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Common structural constituents confer IκB activity to NF-κB p105 and IκB/MAD-3

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The vertebrate NF-κB/c-*rel* inhibitors MAD-3/IκB α, IκB γ/pdI and *bcl-3* all share a conserved ankyrin repeat domain (ARD) consisting of six complete repeats, a short acidic motif and/or an incomplete seventh repeat. We present here a detailed analysis of the domain in p105/pdI and MAD-3/IκB involved in inhibition of DNA binding and in protein interaction with *rel* factors. We demonstrate that in both cases an acidic region and six ankyrin-like repeats are sufficient and required for protein interaction with the *rel* factors. However, for p105/pdI to achieve the high affinity needed to suppress DNA binding, an incomplete seventh repeat is required in addition. Both pdI and MAD-3 associate with *rel* proteins by forming heterotrimeric complexes, as shown by native gel analysis and by cross-linking. Furthermore, we demonstrate that deletion of only three amino acids in the first repeat converts the subunit specificity of the p105 ARD into that of MAD-3/IκB. We conclude that functionally the ARD in these molecules has a modular structure, with different subregions determining the specificity for the NF-κB subunits p50 and p65.

Key words: ankyrin repeats/gene expression/nuclear factor κB/signal transduction

Introduction

Nuclear factor κB (NF-κB) (for review see Lenardo and Baltimore, 1990; Baeuerle, 1991) belongs to a multigene family of transcription factors including the *rel* (proto)oncogene and the *Drosophila* morphogen *dorsal*. These proteins are involved in signal transduction and are subject to nuclear translocation control mediated by a class of cytoplasmic inhibitors (IκBs) (reviewed in Grilli *et al.*, 1993). Cloning of the genes encoding NF-κB subunits revealed a highly conserved DNA binding and dimerization domain shared by the NF-κB, *rel* and *dorsal* proteins (for review see Blank *et al.*, 1992). The p50 subunit is encoded as a 105 kDa precursor (p105), from which p50 must be produced by processing (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Fan and Maniatis, 1991; Meyer *et al.*, 1991). p105 contains in its C-terminal half ankyrin-like repeats, which were also found in p100, a protein highly related to p105 (Neri *et al.*, 1991; Schmid *et al.*, 1991; Bours *et al.*, 1992; Mercurio *et al.*, 1992). Several genes encoding IκB molecules have been isolated and their products also contained ankyrin-like repeats (for a review, see Nolan and Baltimore, 1992). The number of repeats varies between six in human MAD-3/IκB (Haskill *et al.*, 1991), and its avian and rodent homologues pp40 and RL/IF-1 (Davis,N. *et al.*, 1991; Tewari *et al.*, 1992), and six complete and one incomplete seventh repeat in *Bcl-3* (Ohno *et al.*, 1990; for sequence alignment see Hatada *et al.*, 1992). *Cactus*, the IκB-like regulator of the *Drosophila* morphogen *dorsal*, also contains six repeats (Geisler *et al.*, 1992; Kidd, 1992).

We and others showed that the C-terminal half of p105, when expressed as a separate molecule, can inhibit DNA binding of NF-κB in an IκB-like fashion (Hatada *et al.*, 1992; Inoue *et al.*, 1992a; Liou *et al.*, 1992). Protein products encoding this activity were identified only in murine lymphoid cells, and could represent a tissue-specific IκB form (Inoue *et al.*, 1992a). In contrast, no abundant C-terminal products of p105 have so far been identified in human cells (Fan and Maniatis, 1991; Hatada *et al.*, 1992). Recently, it has been demonstrated that the entire p105 protein displays IκB-like activity and associates in the cytoplasm with other members of the *rel* family (Rice *et al.*, 1992; Naumann *et al.*, 1993a). This property is also common to the precursor p100/lyt-10 (Naumann *et al.*, 1993b).

Molecules with IκB-activity derived from p105 require an intact ankyrin repeat domain (ARD) (Hatada *et al.*, 1992); for the proto-oncogene *bcl-3* (Ohno *et al.*, 1990), the ARD was reported to be sufficient for this function (Wulczyn *et al.*, 1992). In contrast, it has recently been claimed that pp40 and the *bcl-3* product require further C-terminal or N-terminal sequences for their IκB-like activities on p65 and p50, respectively (Inoue *et al.*, 1992b; Kerr *et al.*, 1992).

Because knowledge of the mode of interaction between *rel*-homologous factors and proteins with IκB activity is of crucial importance for understanding regulatory aspects, we set forth to characterize the minimal domains in MAD-3 and in p105 that are required for association with the target proteins and for inhibition of DNA binding. The stoichiometries with which the IκB molecules associate with the *rel* homology domain of the target transcription factors were analysed both by native gel electrophoresis and by crosslinking. We show here that both MAD-3/IκB and the p105-derived inhibitor bind only to dimerized *rel* factors, yielding heterotrimeric complexes.

By analysing a number of mutant proteins, we show for both p105/pdI and MAD-3 that six repeats followed by an acidic motif make up the smallest structure able to interact with NF-κB and, for MAD-3, to inhibit DNA binding. Strikingly, removal of the acidic stretch resulted in complete inactivation in both cases. For p105/pdI, an additional incomplete seventh repeat was required for inhibition of DNA binding. Furthermore, we demonstrate that the target specificity of p105/pdI can be switched to that of MAD-3/IκB by deleting only three amino acids. This deletion abrogates the ability of pdI to recognize p50 efficiently, without influencing recognition of p65. We

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therefore propose as a model that IκB domains are modular and that discrete subregions confer contacts to individual *rel* subunits in a dimeric transcription factor.

Results

The DNA binding inhibition domains of NF-κB p105 and MAD-3/IκB

We have demonstrated previously that the NF-κB precursor p105, as well as its C-terminal half (pdI, IκB γ), inhibit DNA binding of NF-κB p50, and that the conserved ankyrin repeats are required to confer this activity (Hatada *et al.*, 1992; Naumann *et al.*, 1993).

To delineate the domains of p105 and MAD-3 which inhibit the DNA binding of p50 and p65, we investigated the ability of various p105 and MAD-3 constructs to dissociate DNA complexes. Figure 1A shows the p105 constructs schematically and summarizes their relative efficiencies of inhibition of DNA binding, as determined by electrophoretic mobility shift assays. Figure 1B shows the bacterially expressed proteins. The entire C-terminal half of the p105 precursor was the most efficient molecule and suppressed DNA binding of p50 and Δp65 to the same extent (construct A). Its affinity for p65 is comparable to that of MAD-3/IκB (see below). A deletion of 74 residues at the C-terminus (construct B) reduced the inhibitory effect by ~5-fold. Another reduction was observed with further C-terminal (F) or N-terminal deletions (constructs M, G and H), all displaying similar affinity as long as the ankyrin repeat domain (ARD) remained intact. In fact, the ARD alone (mutant H), which consists exclusively of six repeats followed by a short acidic stretch and a seventh incomplete repeat, could still suppress DNA binding of both p50 and p65. Disruption of the ARD by deletion of either the seventh repeat (mutant J), or of both the seventh repeat and the acidic region preceding it (mutant K), or of the first ankyrin repeat (mutant L) led to complete inactivation.

Thus, the ability to dissociate DNA complexes of p50 and p65 indistinguishably is conferred to p105 by residues 540–810, encoding six ankyrin repeats, one incomplete repeat and a short acidic region.

To determine whether pdI/p105 and MAD-3/IκB require comparable or distinct domains, we next investigated the region of MAD-3 required to inhibit DNA binding of p65. MAD-3 has six ankyrin repeats followed by an acidic region, a structure which is also conserved in pp40, *cactus* and p105 (for repeat alignment see Hatada *et al.*, 1992 and Kidd, 1992). MAD-3 constructs with deletions N-terminal to the first ankyrin repeat or C-terminal to the acidic region had only very slightly lower DNA inhibition efficiencies than the full-length molecule (Figure 2A, constructs Q, R and S). However, deletion of the acidic region (protein T) led to a total inactivation of the IκB molecule. Therefore, the minimal inhibitory domain in MAD-3 comprises the six ankyrin repeats and the acidic region, as represented by deletion construct S.

A deletion of three amino acids in the first ankyrin repeat alters subunit recognition

Much to our surprise, a deletion of only three amino acids at the beginning of the first repeat of pdI/p105 (Figure 1A, mutants I versus H) led to a sharp discrimination between p50 and p65: while inhibition of p65 DNA binding was unaltered, this mutant protein was entirely inactive on p50.

Thus, the newly generated subunit specificity resembles that known for IκB/MAD-3 (Haskill *et al.*, 1991). In fact, construct H of the pdI protein (pdI-H) was active on all three DNA binding proteins including c-*rel* (Figure 3A, upper panel), whereas removal of its first three residues (producing pdI-I; Figure 3A, middle panel) led to a loss of affinity for

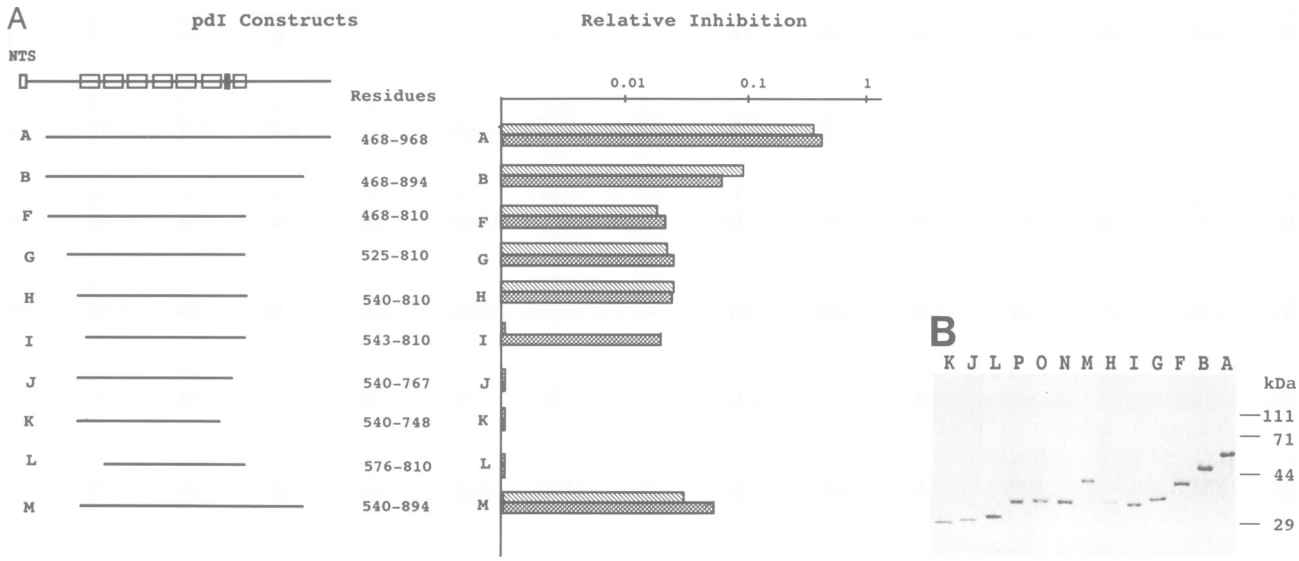

Fig. 1. (**A**) Schematic diagram of the pdI constructs and their relative efficiencies of inhibition on p50 and Δp65. The upper line to the left represents the C-terminal half of p105 (pdI), starting at the nuclear translocation signal (NTS), and containing ankyrin-like repeats (open boxes) and an acidic stretch (black box). The numbers refer to amino acid residues in the p105 precursor (Meyer *et al.*, 1991). The graph to the right represents the relative efficiencies of inhibition of each of the pdI constructs on p50 (upper hatched bars) and Δp65 (lower crisscrossed bars) DNA binding. Bacterially expressed pdI mutants were titrated against constant amounts of the NF-κB subunits (0.15 pmol and 0.2 pmol for p50 and Δp65, respectively), which were preincubated with DNA. EMSAs were quantified and the relative inhibition rates calculated as the molar ratios of *rel* dimer/inhibitor required for 90% inhibition of DNA binding. Each bar is the average of three or four experiments. (**B**) SDS–PAGE of bacterially expressed proteins: pdI deletion mutants (A, B, F, G, I, H, M, L, K and J) and point mutants (N, O, P).

p50, even at a 60-fold molar excess, but the recognition of DNA complexes with p65 or c-*rel* was unchanged. As a comparison, MAD-3 (construct R) effectively dissociated DNA complexes with p65 and c-*rel*, but not with p50 (Figure 3A, lower panel). That MAD-3 has a higher affinity than the p105/pdI deletion mutants is probably due to the loss of the flanking protein sequences of p105/pdI, as shown in Figure 1.

Thus, specific recognition of different *rel* factors can be altered by a short deletion and, consequently, must involve separate sub-regions of the ARD.

To rule out a potential influence of the unique C-terminal sequences in p65, which are deleted in Δp65, we analysed inhibition of full-length p65 and native NF-κB. Both pdI-A and MAD-3 also interacted efficiently with full-length p65 (Figure 3B, upper panel) or with TPA-induced nuclear NF-κB (lower panel). The latter was also inhibited by both pdI-H and pdI-I, presumably due to the presence of the p65 subunit in native NF-κB.

To determine whether conversion of the three specificity-determining residues (Asp-Glu-Asn) in p105 to those in MAD-3 (Thr-Glu-Asp) would change the target specificity, we tested pdI mutant proteins containing the sequences Thr-Glu-Asn, Asp-Glu-Asp and Thr-Glu-Asp (mutants N, O and P, respectively, in Figure 1B). Surprisingly, all point mutants displayed unchanged interaction with both p50 and p65 (data not shown), suggesting that the deletion did not simply remove residues directly involved in contacting p50. We assume that the deletion led instead to a conformational effect on adjacent sequences.

We also analysed MAD-3 for the effect of a deletion of the three amino acids. In fact, this deletion completely abolished the interaction with p65 (Figure 2A, mutants V versus S). A mutation of only the first amino acid (from Thr-Glu-Asp to Asp-Glu-Asp) was sufficient to reduce the inhibition efficiency drastically (mutants U versus S). This is surprising, because in pdI/p105 the corresponding point mutation did not affect repression of p65 DNA binding. These results indicate that further, possibly non-identical residues in pdI and MAD-3 are involved in determining subunit recognition of p65.

Subunit composition of rel–IκB complexes

The ability of IκB molecules to dissociate NF-κB–DNA complexes *in vitro* (Baeuerle and Baltimore, 1988; Zabel and Baeuerle, 1990), suggestive of a nuclear role for the inhibitors, appears inconsistent with their major function *in vivo*, which is to control nuclear translocation. Although the relationship between these activities has not yet been established, it will be essential to determine the subunit composition of NF-κB–IκB complexes in order to understand the two processes.

After having defined the domains of p105 and MAD-3 required for inhibition of DNA binding, we then asked in what molar proportions the inhibitors interact with the NF-κB subunits. For this purpose, a high pH native PAGE system was used (see Materials and methods). A p50 mutant (p42), which contains only the conserved *rel* homology domain RHD (amino acids 1–368, Kretzschmar *et al.*, 1992) and has a high pI value (7.23), migrated slowly (Figure 4A, lane 1). Ferguson plot analysis (Hedrick and Smith, 1968) indicated that this mutant migrates as expected for a dimer (not shown). The p105/pdI mutants H and B, which have acidic pIs, migrated rapidly towards the anode (Figure 4A, lanes 3 and 5, respectively). Incubation of the p50 RHD and pdI-H together resulted in a complex with intermediate mobility which sequestered all of the p50, leaving only a small excess of free inhibitor (lane 2). Similarly, p50 and pdI-B formed a complex with mobility intermediate to that of p50 and the p50–pdI-H complex (lane 4). To determine whether one or two pdI molecules could bind to a p50 dimer, both pdI-B and pdI-H were added to p50. Only two complexes were observed, corresponding in mobility to those seen with p50 and pdI-B, and p50 and pdI-H; in addition, there was a small amount of excess pdI-H (lane 6). Since no complexes with intermediate mobility were observed, this indicates that only one pdI molecule can interact with a p50 dimer.

This conclusion was supported by analysing the complexes in a 2D-gel (Figure 4B). The complexes generated by p50 and pdI-H and -B were initially separated in a native gel (as in Figure 4A, lane 6). The lane was excised and further analysed in the second dimension by SDS–PAGE (Figure 4B). As expected, the upper complex consisted of p50 and pdI-B, whereas the lower complex contained p50 and pdI-H. The stoichiometries in the complexes, as estimated by Coomassie staining, appeared consistent with two molecules of p50 bound by one molecule of pdI. Therefore, we conclude that p50 dimers associate with pdI to form heterotrimeric complexes. The clear separation between B and H in the second dimension electrophoresis excludes the formal possibility that a heterotetrameric $(p50)_2$–pdI-B–pdI-H form was migrating very close to one of the complexes in the native gel.

Surprisingly, incubation of p50 with pdI-I, which was inactive on p50 in band shift assays (see above), also led

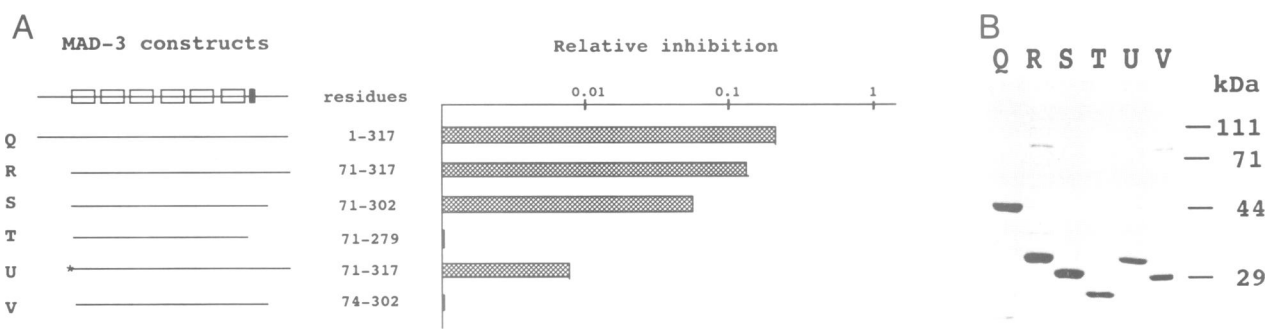

Fig. 2. The minimal inhibitory domain of MAD-3 includes an acidic region. (**A**) Schematic diagram of the MAD-3 constructs and their relative efficiencies of inhibition on Δp65 DNA binding, as described in the legend for Figure 1. Open boxes indicate ankyrin-like repeats and the black box, an acidic stretch. (**B**) SDS–PAGE of bacterially expressed MAD-3 mutants.

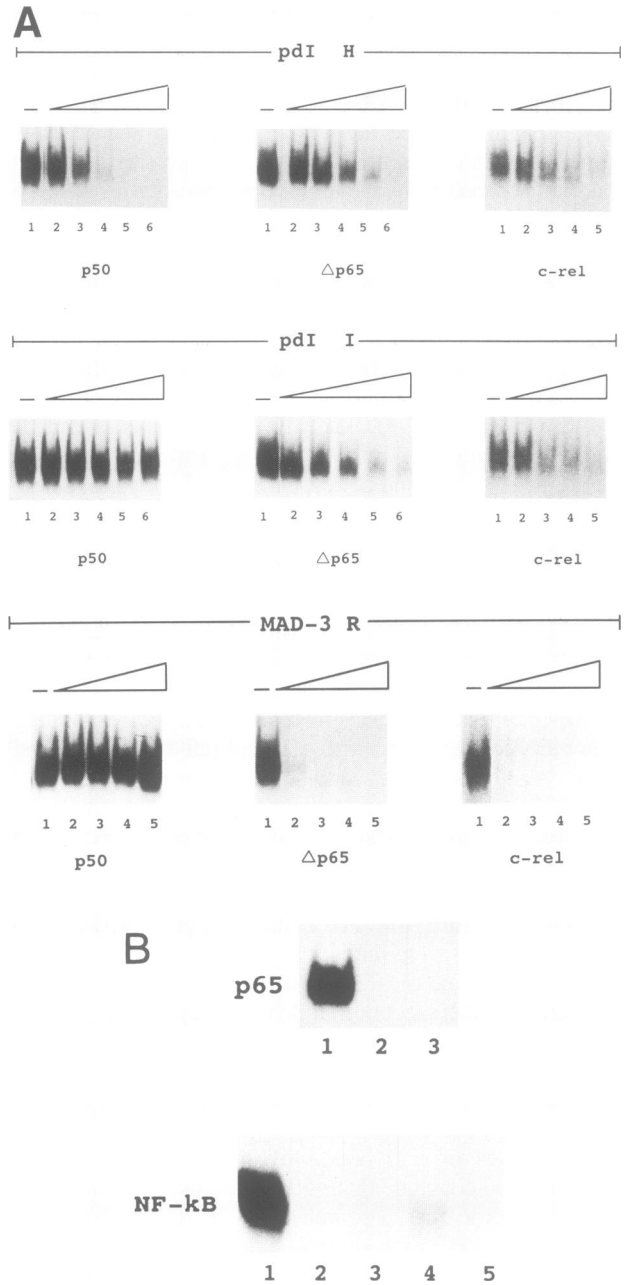

Fig. 3. (**A**) A three amino acid deletion in the ARD of pdI alters its substrate specificity. (Upper panel) The minimal repeat domain in pdI is an active inhibitor. Increasing amounts of pdI H (1, 3, 10, 30 and 100 pmol in lanes 2, 3, 4, 5 and 6, respectively) were added to p50, Δp65 and c-*rel*, in EMSAs. (Middle panel) A deletion of the first three residues in the ARD of pdI changes its substrate specificity to that of MAD-3. pdI mutant I was added (1.4, 4.6, 14, 46 and 92 pmol in lanes 2, 3, 4, 5 and 6, respectively) to the DNA binding reaction. (Lower panel) MAD-3 suppresses DNA binding of p65 and c-*rel* but not p50. Deletion mutant R of MAD-3 was mixed in increasing amounts (1.4, 4.2, 14 and 42 pmol in lanes 2, 3, 4 and 5, respectively) with the same DNA binding factors. The amounts of NF-κB/*rel* proteins were 0.15 pmol for p50, 0.2 pmol for Δp65 and 30 pmol for c-*rel*. Free DNA is not shown. (**B**) (Upper panel) The unique C-terminal sequences in full-length p65 do not affect inhibition by MAD-3 or pdI. pdI-A (7 pmol, lane 2) or MAD-3 Q (10 pmol, lane 3) were incubated with 0.5 pmol of p65 (lanes 1–3) and subjected to EMSA. (Lower panel) pdI-H and -I proteins have the same effect on cellular NF-κB as on Δp65. Nuclear extract from TPA-stimulated HeLa cells was incubated without any inhibitor (lane 1) or with pdI-A (1.8 pmol, lane 2), pdI-H (3.4 pmol, lane 3), pdI-I (3.4 pmol, lane 4) or MAD-3 Q (2.5 pmol, lane 5) and subjected to EMSA.

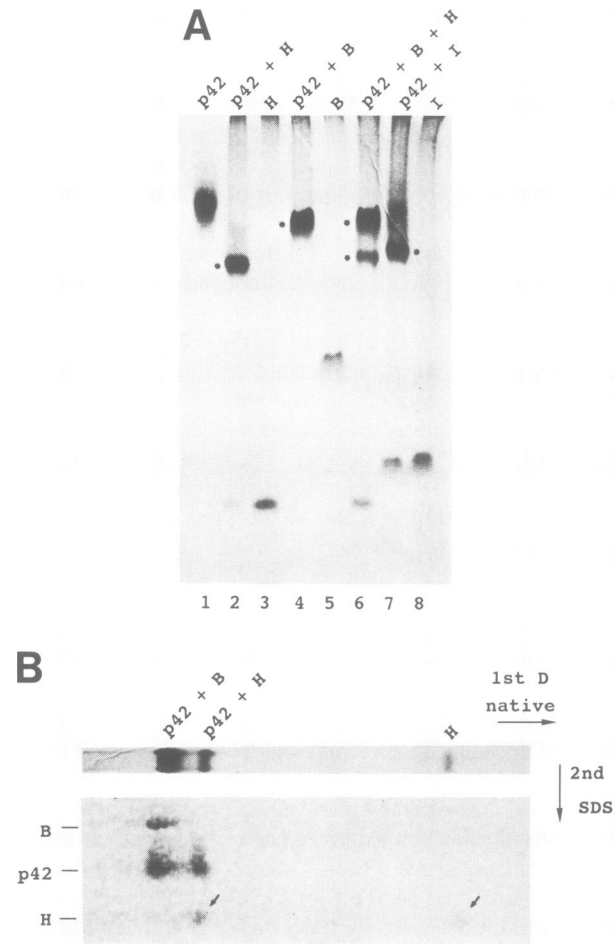

Fig. 4. Detection of heterotrimer formation between pdI and p50. (**A**) pdI mutants B (112 pmol), H (140 pmol) and I (195 pmol) were incubated with 70 pmol (dimer) p42, a shorter form of p50, and applied to a native gel. The resulting complexes are indicated by asterisks. In panel (**B**), a lane equivalent to that shown at the top was excised from a native gel and overlaid on a second dimension SDS–PAGE. The arrows at the top right corner point to the direction of migration in each dimension. The individual components of each complex are indicated to the left. The positions of the fainter bands of mutant H are marked by small arrows. The gels were stained with Coomassie brilliant blue.

to the formation of a complex (Figure 4, lane 7) but with apparently lower affinity, because there were residual amounts of free p50 and pdI. Due to the high protein amounts used, native gels thus represent a very sensitive system for detecting even low affinity interactions and may reveal associations not observed in band shift assays.

Ferguson plot analysis (data not shown) of the native gels indicated that pdI-I and -H migrated as monomers and p50 as dimers, suggesting a close to globular shape. Complexes of p50 and pdI migrated more slowly than expected for heterotrimers, which can be attributed to a non-globular structure of the complexes.

To rule out the influence of molecular shape in the native gel system, we complemented our analysis with cross-linking assays and investigated the interaction of pdI/p105 and of MAD-3/IκB with p65 (Figure 5). Δp65, containing almost exclusively the conserved *rel* homology domain, was cross-linked to form homodimers (lanes 1+2 and 7+8), whereas pdI-H was essentially monomeric (lanes 5 and 6). When Δp65 was mixed with pdI-H (lanes 3 and 4), two complexes

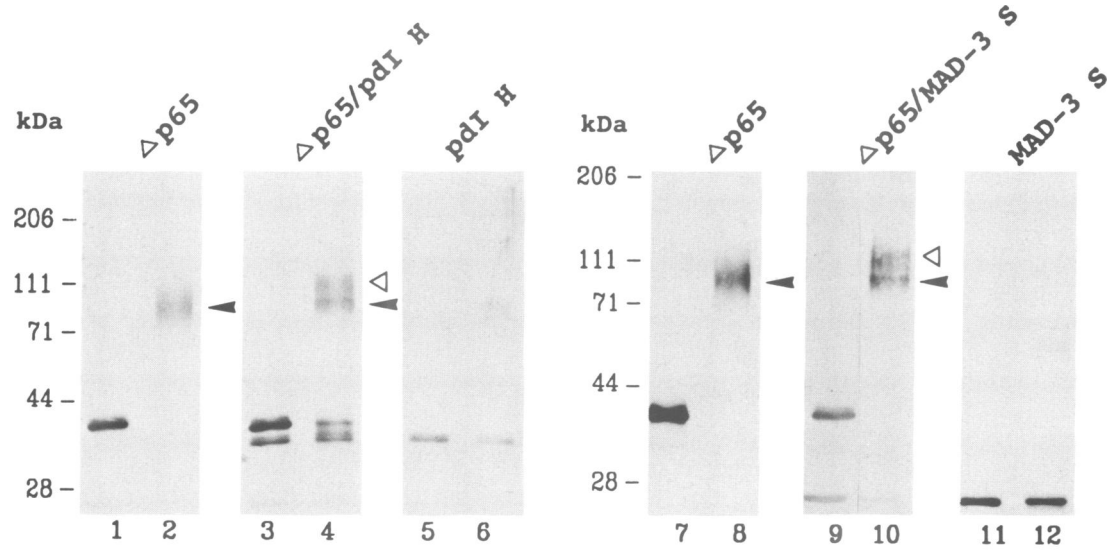

Fig. 5. Cross-linking analysis of the complexes between Δp65 and pdI and MAD-3. Δp65, pdI-H and MAD-3 S were subjected to glutaraldehyde cross-linking, separated in an SDS gel, blotted and visualized by an antibody against the gene 10 epitope, as described in Materials and methods. The odd-numbered and even-numbered lanes show the proteins without and with glutaraldehyde, respectively. Black arrowheads indicate Δp65 dimers, whereas triangles show the complexes between Δp65 and pdI or MAD-3. The molecular weight markers are indicated to the left.

with apparent molecular weights of ~85 and ~120 kDa were observed, which are consistent with a dimer of Δp65 and a heterotrimer of the composition $(\Delta p65)_2$–pdI-H, respectively. Similarly, MAD-3 (construct S), did not form homodimers (lanes 11 and 12), but formed a complex with Δp65 of ~110 kDa, corresponding to a heterotrimer, which migrated more slowly than dimeric p65 (lanes 9 and 10). The controls with pdI and MAD-3 alone show a faint amount of homodimerization in both cases (lanes 6 and 12), which may indicate a weak interaction of the IκB molecules with themselves.

In summary, our native gel electrophoresis system and the chemical cross-linking analysis support the model of a heterotrimeric association of both p105/pdI and MAD-3/IκB with dimeric NF-κB subunits.

An acidic region in both pdI and MAD-3 is essential for interaction with p50 and p65

In order to determine whether the domain required for inhibition of DNA binding of *rel* factors coincides with a minimal protein interaction domain, we further analysed pdI and MAD-3 mutants in the native gel system for their affinity for p50 or p65 (Figure 6). p105/pdI protein J, lacking the seventh repeat, and pdI-K, devoid of the seventh repeat and the acidic region preceding it, neither of which inhibited DNA binding (Figure 1A), were analysed for interaction with p50. Although most of the p50 and pdI-J molecules did not react, a small amount of a new complex could be detected (Figure 6, lane 3, asterisk), whereas pdI-K was totally inactive (lane 2). Thus, even though pdI-J cannot inhibit DNA binding, it can still interact weakly with its substrate. However, removal of the acidic region in pdI-K abrogates any interaction.

The same holds true for MAD-3, in that a construct with six repeats, but without the acidic region (MAD-3-T) (Figure 6, lane 9) did not associate with Δp65 (MAD-3-T was not well stained with silver, even though it was present in the same molar amount as the other truncated MAD-3 proteins). In contrast, mutant S, consisting of six repeats plus the acidic region, interacted strongly with Δp65 (lane 7),

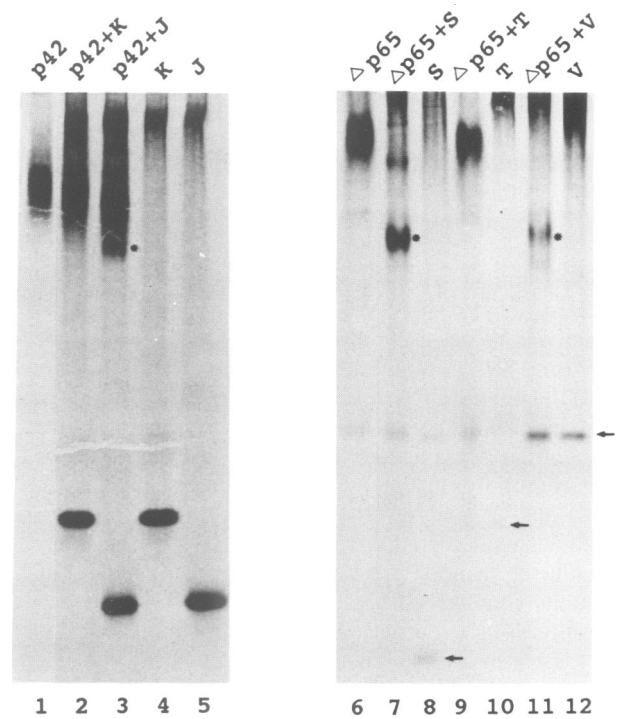

Fig. 6. The acidic region in both pdI and MAD-3 is necessary for physical interaction with NF-κB. pdI mutants K (350 pmol, lanes 2 and 4) and J (250 pmol, lanes 3 and 5) were incubated with 140 pmol of p42 (lanes 1–3) and applied to an 8% acrylamide native gel. In the same manner, Δp65 (35 pmol in lanes 7, 9 and 11; in lane 6, 70 pmol) was mixed with 60 pmol each of the MAD-3 constructs S (lanes 7 and 8), T (lanes 9 and 10) and V (lanes 11 and 12). The proteins were visualized by Coomassie blue (left panel) or by silver staining (right panel). The arrows point to the MAD-3 mutant protein bands, and the asterisks, to the p42–pdI and Δp65–MAD-3 complexes.

in accordance with its efficiency of inhibition in EMSAs. Interestingly, MAD-3-V, in which the first three residues of the first ankyrin repeat were deleted, was able to interact weakly with Δp65 (lanes 11 and 12), although it had completely lost its inhibitory function in EMSAs. This is

similar to pdI-I, which did not inhibit DNA binding, but still could interact weakly with p50 (Figure 4A, lanes 7 and 8).

Thus, pdI and MAD-3 require the same domain for protein–protein interaction with p50 or p65, consisting of six ankyrin repeats and the acidic region. In both cases, deletion of the three first amino acids of the first repeat reduces the affinity. One may refer to this common motif as *rel* recognition domain (RRD). For p105/pdI, the protein interaction domain is not identical to the inhibitory domain, since pdI needs in addition the seventh incomplete repeat to inhibit DNA binding. The seventh repeat seems to be required to achieve the high affinity needed for inhibition of DNA binding. Therefore, it appears that the strength of protein–protein interaction of the RRD with *rel* factors determines abrogation of DNA binding. Moreover, it seems to be impossible to separate a protein association domain from a DNA dissociation moiety, since in no case were upshifted complexes observed.

Discussion

All IκB molecules known to date share a conserved structural element: the ankyrin-like repeat domain. To delineate functionally relevant domains and to determine their mode of interaction, we analysed the mechanism by which the IκB molecules MAD-3 and pdI/IκBc/p105 interact with NF-κB proteins. We found that a minimal structure, comprising six ankyrin-like repeats and a short acidic region, is in both cases required and sufficient to recognize specifically NF-κB proteins and is therefore referred to as *rel* recognition domain (RRD). Whereas this domain alone mediated inhibition of DNA binding by MAD-3, p105 additionally required a seventh repeat for acquiring the affinity needed to inhibit DNA binding.

Short deletions of three amino acids in the first repeat selectively reduced the affinity of pdI for p50, but not for p65 or c-*rel*. A similar deletion reduced the affinity of MAD-3 for p65. The exclusive effect on only one target protein for pdI suggested that separate subregions of the *rel* recognition domain may bind to p50 or p65 and c-*rel*.

Furthermore, native gel electrophoresis and chemical cross-linking analyses showed that both MAD-3 and pdI bound as monomers to p65 or p50 dimers, respectively, to form heterotrimers, presumably by contacting both NF-κB subunits simultaneously.

Ankyrin-like repeats have been found in a variety of proteins with diverse functions, including cell cycle control proteins, where they were first described (Breeden and Nasmyth, 1987), in erythrocyte ankyrin (Lux *et al.*, 1990) and GA binding protein β (GABP-β; Thompson *et al.*, 1991). They seem to constitute a scaffold structure which provides contacts to other molecules. Except for IκB molecules, ankyrin repeat domains have so far been analysed only in erythrocyte ankyrin and GABP-β, which contain 22 and four continuous repeats, respectively. Interaction of erythrocyte ankyrin with the anion exchanger heavily depended on the last two repeats (Davis,L. *et al.*, 1991). GABP-β required four repeats to interact with GABP-α efficiently, but the first two repeats were dispensable for a somewhat weaker interaction (Thompson *et al.*, 1991).

The fact that IκB molecules require at least six conserved repeats for interaction, together with the fact that dimerized NF-κB proteins were bound, suggest that the rather large region contacts the two *rel* subunits simultaneously.

The constructs tested in this study were based on our previous sequence alignment (Hatada *et al.*, 1992) of the ankyrin domains of p105, MAD-3 and *bcl-3*, which is thereby now experimentally verified. Inoue *et al.* (1992b) and Kerr *et al.* (1992) came to the conclusion that the repeat domain is not sufficient for interaction with *rel* factors. The discrepancy with our data can be explained by the fact that their constructs did not include the sixth repeat and the acidic region (in the case of pp40) or the sixth and the seventh repeats (in the case of *bcl-3*) according to our alignment (Hatada *et al.*, 1992). The *cactus* gene product also encodes six repeats, followed by an acidic motif, and in agreement with our data, proteins containing this structure were able to bind to *dorsal* and inhibit DNA binding (Geisler *et al.*, 1992; Kidd, 1992). Strikingly, the acidic region in *cactus* seemed not to be required for interaction with *dorsal* (Kidd, 1992), which may reflect the divergence between the NF-κB and *dorsal* systems. Our data are also in accordance with those of Blank *et al.* (1991), who showed that internal deletion of either the seventh repeat or the acidic motif in p105, which normally cannot bind to DNA, led to cytoplasmic complexes with strong DNA binding activities in transfected cells. Thus, removal of these regions could loosen the intramolecular contacts between the *rel* homology domain and the ARD in the precursor (Henkel *et al.*, 1992).

Whereas both p105 and MAD-3/IκB require the acidic motif for conferring protein interaction, *bcl-3* contains six complete and one incomplete seventh repeat (Ohno *et al.*, 1990) and this structure is both required and sufficient for binding to p50 and for inhibiting DNA binding (Wulczyn *et al.*, 1992). The incomplete seventh repeat of *bcl-3* is similar to that of p105 (Hatada *et al.*, 1992) and it might functionally replace a (lacking) acidic region in *bcl-3*. It is therefore possible that the acidic region and the seventh repeat contact distinct regions on the *rel* dimers and participate in discrimination of the target proteins. For example, MAD-3, containing an acidic motif, binds well to p65 and poorly to p50, whereas *bcl-3*, containing a seventh repeat, binds well to p50 but weakly to p65; p105, containing both substructures, binds efficiently to both p65 and p50. Alternatively, the absence of an acidic motif in *bcl-3* may indicate some functional peculiarities of *bcl-3*, such as the ability to form supershifts with NF-κB complexes under certain conditions (F.G.Wulczyn and C.Scheidereit, unpublished observations).

The conserved acidic motif in human, murine and avian p105 and in MAD-3, pp40 and RL/IF-1 comprises 20–23 residues with 9–12 net negative charges and is predicted to be α-helical in structure (not shown). Acidic regions are also known as activation domains in transcription activators and are believed to mediate protein interactions (Ptashne and Gann, 1990).

Intriguingly, the proteins of the *notch* group, consisting of *notch* (Wharton *et al.*, 1985), *xotch* (Coffman *et al.*, 1990), *tan-1* (Ellisen *et al.*, 1991), *int-3* (Jhappan *et al.*, 1992), *glp-1* and *lin-12* (Yochem and Greenwald, 1989), also have six ankyrin-like repeats, followed by a highly conserved sequence of 27 mainly hydrophilic amino acids. Whereas the repeats clearly fall into a different class as compared with the IκB repeats, the similar organization of the *notch* domains suggests that they might also require their entire six repeats and the proximal 27 amino acids to interact with their target

proteins, which might also be dimerized transcription factors.

Since the IκB molecules tested so far do not detectably bind monomeric *rel* subunits (E.N.Hatada, unpublished) and do not dissociate dimers, they may contact both subunits in a dimer. This is also supported by the observations that the minimal interaction domain for *bcl-3* on p50 coincides with the dimerization domain of p50 (Wulczyn *et al.*, 1992), and that a dimerization-deficient p65 mutant is not bound by MAD-3 (Beg *et al.*, 1992). We propose that IκB molecules in general may contact both *rel* subunits through common and specific subregions of the *rel* recognition domain (RRD).

By assuming a two-fold rotational symmetry of the *rel* dimer, the surfaces of each subunit, corresponding to opposite sides of non-dimerized proteins, would be exposed on one side. It is feasible that the RRD recognizes these surfaces, both of which would be required for stable association. Whereas some residues generally conserved in the *rel* subunits would provide some interaction, others are subunit-specific and are needed for high affinity interaction. This could explain why MAD-3 does associate with $(p50)_2$, but weakly, whereas both p50–p65 and $(p65)_2$ are recognized efficiently. Further experiments will be required to test this hypothesis.

We observed that a deletion of three residues at the beginning of the repeat domain of pdI generated a molecule with a subunit specificity similar to that of MAD-3 (Beg *et al.*, 1992). We do not believe that this is physiologically relevant, because no major C-terminal products of p105 were yet observed in human cells (Hatada *et al.*, 1992). The results point out, however, that minor alterations of the ARD can lead to an altered specificity of subunit recognition and hence, that subunit specificity appears to be a poor criterion for identifying IκB molecules. Furthermore, it cannot be excluded that post-translational modifications of IκBs may alter their target specificities.

We found that pdI and $(p50)_2$ interact to form heterotrimers. This observation is consistent with our earlier data that p105 associates with p50 to form heterodimers (Naumann *et al.*, 1993a). A p105–p50 heterodimer may form an intramolecular loop, bringing together the dimerized *rel* domains with the ankyrin repeat domain. It may thus resemble a $(p50)_2$–pdI complex, except for the covalent linkage of the pdI and one of the p50 moieties.

We failed to detect heterotetramer formation between p50 and pdI or MAD-3 and p65 in our experiments, whereas we showed recently that *bcl-3* can form both heterotrimers and heterotetramers with p50 (Wulczyn *et al.*, 1992). It is possible that under native gel conditions such tetramers are unstable. Alternatively, *bcl-3* may interact with p50 by an inherently different mode. The possibility that *bcl-3* can interact with heterotrimers containing MAD-3 to form heterotetramers is currently under investigation.

Materials and methods

DNA constructs

The p50 expression vector has been described elsewhere (Hatada *et al.*, 1992). Construction and characterization of p42, a shorter form of p50 comprising residues 1–368 of the precursor p105 protein, has been described (Kretzschmar *et al.*, 1992). For c-*rel*, the cDNA was digested with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of pET3c (Novagen). Full-length p65 and Δp65 (residues 1–310; Ruben *et al.*, 1991), and the pdI and MAD-3 (Haskill *et al.*, 1991) constructs were generated from cDNA clones by PCR using appropriate oligonucleotides and inserted into the *Bam*HI site of pET3c. The pdI and MAD-3 point mutants were generated by PCR using primers containing the desired changes. All PCR primers had *Bam*HI sites at their ends, preceded by a stop codon in the antisense oligonucleotides. The clones were verified by double-stranded DNA sequencing at their fusion points, which also confirmed the introduced point mutations. Proteins expressed from all the above constructs contained a gene 10 tag leader sequence at their N-termini, as a result of the cloning strategy.

Proteins were expressed in *Escherichia coli* BL21(DE3)pLysS, SDS gel-purified and renatured (Hager and Burgess, 1980) or isolated from Triton-washed inclusion bodies and renatured (Hatada *et al.*, 1992). Bacterial p42 was purified over BioRex-70 and one affinity step essentially as described for NF-κB purification (Meyer *et al.*, 1991), whereas Δp65 was only partially purified over Biorex-70.

Gel retardation assays/EMSAs

Gel retardation assays were performed as described by Kawakami *et al.* (1988). The DNA binding proteins were mixed with the probe, a double-stranded oligonucleotide containing the κB site from the mouse κ light chain enhancer (Kawakami *et al.*, 1988), prior to addition of the inhibitors. Full-length p65 was assayed in the presence of one-third of the usual concentration of poly(dI–dC).

Native gels

Complexes formed between *rel* factors and inhibitors were analysed in non-denaturing 8% gels. This discontinuous buffer system was essentially as described by Laemmli (1970), except that SDS was omitted from the solutions (Garfin, 1990). The samples were electrophoresed at 5 V/cm for 15 h. The gels were stained with silver or with Coomassie brilliant blue, depending on protein amounts. pI values were calculated using the GCG software (University of Wisconsin).

For Ferguson plots (Hedrick and Smith, 1968), the protein complexes were run on native gels with 6, 8, 10 and 12% total acrylamide concentration, using as standards ferritin, catalase, lactate dehydrogenase and albumin (Sigma).

Glutaraldehyde cross-linking

Cross-linking was essentially as described by Wulczyn *et al.* (1992) and Naumann *et al.* (1993a). Briefly, bacterially expressed proteins were incubated with 0.0025% glutaraldehyde for 1 h at room temperature. 1 pmol of pdI-H, MAD-3 S and Δp65 (calculated as monomer) were used. After quenching with 1/25 vol diethanolamine, samples were separated on SDS–PAGE and transferred to PVDF membrane (Millipore). The protein complexes were visualized by an antibody raised against the gene 10 epitope (Novagen).

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