amino acids. The N-terminal (N) portion of p110 encompasses p50 while the C-terminal (C) portion contains IκB-γ. The NRD motif is shown as bold boxes and the eight SWI6/ANK repeats as filled boxes. Repeats 1 and 8 are more degenerate than the other six repeats. The NLS of p50 is boxed and a putative processing site indicated by an arrowhead. The lower left part of the panel shows a three-dimensional model of p110 highlighting the flexible linkage of p50 and the C-terminal domain such that it can properly mask the NLS upon intramolecular folding (lower right panel). Note that for interference with dimerization (NRD motif) fewer repeats are required, consistent with data from deletion experiments.

1992), p65 was at low concentrations cytoplasmic, but nuclei could be 'filled up' with p65 when higher amounts of p65 were expressed (Zabel et al., 1993). It seemed that p65 was retained by an endogenous activity in the cytoplasm (IκB?) which could be titrated out by overexpression of p65. Mutational alterations of basic amino acids in the putative NLS sequences of p50 and p65 into uncharged residues resulted in the accumulation of p50 and p65 in the cytoplasm, even when the proteins were expressed to high levels. This shows that the cluster of positively charged amino acids, which is conserved among all members of the NRD family of proteins, is indeed part of a NLS.

Isolation of the cDNA coding for p50 led to another surprise: p50 is not synthesized as active DNA-binding protein, but is contained in the N-terminal half of a non-DNA-binding precursor of 110 kDa, referred to as p110 (human p110 has 969 amino acids) (Kieran et al., 1990; Ghosh et al., 1990; Bours et al., 1990; Meyer et al., 1991) (Figure 3). The C-terminal half of p110 contains yet another sequence motif discovered earlier in several proteins involved in cell cycle control and cell architecture (reviewed in Blank et al., 1992). This sequence motif with a length of 30–33 amino acids, called SWI6/ankyrin (ANK) repeat, is reiterated eight times in p110. The precise cleavage site in p110 has not yet been identified. Experiments using recombinant p50 must therefore rely on a p50 product with an estimated C-terminus. The protease responsible for p110 processing is thought to be related to a ubiquitin-dependent enzyme (Fan and Maniatis, 1991). The natural cleavage site of p110 is close to a sequence highly enriched in glycine residues that could potentially serve as a 'hinge' region between the two halves of p110 (Figure 3; see below).

Are there functional differences between p50 and p65? The p50 molecule has, apart from the NRD domain, very little extra sequence, whereas p65 has a C-terminal extension of unique protein sequence (Figure 2). In addition to DNA-binding properties, transcription factors are known to require *trans*-activation domains, which are thought to interact with components of the basic transcription machinery. We therefore tested p50 and p65 for their *trans*-activating potential. This was perfomed in two ways. Firstly, *trans*-activation of a κB-controlled reporter gene construct by overexpressed p50 and p65 homodimers was tested. Secondly, p50 and p65 sequences were fused to the DNA-binding domain of the yeast GAL4 protein and the fusion proteins examined for *trans*-activation of a GAL4-controlled reporter gene construct.

Upon separate overexpression of p50 and p65 in COS cells, the respective p50–p50 and p65–p65 complexes were detected by EMSA (Schmitz and Baeuerle, 1991). Although the cell line contained endogenous NF-κB, this was not active and the endogenous NF-κB subunits could not detectably form heterodimers with the overexpressed proteins. In transient transfection assays using a chloramphenicol acetyltransferase (CAT) reporter plasmid with two κB sites, *trans*-activation was

exclusively observed with p65. This was also reported by other laboratories (Ruben et al., 1992; Perkins et al., 1992; Ballard et al., 1992). Only when the *trans*-activation domain of the herpes virus protein vp16 was fused to the C-terminus of p50 could the protein induce κB-dependent reporter gene expression (Perkins et al., 1992; S. Grimm and P. Baeuerle, unpublished work). Also, when sequences of p50 and p65 were linked in full length or as fragments to the GAL4 domain, only sequences derived from p65 showed induction of a GAL4-controlled CAT reporter gene (Schmitz and Baeuerle, 1991; Ruben et al., 1992). The NRD domains of p50 and p65 were inactive, whereas C-terminal sequences from p65 could strongly stimulate transcription.

A fine mapping identified the most C-terminal 30 amino acids of p65 as a strong and independent *trans*-activation domain, called TA_1. The N-terminally adjacent 100 amino acids also showed activity and were referred to as TA_2. TA_2 contains a sequence motif homologous to TA_1 (TA_1') which is, however, more dependent on flanking sequences for independent activity than is TA_1 (M. L. Schmitz and P. Baeuerle, unpublished work). The negatively charged TA_1 and TA_2 have a high probability to be present in an α-helical conformation. In the case of TA_1 this structure prediction was supported by c.d. analysis of a synthetic peptide (M. L. Schmitz, M. dos Santos Silva and P. Baeuerle, unpublished work). Breakage of the α-helix by introduction of a proline residue or changing its surface by addition or deletion of one alanine residue in its middle strongly impaired the activity of TA_1, suggesting that the α-helix is an important element for recognition of p65 by adaptor proteins. The 'squelching' effect observed upon expression of vp16 indicated that TA_1 and TA_1' belong, like vp16, to the class of acidic activators.

When p50 is expressed in COS cells to higher levels than p65, a strong repression of the κB-dependent *trans*-activation is observed (Schmitz and Baeuerle, 1991). Because transcriptionally inactive p50 homodimers bind to the same sites as the *trans*-activating p50–p65 and p65–p65 complexes, this effect might come from p50 homodimers occupying limited binding sites. This idea found strong support in the observation that a GAL4–p65 chimaera, which displays a dual DNA-binding specificity, was only affected by p50 overexpression in its κB-dependent but not GAL4-dependent *trans*-activating activity (Schmitz and Baeuerle, 1991). The negative regulatory effect of p50 homodimers on IL-2 promoter activity was recently proposed to play a physiological role during T cell activation (Kang et al., 1992).

An intriguing finding is that p50 homodimers are transcriptionally active in cell-free transcription systems (Kretzschmar et al., 1992; Fujita et al., 1992). The addition of recombinant p50 produced in *Escherichia coli* or insect cells to *in vitro* assays strongly stimulated κB-dependent initiation of mRNA synthesis. The stimulation was strongest with the palindromic motif from the MHC class I enhancer and almost undetectable with that from the β-interferon enhancer (Fujita et al., 1992). It was proposed that the binding sites influenced the conformation of bound p50 such that a *trans*-activation domain is either exposed or sequestered. A conformational alteration of p50 dimers was indeed evident from the distinct protease susceptibility of p50 complexes formed on different cognate sequences. The reason for the opposite activities of p50 in assays *in vitro* and transient transfection assays using intact cells remains unclear.

In conclusion, the p65 subunit in NF-κB serves for strong transcriptional activation of genes, whereas a major function of the p50 subunit is to associate with p65 in order to form a heterodimer that binds with increased affinity to DNA. Thus, p50 can be considered as a 'helper' subunit imposing a limited regulation on the *trans*-activating p65 subunit by increasing its affinity for DNA. This strategy is not without precedent. c-Myc, a *trans*-activator and proto-oncogene product, binds poorly to DNA (Blackwood et al., 1992). For high-affinity DNA binding it requires Max, a transcriptionally inactive heterodimerization partner. In the following section, additional subunits of NF-κB will be described that impose a very tight negative control on the activity of the DNA-binding subunits.

THE INHIBITORY IκB SUBUNITS: STRUCTURE AND FUNCTION

Purification and specificity

In non-stimulated cells, NF-κB DNA binding activity is not detectable in nuclear, cytosolic or membrane fractions. However, if cytosolic fractions are treated with the ionic detergent sodium deoxycholate, followed by a chase with the non-ionic detergent Nonidet P-40, the DNA-binding activity of a κB-specific factor can be generated. This *in vitro*-activated cytoplasmic factor is identical to the NF-κB found in nuclei of activated cells, as shown by purification, DNA-binding analyses and partial protein sequencing of p50 and p65 subunits (Baeuerle and Baltimore, 1989; Kieran et al., 1990; Ghosh et al., 1990; Zabel et al., 1991; Nolan et al., 1992). The treatment with deoxycholate released an activity from NF-κB that, upon re-addition, reversibly inhibited the DNA-binding activity of NF-κB (Baeuerle and Baltimore, 1988b). The inhibiting factor, termed inhibitor of NF-κB (IκB), could not interfere with the DNA-binding of any other nuclear factor tested. Treatment with deoxycholate (Baeuerle and Baltimore, 1989) or low pH (Zabel and Baeuerle, 1991) allowed dissociation of NF-κB and IκB and subsequent purification of NF-κB from cytosol by DNA affinity chromatography, as well as purification of IκB by conventional column chromatography methods (Zabel and Baeuerle, 1990; Ghosh et al., 1990; Ghosh and Baltimore, 1990; Link et al., 1992).

In our laboratory, two chromatographically distinct IκB variants were isolated from human placenta (Zabel and Baeuerle, 1990). IκB-α had an apparent molecular size of 37 kDa, very similar to the IκB variant isolated from rabbit lung (Ghosh and Baltimore, 1990), while IκB-β had a size of 43 kDa (Link et al., 1992). Both isoforms had isoelectric points between 4.8 and 5. A c-Rel-associated IκB was immunoisolated from chicken cells. The 40 kDa phosphoprotein, termed pp40, was immunologically related to human IκB-β (Kerr et al, 1991). There are two substantial differences between IκB-α and -β, suggesting that the proteins come from different genes. (i) While IκB-α was specific for NF-κB, IκB-β could, in addition, inhibit the DNA binding of the related c-Rel protein (Kerr et al., 1991). (ii) While *in vitro* treatments with protein kinases A and C abolished the inhibitory activity of both variants, a phosphatase treatment interfered only with the inhibiting activity of purified IκB-β (Kerr et al., 1991; Link et al., 1992). The physiological relevance of the *in vitro* phosphorylation data is presently unknown. The various studies have recently been reviewed in detail (Schmitz et al., 1991).

In NF-κB, both IκB variants bind preferentially to the p65 rather than the p50 subunit. This is evident from the following observations. (i) An excess of p65, but not p50, can prevent inhibition of NF-κB by purified IκB proteins (Urban and Baeuerle, 1990). Likewise, addition of p65 to an inactive complex of NF-κB and IκB leads to the release of active NF-κB. (ii) IκB proteins can only inhibit the DNA binding of NF-κB and p65 homodimers but not that of p50 homodimers (Baeuerle and Baltimore, 1989; Urban et al., 1991). (iii) p65 but not p50 can bind stoichiometric amounts of the IκB protein (see below), as tested by coimmunoprecipitation (Zabel et al., 1993).These results could mean that IκB proteins bind to sequences unique

for p65 and not to the homologous NRD domain. However, Nolan et al. (1991) showed that a portion of p65 encompassing the NRD motif is susceptible to IκB inhibition. This suggests that the NRD domain, in addition to binding DNA and a second DNA-binding subunit, also interacts with IκB. The binding sequences for IκB were mapped to the C-terminal half of the NRD domain (Beg et al., 1992). This explains why IκB-β can inactivate both p65 and c-Rel, which share sequence similarity only within the NRD domain. Interestingly, antibodies to p50 could coimmunoprecipitate small amounts of MAD-3. Furthermore, a high excess of recombinant IκB (MAD-3) interfered with DNA binding (Liou et al., 1992) and nuclear uptake of p50 homodimers (Beg et al., 1993; Zabel et al., 1993). These observations suggest that the NRD domain of p50 also has a weak affinity for IκB proteins which, at physiological concentrations of the proteins, might not be relevant. Bcl-3 and IκB-γ are IκB proteins specifically binding to the NRD domain of p50 (see below).

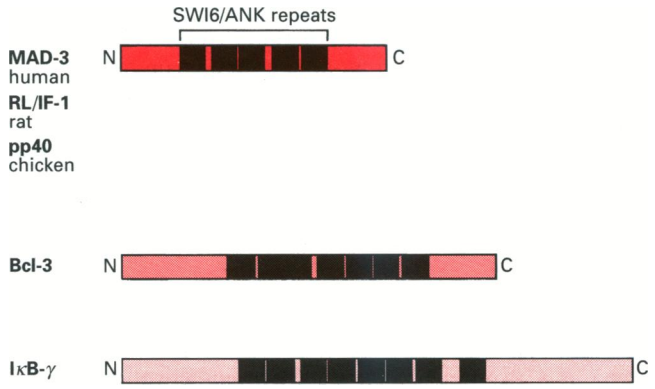

Figure 4 Structures of IκB subunits

The primary structures of IκB proteins are shown. The SWI6/ANK repeats are shown as filled boxes with their real distances. The C-terminal portion of p100 might yet contain another IκB protein, IκB-δ, not listed here. N, N-terminus; C, C-terminus.

Molecular cloning of the IκB proteins

As will be described in a separate section, the first cloned IκB protein was the p50 precursor which contains in its C-terminal half an IκB protein called IκB-γ. IκB-γ can arise from alternative splicing and has specificity for p50. When macrophages adhere to their substratum, they newly express a protein called MAD-3 (Haskill et al., 1991). Molecular cloning and *in vitro* translation revealed that human MAD-3 had an apparent molecular size of 35 kDa and contains five ankyrin repeats. The size of MAD-3, and its sequence similarity to the C-terminal portion of the p50 precursor, prompted the investigators to test MAD-3 for IκB-like activity. The protein could indeed inhibit specifically the DNA-binding activity of NF-κB and c-Rel *in vitro*. Tewari et al. (1992) isolated a MAD-3-encoding cDNA clone from rat liver as one induced upon hepatectomy, and called the protein RL/IF-1. In parallel, a cDNA clone encoding the chicken pp40 protein was isolated by immunoscreening of an expression library (Davis et al., 1991). pp40 was highly homologous to human MAD-3 and showed the same inhibiting specificity, suggesting that pp40 is the chicken homologue of MAD-3. Partial amino acid sequence indicated that the IκB protein purified earlier from rabbit lung (Ghosh and Baltimore, 1990) was highly related if not identical to MAD-3.

Another protein tested for IκB-like activity because of its SWI6/ANK repeats is the proto-oncogene product Bcl-3. Bcl-3 was discovered as being encoded adjacent to a translocation breakpoint on human chromosome 19 associated with chronic lymphocytic leukaemia (Ohno et al., 1990). The protein has an apparent molecular size of 47 kDa and contains seven SWI6/ANK repeats. Also Bcl-3 showed IκB-like activity (Hatada et al., 1992; Wulczyn et al., 1992). It was, however, specific for the p50 homodimer and could not significantly inhibit formation of complexes of NF-κB or c-Rel with DNA. Phosphopeptide mapping with *in vitro* $^{32}PO_4$-labelled Bcl-3, MAD-3 and placental IκB-α showed that IκB-α was unrelated to MAD-3 but shared phosphopeptides with Bcl-3 (Kerr et al. 1993). In strong support for the idea that IκB-α is part of Bcl-3, N-terminal truncation of Bcl-3 resulted in alteration of its inhibiting specificity. A shortened form of Bcl-3 was a specific inhibitor of p65 but not of c-Rel or p50 homodimers, as was reported earlier for IκB-α (Kerr et al., 1991). A truncation seems to occur in intact cells because Bcl-3-specific antibodies immunoprecipitated a 37 kDa protein apart from the 50 kDa Bcl-3. Future studies have to explore by what mechanism (proteolysis or alternative splicing) Bcl-3 is processed to yield IκB-α-like activity. Recently we observed that monospecific antibodies to MAD-3 immunodeplete IκB-α but not IκB-β activity (Zabel et al., 1993). This suggests that IκB-α is highly related to MAD-3 and that IκB-β might not yet be cloned. Further studies are required to define the relationship between IκB-α, MAD-3 and Bcl-3. An interesting possibility is that Bcl-3 serves as a gene activator by relieving the negative regulatory effect of p50 homodimers occurring when p50 dimers occupy binding sites for transcriptionally active complexes. In transfection experiments using a reporter gene controlled by the HIV-1 enhancer, this activity of Bcl-3 was indeed demonstrated (Franzoso et al., 1992).

Structural features of IκB proteins

The primary structures of MAD-3 (pp40; RL/IF-1) and Bcl-3 have a few common features (Figure 4). The SWI6/ANK repeats are clustered in the middle of the molecules and are flanked by sequences rich in acidic, hydroxyl, proline and glycine residues. Nolan and Baltimore (1992) noted upon aligning the SWI6/ANK repeat domains from IκB-like proteins that the repeats show much greater sequence similarity when compared with respect to their position among the different proteins then when compared within one molecule. For instance, the first repeats in each protein were much more similar to each other than repeat 1 and 2 within the same protein. This might indicate that the repeats have individual functions, a question that can be addressed by swopping experiments.

The pp40 protein was subjected to a deletion and mutational analysis (Inoue et al., 1992a). The most highly conserved sequence within the SWI6/ANK repeats was mutated into a stretch of alanine residues. With the exception of repeat 3, this abolished in each case the capability of pp40 to inhibit DNA binding and to associate with c-Rel and p65. A portion of the molecule encompassing solely the SWI6/ANK repeats was inactive. Only when the C-terminal portion was present in addition to the repeats was pp40 active. Apparently the N-terminal portion was dispensable for the tested activities. These results show that the SWI6/ANK repeats are necessary but not sufficient for the activity of pp40. Very similar results were obtained with MAD-3 (T. Henkel and P. Baeuerle, unpublished work).

The various functions of IκB proteins

It is now well-established that IκB proteins inhibit the DNA-binding activity of proteins with NRD domains. The molecular

mechanism of inhibition is, however, poorly understood. There is preliminary evidence that IκB proteins neither simply mask the DNA-binding domain nor interfere with dimerization of DNA-binding subunits. If IκB disrupts the association between p50 and p65, p50 would be released and become detectable as DNA-binding p50 homodimer. This is not the case. Moreover, sizing data suggested that the cytoplasmic NF-κB complex is a heterotrimer composed of p50, p65 and IκB molecules (Baeuerle and Baltimore, 1988b; Zabel and Baeuerle, 1990). It is, however, possible that IκB, by binding to p65, alters its association with p50 resulting in a reduced DNA-binding affinity or altered specificity. Future studies using recombinant proteins should therefore investigate whether the NF-κB–IκB complex retains some novel DNA-binding properties. The related c-Rel protein can apparently form a complex with DNA which contains IκB proteins, as was evident from the immunoreactivity of a c-Rel–DNA complex with antibodies to pp40 (Kerr et al., 1991).

We have observed that IκB proteins can reduce the half life of a NF-κB–DNA complex from 45 min to less than 7 min (Zabel and Baeuerle, 1990). This dissociation followed higher-order kinetics. In cell-free transcription assays, IκB could even disrupt a transcription initiation complex induced by NF-κB, thereby specifically terminating *in vitro* transcription (Kretzschmar et al., 1992). The findings indicate that IκB proteins do not simply cover the DNA-binding domain of NF-κB but rather exert an allosteric effect on the heterodimer. The IκB protein MAD-3 was found to be predominantly in the nucleus (Zabel et al., 1993). Taken together, these properties would allow IκB proteins to function in the nucleus as inhibitors of NF-κB-dependent transcription.

Apart from inhibiting DNA binding, another function of IκB proteins is to control the nuclear uptake of associated DNA-binding proteins. This was first evident from the findings that the complex of NF-κB with IκB was cytoplasmic upon subcellular fractionation and could not be removed by enucleation procedures from living cells (Baeuerle and Baltimore, 1988b). More direct proof was obtained by indirect immunofluorescence labelling of cells overexpressing MAD-3 and the DNA-binding subunits of NF-κB (Beg et al., 1992; Zabel et al., 1993). When MAD-3 was overexpressed on its own it was present in both cytoplasm and nucleus. It is possible that the protein passively entered the nucleus because its size of 37 kDa is below the cut-off of nuclear pores. When MAD-3 was coexpressed with p65, it completely prevented nuclear uptake of p65. Likewise, p65 interfered with nuclear appearance of MAD-3, suggesting that the two subunits mutually control their access to nuclei. Also, p50 and MAD-3 mutually affected their nuclear uptake; however, a high excess of MAD-3 was required, which is consistent with the much weaker affinity of MAD-3 for p50 (see above).

Because both p50 and p65 contain functional NLS signals, we tested, by the use of antibodies recognizing the NLS epitopes in p50 and p65, whether IκB proteins interfere with the accessibility of NLS sequences for NLS receptors involved in targetting proteins to nuclear pores. Recombinant MAD-3 or purified IκB-α could indeed prevent immunoprecipitation of p65 by anti-p65NLS IgG. Immunoprecipitation with another p65-specific antibody was not influenced by MAD-3. MAD-3 could also not block immunoprecipitation of p50 by anti-p50 NLS. The reactivity of anti-p50 NLS IgG was however affected by MAD-3 when p50 was in complex with p65. This suggests that in the p50–p65 heterodimer one IκB molecule can mask the NLS in both p65 and in p50, although it is bound to only one of the two subunits. Two observations by Beg et al. (1992) are consistent with NLS masking by IκB. These investigators showed that addition of a second NLS from SV40 large T antigen to the N-terminus of p65 can over-ride the inhibiting effect of IκB on nuclear uptake. Moreover, mutation of all four basic residues in the NLS of p65 abolished binding of IκB. This indicates, but not necessarily proves, that IκB directly binds to the NLS of p65.

In conclusion, IκB proteins are proteins specialized in negatively controlling the DNA-binding subunits of NF-κB/Rel proteins. A particular advantage of such regulatory subunits is that they allow a post-translational induction of transcription factors via mechanisms simply releasing the inhibitors. This is much less time- and energy-consuming than *de novo* synthesis of transcription factors, as observed with c-Jun, c-Fos and c-Myc. Moreover, there is no requirement for primary transcription activators. Two well-studied functions of IκB proteins are inhibition of DNA binding and nuclear uptake of DNA-binding subunits. A third potential role of IκB proteins is downregulation of κB-dependent gene expression in the nucleus, but this possibility has to await further studies with intact cells.

THE PRECURSOR FOR p50: DNA BINDING AND INHIBITORY SUBUNIT IN ONE MOLECULE

While the IκB subunits for the *trans*-activating p65 subunit are produced by separate genes, one of the inhibitory subunits for p50 is produced in *cis* as the C-terminal part of the precursor molecule p110 (Figure 3). An obvious advantage of this strategy is that the inhibitor is always produced in a 1:1 ratio with its target p50. As a consequence, p50 cannot readily appear as active DNA-binding protein and, therefore, cannot operate after its synthesis as constitutive nuclear suppressor of transcription. This is of particular importance in view of the fact that the p50/p110 gene is transcriptionally upregulated by NF-κB (Ten et al., 1991), while the gene coding for the p65 subunit is apparently not (Ruben et al., 1991).

If the C-terminal portion of p110 (IκB-γ) is indeed functionally equivalent to separately encoded IκB proteins, one would expect that it could also interact with p50 in *trans*. This was demonstrated by coimmunoprecipitation of IκB-γ with p50, and by inhibition of the DNA-binding activity of p50 by bacterially expressed IκB-γ (Hatada et al., 1992; Inoue et al., 1992b; Henkel et al., 1992; Liou et al., 1992). An mRNA species encoding solely IκB-γ was detected in B cell lines (Inoue et al., 1992b; Liou et al., 1992). The alternative splice product allows overproduction of IκB-γ relative to p50 and could serve to control p50 homodimers that have escaped control by the coproduced IκB-γ. A second IκB protein specifically controlling p50 homodimers is Bcl-3 (see above). Future studies have to explore whether there are physiological stimuli that can release IκB-γ or Bcl-3 from p50 in order to allow formation of negative regulatory p50 homodimers.

The association of p50 and IκB-γ shows that there is no requirement for covalent linkage of the two parts of p110 in order to form an inactive complex. A proteolytic event generating p50 and IκB-γ from p110 is undoubtedly required for formation of p50 dimers (or p50–p65 heterodimers), but it appears insufficient. Therefore, protease(s) cleaving p110 must not necessarily be controlled or directly involved in the activation. Consistent with this idea, Fan and Maniatis (1991) presented evidence for the involvement of the constitutive, ubiquitin-dependent protease(s) in processing of p110. Moreover, we were so far unsuccessful in finding a treatment of cells that would enhance the slow conversion of p110 into p50 observed in cells overexpressing p110 (T. Henkel and P. Baeuerle, unpublished work). In such cells, an abundant cytoplasmic non-DNA-binding form of p50 is found, presumably a p50–IκB-γ complex (Henkel et al., 1992) or a p50–p110 complex (Rice et al., 1992). The step ultimately controlling formation of homo- and hetero-dimers

containing p50 must therefore involve a mechanism dissociating IκB-γ or p110 from p50.

The protease encoded by HIV-1 was shown to cleave p110 at position 412 *in vitro* and upon HIV-1 infection of cells, which is N-terminal to the physiological site of cleavage (Rivière et al., 1991). Cells infected with HIV-1 showed however no increased amounts of p50 homodimers or nuclear NF-κB, suggesting that the cleaved precursor remained in an inactive form.

p110 sediments through a glycerol gradient with an *s* value of 5.2 S, indicating that it is present as a monomer (provided it has a globular shape). If this is indeed the case, association of p50 and IκB-γ portions would occur intramolecularly. A glycine-rich stretch of 30 amino acids, positioned precisely between the two functionally distinct portions of p110, could provide, as 'hinge', the molecular basis for a spatial approach and intramolecular association of the two functional domains. An alternative model would be that two p110 molecules dimerize via their NRD domains. The IκB-γ portions could then exert their function in *cis* or *trans*. Deletion of an acidic region between SWI6/ANK repeats 7 and 8 of p110 created a mutant protein with DNA-binding activity (Blank et al., 1991). Apparently, the mutation disturbed the presumed intramolecular association of the p50 and IκB-γ portions and caused an 'opening' of the molecule. This allowed dimerization of the NRD domains and DNA binding. Figure 3 shows a model of p110 in 'open' and 'closed' conformation.

Direct evidence for masking of domains within p110 came from an immunological study (Henkel et al., 1992). An antipeptide antibody raised against a C-terminal epitope of p50 was immunoreactive with p50, but not with p110. Only after denaturation or C-terminal truncation of p110 could the antibody recognize the p50 epitope in p110. The observation that the C-terminal 200 amino acids of p110 were sufficient to mask an epitope in the p50 portion that is separated by a linear distance of more than 400 amino acid residues (including a 'flexible' domain) argues strongly for an intramolecular association of large, independent domains in p110.

Deletion analysis of p110 showed that only one SWI6/ANK repeat has to remain with the IκB-γ portion in order to suppress DNA binding of the p50 portion (Kieran et al., 1990). This is surprising given the fact that IκB-γ produced in *trans* has to contain all of its ankyrin repeats in order to maintain its inhibiting activity (Hatada et al., 1992). Possibly, it is the covalent linkage of shortened IκB-γ sequences to p50 that can stabilize, due to the lack of diffusion control, their weak interaction with the p50 portion.

As shown by immunofluorescence studies, p110 is a cytoplasmic protein (Blank et al., 1991; Henkel et al., 1992). Its diffuse cytoplasmic distribution is identical to that of a SV40 large T antigen mutant protein impaired in nuclear transport, and there is no resemblance to the immunostainings observed with antibodies to cytoskeletal proteins. Upon subcellular fractionation, p110 partitions into a 100000 ***g*** supernatant, suggesting that it is a cytosolic protein. The exclusion of p110 from nuclei was unexpected since p110 contains the NLS sequence of p50. Studies with an antipeptide antibody specific for a sequence overlapping the NLS showed that in p110 this epitope was not accessible, unless the protein was treated with the ionic detergent SDS. The antibody is thought to mimic a physiological receptor(s) involved in recognizing NLS sequences and targetting proteins to nuclear pores. The observation that it reacts with p50, but not p110, suggests that the IκB-γ portion masks the NLS epitope. Immunoprecipitation of C-terminally truncated forms of p110 with the antibody showed that immunoreactivity was restored when only 200 amino acids were deleted from the C-terminus, which removes only one SWI6/ANK repeat from IκB-γ. When this truncated form of p110 was expressed in cells, it was partially taken up into nuclei. A C-terminal domain of 200 amino acids is apparently sufficient to mask the NLS. The repeats seem not to be directly required for cytoplasmic retention, but might serve to properly position the C-terminal domain, as detailed in Figure 3.

p110 is found in complexes containing v-Rel and c-Rel (Capobianco et al., 1992; Kochel et al., 1991). It will be interesting to find out the stoichiometry and physiological significance of this interaction and what sequences of p110 are binding v-Rel. Can p110 unfold and use its dimerization domain to bind v-Rel, or does the IκB-γ portion have free valencies? Very recent studies provided evidence that the entire p110 molecule has an IκB-like function and controls DNA binding and nuclear uptake of c-Rel and p65 (Rice et al., 1992).

In conclusion, p110 appears as an unusual molecule. It is a very rare example of a non-viral cytoplasmic precursor protein requiring proteolysis for maturation. p110 combines two opposite functions in one molecule: a DNA-binding function with nuclear affinity and a specific inhibitor of the DNA-binding portion and its nuclear affinity. The sequences of p110 containing the two activities can apparently interact within the same molecule. The interaction of p110 with c-Rel and p65 is an intriguing finding; inducible cytoplasmic complexes could form in a single-step reaction.

BIOGENESIS OF NF-κB

An inducible transcription factor composed of several subunits that are encoded by different genes relies very much on a co-ordinate production and assembly of the subunits. In the case of NF-κB, overproduction of p50 would result in a constitutive DNA-binding protein with no or low *trans*-activating potential. On the other hand, overproduction of p65 would result in a constitutive activator bypassing the inducible control imposed by limited IκB. Overproduction of IκB might not be as deleterious as long as reactions inactivating NF-κB-bound IκB are not inhibited by an excess of free IκB. As discussed above, p50 brings along its own inhibitor within a precursor molecule. Therefore, it does not matter that the p110 gene is transcriptionally upregulated by NF-κB (Meyer et al., 1991; Ten et al., 1991); all p110 that is produced in excess over p65 would accumulate in an inactive form and remain as such in the cytoplasm, even after proteolytic processing (Figure 5). The gene encoding p65 seems to be expressed at a very low level (Nolan et al., 1991; Ruben et al., 1991), which is consistent with our finding that p65 protein is barely detectable in Western blots using total protein from various cell types. On the other hand, the IκB protein MAD-3 gives a much stronger signal in Western blots, suggesting that it is present in excess over p65. This imbalance would ensure a tight control of IκB over the *trans*-activating p65. There is good evidence that IκB proteins can inhibit DNA binding and nuclear uptake of p65 that is not yet complexed with p50 (Urban et al., 1991; Beg et al., 1992; Zabel et al., 1993).

The complexes ultimately used for assembly of the inducible heterotrimeric p50–p65–IκB complex in the cytoplasm would then be p50–IκB-γ and p65(homodimer)–IκB(-α or -β/MAD-3) (Figure 5). Future studies must explore whether there is a spontaneous exchange reaction releasing IκB-γ when p50–IκB-γ encounters p65–IκB, or whether additional proteins are required to control the process of assembly. In several studies, intact IκB-γ could not be detected in cells (Fan and Maniatis, 1992; Inoue et al., 1992b; Henkel et al., 1992), which could be explained by an extreme lability of the protein within living cells. By selective

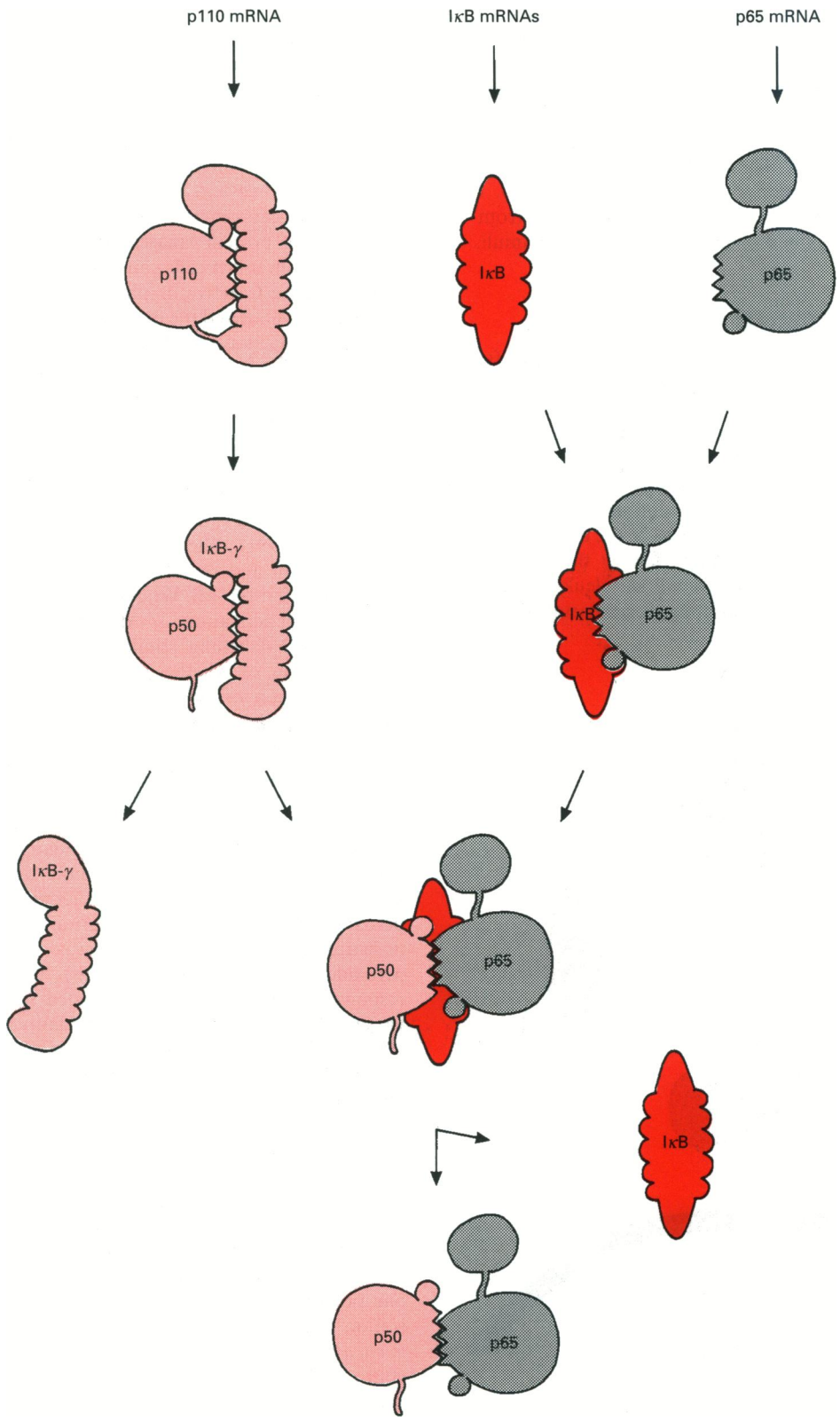

Figure 5 A model for the biogenesis of the inducible form of NF-κB

The inducible cytoplasmic form of NF-κB is assembled from the products of at least three different genes, encoding p110, IκB and p65. While the p110 and MAD-3 genes are inducible, the p65 gene appears to be constitutively expressed at low levels. For reasons of simplicity, the model shows a dimer of p65 and IκB, but a p65 homodimer with one or two bound IκB molecules is more likely to occur. p110 is shown as an intramolecularly folded molecule that, even after proteolysis, does not release p50. This assures that no inhibiting p50 homodimers are formed that could constitutively enter the nucleus. By an as yet unknown process, p50 is released from IκB-γ and incorporated into the complex with p65 and another IκB. Release of the IκB protein finally triggers gene activation by the p50–p65 heterodimer. An alternative pathway is direct association of p65 with p110. This complex must then undergo proteolysis prior to or during activation in order to yield p50.

degradation of IκB-γ, the reaction between p50–IκB-γ and p65–IκB could be shifted towards formation of the p50–p65–IκB trimer.

An alternative and much simpler assembly of an inducible cytoplasmic NF-κB complex could occur via p110. Newly synthesized p65 would in this model associate with p110 present in excess in the cytoplasm. The p110–p65 complex has then to undergo proteolysis in order to generate functional p50. It is presently unknown whether IκB-γ is released from a p50–p65–IκB-γ complex in response to extracellular stimuli.

CONTROL OF NF-κB ACTIVATION

In centre stage of the control of NF-κB transcription factor activity are inhibitory subunits rather than pretranslational regulatory steps. Activation of the factor appears to simply require disruption of the interaction between IκB and DNA-binding subunits (Figure 6). Both types of subunits could potentially serve as targets for dissociating reactions. Because *in vivo* activated nuclear p50–p65 is susceptible to inhibition by purified IκB, IκB rather than p65 seems to be the target (Baeuerle and Baltimore, 1988b). This is supported by the very recent finding that stimulation of pre-B cells with interleukin-1, tumour necrosis factor, phorbol ester and lipopolysaccharide all induce a discrete mobility decrease of IκB followed by a depletion of the protein (T. Henkel and P. Baeuerle, unpublished work).

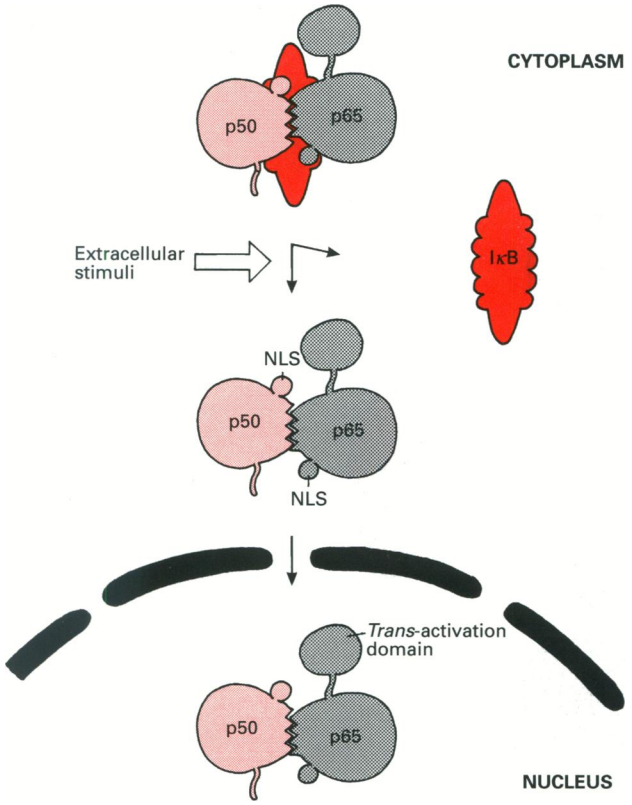

Figure 6 A model for the activation of NF-κB in response to extracellular stimuli

For details see the legends to Figures 1 and 5. The nuclear location signals of p50 and p65 are shown masked by a single IκB subunit. IκB is perhaps exclusively bound via the p65 subunit. Stimulation by a great variety of extracellular signals releases IκB, a reaction not only unmasking the NLS on both p50 and p65 but also restoring nuclear uptake and high-affinity DNA binding of the heterodimer. Finally, binding of NF-κB to enhancer elements in nuclear DNA initiates transcription, a process requiring the strong *trans*-activation domain in the p65 subunit.

In cell-free systems, various reactions were reported to activate NF-κB through release of IκB, including treatments of NF-κB–IκB complexes with sodium deoxycholate, low pH, protein kinases and protein phosphatases (see above). While detergents and low pH might not be physiological activators, phosphorylation events are known to control protein activities in many biological systems. Pharmacological support for an involvement of protein kinases and phosphatases in NF-κB activation within intact cells comes from the inducing effect of the protein kinase C activator phorbol myristate acetate (Sen and Baltimore, 1986b) and the protein phosphatase inhibitor okadaic acid (Thévenin et al., 1991), and from the inhibiting effect of the tyrosine kinase inhibitor herbimycin A (Iwasaki et al., 1992). However, these findings do not prove a direct phosphoryl transfer onto IκB, as observed by *in vitro* kinase experiments (Ghosh and Baltimore, 1990). The only direct evidence that phosphorylation of IκB proteins influences IκB activity in intact cells comes from the observation that a phosphatase treatment of purified IκB-β and pp40 abolishes their inhibiting activity (Link et al., 1992; Kerr et al., 1991). The proteins were apparently purified in a phospho form and required the bound phosphate in order to bind and inhibit NF-κB. Whether this modification is involved in the process of activation, or rather has a modulatory role, is not known. $^{32}PO_4$-labelling studies with intact cells will allow a demonstration of whether there are changes in the state of phosphorylation of IκB proteins in response to physiological stimuli. Mutational analysis of sites must finally demonstrate the functional significance of any modification identified. Of particular interest will be the question whether the various IκB proteins respond differently to various inducing conditions.

One common intracellular reaction induced by many, if not all, NF-κB-activating stimuli is oxidative stress (reviewed in Schreck et al., 1993). There are now three lines of evidence suggesting that reactive oxygen intermediates, most probably peroxides, play a role in mobilization of NF-κB. (i) NF-κB is post-translationally activated by low concentrations of hydrogen peroxide (Schreck et al., 1991). (ii) Activation of NF-κB in response to all inducing agents tested so far is blocked by a variety of chemically distinct antioxidants (Schreck et al., 1991, 1992, 1993). (iii) Reports in the literature describe induction of oxidative stress by many agents activating NF-κB, for instance, tumour necrosis factor, interleukin-1, phorbol ester, lipopolysaccharide, anti-IgM and u.v. light (reviewed in Schreck et al., 1993). These observations suggested that NF-κB is an oxidative stress responsive transcription factor, and that reactive oxygen intermediates play a messenger function in the activation of the factor. How they can cause the release of IκB is not understood. Direct oxidative modification of IκB, as demonstrated for the prokaryotic factor oxyR (Storz et al., 1991), is one possibility; but the rather slow kinetics of NF-κB mobilization in response to H_2O_2 and the failure to activate NF-κB *in vitro* by treatment with reactive oxygen intermediates (Schreck et al., 1991) calls for other proteins sensing and transducing the signal to the cytoplasmic NF-κB complex. After all, (oxidative stress-responsive) protein kinases are very likely to be involved.

HETEROTYPIC DIMERIZATION AND THE RELATIONSHIP OF NF-κB TO OTHER MEMBERS OF THE FAMILY

There are currently five proteins in higher vertebrates known to contain the NRD domain (for reviews see Blank et al., 1992; Nolan and Baltimore, 1992). p50/p110 and p65 have been described in detail here. p49/p100 (also called p50B) is a protein

highly related to p50/p110 (Schmid et al., 1991; Bours et al., 1992; Neri et al., 1992; Mercurio et al., 1992). It is therefore possible that p49/p50B fulfils the same 'helper' function as p50, and that p100 contains an inhibitory activity in its C-terminal half. A protein highly related to p65 is the proto-oncogene c-Rel (reviewed in Gilmore, 1990). Also c-Rel has strong *trans*-activating potential (Bull et al., 1990). Depending on the investigators, the fifth protein is called Rel-B (Ryseck et al., 1991) or I-Rel (Ruben et al., 1992). Rel-B was identified as immediate-early serum responsive factor and found to have *trans*-activating activity. A cDNA encoding I-Rel was isolated with the help of a PCR product amplified with degenerate DNA primers homologous to the NRD domain of p50. Although Rel-B and I-Rel seem to be identical, I-Rel was reported to have inhibitory activity. Future studies should address this controversy.

Apart from forming homodimers, it seems that most NRD family members can *in vitro* form heterodimers among each other, as tested with recombinant or *in vitro* translated proteins by immunoprecipitation or EMSA. Furthermore, all five NRD proteins (and various combinations thereof) can form complexes with the κB motif 5′-GGGACTTTCC-3′ and positively or negatively affect *trans*-activation from κB-controlled reporter genes in transient transfection assays. This led to confusion about the composition of 'NF-κB-like complexes' detected in nuclear extracts by EMSA. A similar problem was encountered earlier with the factor AP-1 when it became clear that there is extensive heterotypic dimerization between members of the Jun/Fos/Fra/CREB family of basic/leucine zipper *trans*-activators (reviewed in Lamb and McKnight, 1992). Therefore, in future experiments, the following questions should be addressed. (1) Do all possible heterodimers (and homodimers) of NRD proteins exist in living cells? Or, perhaps, does the controlled assembly of subunits only allow certain subunit combinations? As detailed in this Review, there is very good evidence that the combination of p50 and p65 is of physiological relevance. Also, p65 and c-Rel can apparently form heterodimers in cells, but these seem to have a DNA-binding specificity markedly distinct from that of p50–p65 (Hansen et al., 1992). U.v.-crosslinking studies suggested that p50 and c-Rel can form complexes in activated T cells during a later stage of the activation process (Molitor et al., 1991). (2) Do the NRD proteins vary in their tissue- and cell type-specific expression? If so, only certain combinations would occur in a given cell type, the number of which might be further limited by a controlled assembly. (3) Do physiologically relevant complexes of p50 and p65 with other members of the NRD family have the same sequence specificity as NF-κB and do they bind to κB motifs with the same high affinity? The determination of affinity constants and assay methods to detect DNA target sequences will be required to address this question.

CONCLUSIONS

Future studies on the subunits of the NF-κB system will focus on the fine structure and sequence motifs of the subunits. X-ray crystallography and n.m.r. techniques are required to understand in detail how DNA-binding subunits of NF-κB homo- and hetero-dimerize and how they contact DNA. The techniques will also be helpful to solve the structure and function of SWI6/ANK repeats in the specific interaction of IκB proteins with DNA-binding subunits. Important questions that need to be addressed in the near future are the following. What combinations of NRD proteins do really exist in living cells and what is their specificity? By what mechanism is IκB released from NF-κB upon stimulation? Finally, what is the role of NF-κB and IκB proteins in growth control?

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