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The NF-κB p50 precursor, p105, contains an internal IκB-like inhibitor that preferentially inhibits p50

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The p50 subunit of NF-κB is apparently synthesized as a precursor molecule of 105 kDa (p105); subsequent processing releases the amino-terminal p50 polypeptide with *rel* homology, DNA binding activity and transcriptional activation potential. The carboxy-terminal region of p105 contains seven copies of an ankyrin-related sequence previously found in several genes involved in differentiation and cell cycle control. Two proteins with IκB activity, MAD-3 and pp40, have been cloned and found to contain five obvious ankyrin repeats that align with those in the carboxy-terminus of p105. Both proteins target their inhibitory activity to the p65 subunit of NF-κB and to c-*rel*. Here we show that the bacterially expressed and purified carboxy-terminal region (CTR) of p105 abolishes the binding of p50 homodimers to a κB motif but minimally affects the binding of p65 homodimers and NF-κB. By contrast, MAD-3 inhibits the binding of p65 and NF-κB but not p50. Both the CTR and MAD-3 interact with their respective targets through physical association both *in vitro* and *in vivo*. The CTR can be expressed as an independent entity and thus may play two roles, as a *cis* inhibitor built into the p105 molecule and as a *trans* regulator of p50.

Key words: ankyrin repeats/IκB/NF-κB/p105

Introduction

NF-κB is a DNA binding transcription factor that regulates a variety of cellular and viral genes whose control regions contain κB binding motifs. It is a heterodimeric protein composed of polypeptides of molecular weights 50 and 65 kDa (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1989; Ghosh *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991). The genes encoding the p50 and p65 components share extensive similarity with the oncoprotein v-*rel*, its cellular counterpart c-*rel* and the *Drosophila* morphogen, *dorsal* (Gilmore, 1990). *rel*-related proteins have been physically, functionally or genetically associated with another group of proteins that inhibit their DNA binding activity called IκBs. IκBα and IκBβ are cytosolic inhibitors of NF-κB (Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990; Urban and Baeuerle, 1990; Zabel and Baeuerle, 1990; Kerr *et al.*, 1991); pp40 is an IκB-like molecule that can be co-immunoprecipitated with anti-*rel* antibodies (Simek and Rice, 1988; Tung, 1988); *cactus* is a *Drosophila* protein that has been genetically shown to counteract the function of the *dorsal* morphogen (Roth *et al.*, 1991). These inhibitors may affect subcellular localization of *rel*-related proteins but are assayed by their ability to modulate DNA binding.

Two cDNAs encoding IκB-like proteins have been cloned. MAD-3 was cloned by a substractive screening method; it is a cDNA representing an mRNA that is rapidly induced following adherence of human monocytes. *In vitro* translated MAD-3 protein specifically inhibits the DNA binding activity of NF-κB but not that of p50 dimer (known as KBF-1) (Israël *et al.*, 1987; Kieran *et al.*, 1990). Chicken pp40, which displays IκB-like activity *in vitro*, was also cloned as a cDNA (Davis *et al.*, 1991). Peptide sequences obtained from purified rabbit IκBα confirmed that both proteins are IκBs. MAD-3 and pp40 share extensive homology especially in their middle portions which contain ankyrin-like repeats previously found in several other proteins, including ankyrin, Notch, Lin-12/glp12 of *Caenorhabditis elegans* and CDC10 and SWI4/SWI6 in yeast as well as the GABP transcription factors (Lux *et al.*, 1990; Thompson *et al.*, 1991). The ankyrin repeats are thus present in proteins with diverse functions, such as differentiation and cell cycle control, and may function as general protein–protein interaction domains. So far, all of the IκB activities detected have as their targets the p65 subunit of NF-κB or c-Rel. Inhibitors directed at p50 have not been described.

The NF-κB p50 subunit was molecularly cloned as a full-length cDNA with potential to encode a precursor protein of 105 kDa; the 5′ portion of this cDNA is homologous to c-*rel* while the 3′ end encodes a segment with seven ankyrin repeats (Ghosh *et al.*, 1990; Kieran *et al.*, 1990). Processing of the p105 precursor is apparently required to generate the functional p50 and the carboxy terminus may modulate this processing event (Blank *et al.*, 1991; Fan and Maniatis, 1991). Full-length p105 binds DNA poorly but deletion of the carboxy terminus confers DNA binding capacity on the *rel* homologous region (Ghosh *et al.*, 1990; Kieran *et al.*, 1990). Thus, the carboxy-terminal portion of the precursor protein might directly or indirectly prevent binding of the amino-terminal portion to target DNA. Because the purified physiological inhibitors, IκBs, and the cloned IκB-like proteins, MAD-3 and pp40, also contain ankyrin repeats, we compared the sequence and activity of the carboxy-terminal portion of p105 (the CTR) with MAD-3 IκB. Our studies reveal that the CTR of p105 represents a novel inhibitor for the NF-κB/*rel* family of proteins that shows an inhibitory spectrum distinct from that of MAD-3. We also show that the inhibitors have distinct binding affinities for their targets. The existence of multiple classes of inhibitors for the NF-κB/*rel* family suggests that they may play distinct roles in regulating the activity of the NF-κB/*rel* family of activators.

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Results

Sequence comparison of CTR and MAD-3

The carboxy-terminal region (CTR) of p105 shares extensive homology with the IκB family of proteins. In the ankyrin repeat regions, CTR and MAD-3 have an overall identity of 44%. The homology of CTR to other proteins with ankyrin repeats (i.e. Notch) is considerably lower. The second and the fifth repeats are the most conserved amongst the five repeats (Figure 1A). The homology between each repeat at corresponding positions of different proteins is greater than the homology of different repeats within the same protein (Figure 1B). The apparent conservation of the order and alignment of each repeat suggests that the ankyrin repeats in the NF-κB/rel/IκB family may function as an integrated unit which has a role distinct from that of the repeats found in other proteins. The full-length p105 molecule binds DNA poorly, whereas deletion of the ankyrin repeats of the CTR reveals the DNA binding capacity of the amino-terminal, *rel*-related domain (Ghosh *et al.*, 1990; Kieran *et al.*, 1990). The CTR may therefore act as a *cis* IκB but focus on p50, in contrast to the p65 focus of the other IκBs (Baeuerle and Baltimore, 1989; Davis *et al.*, 1991; Haskill *et al.*, 1991). We therefore investigated the carboxy-terminal repeat domain of p105 for potential IκB-like activities.

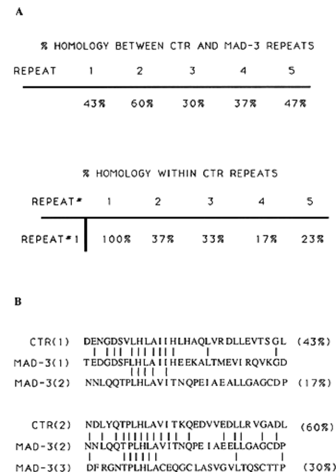

Fig. 1. The ankyrin repeats in the NF-κB/IκB protein family may function as an integrated unit, based on the conserved order and alignment of the repeats. **(A)** Percentage homology among the ankyrin repeats in CTR. Comparison of the ankyrin repeats between p105 CTR and MAD-3 reveals an overall 44% identity. The second and the fifth repeats are the most conserved amongst the five repeats. The homology between each repeat at corresponding positions of different proteins is greater than the homology of different repeats within the same protein. As an example shown at the bottom panel, the repeat 1 of CTR is compared with its repeats 2−5. **(B)** Homology comparison of ankyrin repeats in CTR and MAD-3. Identical amino acids are denoted by vertical bars. The number in parenthesis represents the order of the repeat in the entire molecule. Thus, CTR (1) refers to the first repeat of CTR. % represents the degree of identity when the corresponding peptide sequence is compared with the middle one. As an example shown here, the first repeats of both CTR and MAD-3 are compared with the second repeat of MAD-3. Systematic comparison among repeats of both CTR and MAD-3 shows the same trend that repeats at corresponding positions have a higher degree of homology than when compared with repeats of other positions.

Distinct inhibitory activities for CTR and MAD-3

To assay for effects of CTR on the properties of NF-κB and its subunits, CTR was expressed as an independent molecule consisting of amino acids 484−971 of p105 fused to glutathione-*S*-transferase (GST). This protein includes 54 amino acids 5′ of the first ankyrin repeat, all seven ankyrin repeats and the rest of the p105 C-terminal sequence. It was used in a standard assay of IκB activity, measured as inhibition of NF-κB binding to a DNA target sequence using the electrophoretic mobility shift assay (EMSA) (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988). As comparison and control we similarly expressed the IκB molecule encoded by MAD-3 also fused to GST. Both proteins were purified to near homogeneity (single bands on stained gels).

As targets of potential inhibition, three proteins were used, p50, p65-Δ and NF-κB reconstituted from p50 and p65. p65-Δ is a truncated form of p65 that binds specifically to the κB DNA site and can be inhibited by IκB (Nolan *et al.*, 1991; Fujita *et al.*, 1992). Constant amounts (0.5−1.0 nM, calculated as the monomer) of p50, p65-Δ or NF-κB were incubated with increasing amounts (0.1- to 15-fold molar ratio) of CTR−GST or MAD-3−GST prior to the addition of radiolabeled κB probe (the κB site from the κ light chain gene). Increasing amounts of CTR efficiently abolished the binding of p50 to DNA (left panel of Figures 2A and top panel of Figure 2B). The ratio of CTR to p50 for 50% inhibition was about 0.5. Under the same conditions, MAD-3 only slightly inhibited p50 DNA binding (decrease 22%) up to 5-fold excess molar ratio (Figure 2A, right panel; Figure 2B, top panel). The amount of MAD-3 for 50% inhibition of p50 could not be determined in the EMSAs, but estimation based on linear correlation suggested that MAD-3 had roughly a 50-fold lower affinity for p50 than CTR. Thus, CTR is a potent inhibitor for p50. By contrast and consistant with previous observations (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Haskill *et al.*, 1991), MAD-3 has a higher inhibitory activity for p65-Δ and NF-κB than does CTR (Figure 2B, middle and bottom panels). The molar ratios necessary for MAD-3 to reach 50% inhibition of binding for p65-Δ and NF-κB were 0.75 and 1.1, respectively. The inhibitory ratios of CTR to p65-Δ and NF-κB to reach 50% inhibition were 5.0 and 6.5, respectively (Figure 2B). CTR thus had a 10-fold lower affinity for p65-Δ and NF-κB than for p50. As a control, GST alone did not inhibit NF-κB binding at all (data not shown). In summary, CTR and MAD-3 have different inhibitory activities for the different NF-κB subunits: CTR inhibits p50 with the highest efficiency whereas MAD-3 has a higher affinity for p65 and NF-κB.

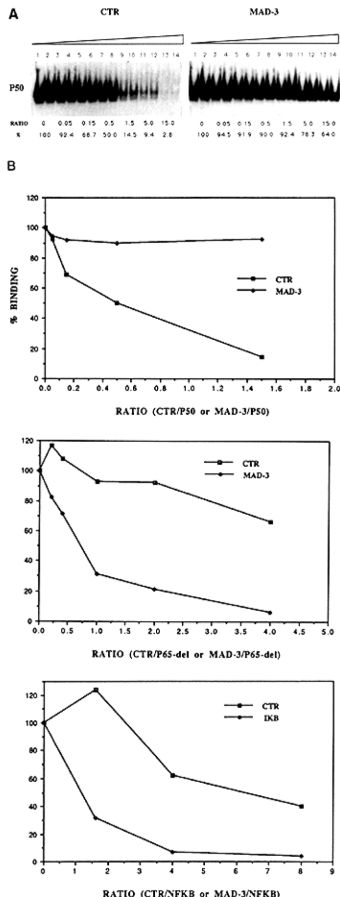

Fig. 2. CTR and MAD-3 have distinct inhibitory activities. **(A)** Differential inhibition of CTR and MAD-3 for p50. A constant amount of p50 protein (40 fmol, calculated as the monomer) was incubated with increasing amount of CTR–GST or MAD-3–GST protein at a molar ratio (CTR–p50 or MAD-3–p50) of 0 (lanes 1 and 2), 0.05 (lanes 3 and 4), 0.15 (lanes 5 and 6), 0.5 (lanes 7 and 8), 1.5 (lanes 9 and 10), 5.0 (lanes 11 and 12) and 15.0 (lanes 13 and 14). Analyses were by EMSA. The average percentage of binding relative to lanes 1 and 2 (control lanes without inhibitor proteins added) is shown below the gel. **(B)** Quantitative plots of EMSA inhibition analyses for p50, p65-Δ and NF-κB by the CTR and MAD-3. The data shown in (A) and other EMSAs of p65-Δ and NF-κB were quantified with a Phosphoimage analyzer. The retarded band of each sample was compared with the control (without any inhibitor protein) and is represented by percentage of binding (*y* axis). On the *x* axis is the ratio of tested inhibitors (CTR or MAD-3) to either p50 (40 fmol, top panel), p65-Δ (25 fmol, middle panel) or NF-κB (25 fmol, bottom panel) in a 20 μl EMSA reaction. Each point is the average of duplicate samples and is represented by square (CTR–GST) or diamond (MAD-3–GST) shaped dot.

Physical association studies

IκB can be co-purified from cells with the NF-κB dimer, suggesting a physical association between these molecules (Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990). The association of CTR with NF-κB subunits was therefore tested *in vitro*. p50 and p65 proteins were radioactively labeled in an *in vitro* translation system and incubated with GST, CTR–GST or MAD-3–GST protein, followed by precipitation with glutathione–agarose beads. CTR–GST clearly precipitated the p50 protein, whereas the control GST alone did not (Figure 3A, lanes 3 and 5). By contrast, MAD-3–GST precipitated only a fraction of input p50 at a high concentration of fusion protein (lane 4). This is consistent with the results obtained from the EMSA quantitative analyses (Figure 2A) in which a large molar excess of MAD-3 protein could only slightly inhibit p50 binding. When *in vitro* translated p65 protein was tested, both CTR–GST and MAD-3–GST could precipitate p65 in large molar excess over the translated p65 protein, whereas control GST alone could not (Figure 3B, lanes 3–5). These results strongly suggest that the inhibitory activities of CTR and MAD-3 proteins for specific *rel* polypetide targets is a function of relative affinity and confirm a direct physical association between inhibitor and target.

Co-immunoprecipitation experiments after co-transfection of both p50 and CTR expression constructs into 293 cells, transformed human embryonic kidney cells, suggests that physical association in cells between these molecules also occurs. p50 was tagged with an influenza virus hemagglutinin antigen epitope (HA). Cells were transfected with either p50–HA or CTR expression constructs or both and labeled with [^{35}S]methionine-containing medium. In lysates containing only transfected p50–HA, the anti-HA antibodies immunoprecipitated three bands, 46, 48 and 52 kDa (Figure 3C, lane 3), which all correspond to p50–HA by Western blot analysis (data not shown). (These three bands probably represent different modified forms of p50–HA protein.) As expected, the anti-HA antibody did not detect any specific complexes in the lysate containing the CTR-expressed protein because the CTR did not contain the HA tag (Figure 3C, lanes 4 and 5). In samples co-transfected with both p50–HA and CTR, the anti-HA antibodies specifically precipitated an additional protein of 56 kDa (similar to the expected size of the CTR protein) in addition to the p50–HA proteins (Figure 3C, lanes 6 and 7). Antibodies made against the CTR also reacted with this 56 kDa band, confirming that the 56 kDa band is indeed the CTR protein (data not shown). The co-immunoprecipitation of CTR protein with p50 suggests that CTR can form a complex with p50 *in vivo*.

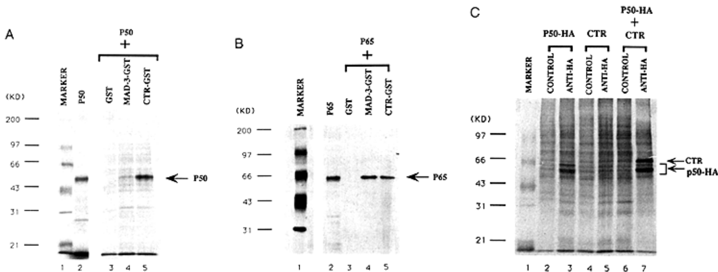

Fig. 3. CTR associates with p50 both *in vitro* and *in vivo*. **(A)** Co-precipitation experiments demonstrate that the CTR can precipitate translated p50 protein *in vitro*. **(B)** Both CTR and MAD-3 can precipitate *in vitro* translated p65 protein. The translated p50 (A) or p65 (B) lysates were incubated with either GST (lane 3) , MAD-3−GST (lane 4) or CTR−GST (lane 5) protein in ELB buffer [250 mM NaCl, 0.01% NP-40, 50 mM HEPES (pH 7.9) and 50 mM EDTA] for 1 h at 4°C. Glutathione−agarose beads were then added to the protein mixture and incubated further for 30 min at 4°C before washing the beads. The components of the resulting complexes were resolved by SDS−PAGE. The *in vitro* translated starting material, p50 or p65, is shown in lane 2. ^{14}C protein size marker (BRL) is shown in lane 1. **(C)** Co-immunoprecipitation of CTR with p50 *in vivo*. The calcium phosphate transfection method was used for transfection of 293 cells. About 5 μg of each construct DNA (CTR−CDM8-neo or p50−HA-CDM8-neo or both) was used per transfection. Two days after the transfection, cells were labeled with [^{35}S]methionine prior to immunoprecipitation. Immunoprecipitation was performed according to standard methods using either control mouse ascites (lanes 2, 4 and 6) or anti-HA ascites (derived from 12CA5 monoclonal hybridoma line) (lanes 3, 5 and 7). The arrows indicate the positions of the p50−HA (three bands) and CTR as determined by independent Western blots with appropriate antibodies (data not shown).

CTR is expressed as an independent RNA

Although the inhibitory activity of CTR for NF-κB might function *in cis*, with the carboxy-terminal portion of p105 preventing the amino-terminal p50 portion from binding to DNA, it is possible that free CTR might be encoded by an independent mRNA. A previous report showed two strongly hybridizing mRNAs, 4.0 and 2.6 kb, which derived from the p105 locus (Ghosh *et al.*, 1990). We therefore investigated the nature of these two transcripts. Poly(A)$^+$ RNAs prepared from the pre-B cell line 230−238, the mature B cell line WEHI 231 and the plasmacytoma cell line S194 were hybridized with probes corresponding to the amino-terminal *rel* homologous (p50) or CTR portions of p105. Both p50 and CTR specific probes hybridized to a 4.0 kb band corresponding to the previously characterized full-length mRNA (Figure 4). By contrast, only the CTR probe hybridized to the 2.6 kb band (Figure 4, right panel). This 2.6 kb RNA was expressed at comparable levels to the 4.0 kb RNA in the pre-B cell line 230−238, but was expressed five times less than the 4.0 kb full-length RNA in the more mature B cell and was almost undetectable in the plasmacytoma cell line. In previous work we had noted that the 4.0 kb RNA was widely expressed in all tested cells (Ghosh *et al.*, 1990). The 2.6 kb RNA was predominantly found in pro-B (LyD9) and pre-B cells (38B9; HY, an immature derivative of 70Z/3 (Schatz *et al.*, 1989; Oettinger *et al.*, 1990; and 230−238), but was barely detectable in all of the more mature B cells tested (unselected 70Z/3, WEHI231, S194 and MPC11) (Ghosh *et al.*, 1990). Thus, the expression of the 2.6 kb mRNA seems to be differentially regulated during B cell differentiation.

Cellular proteins containing CTR epitope

To confirm that the CTR protein can be expressed in cells, we performed immunoblotting analyses using antiserum raised against the non-ankyrin repeat portion of the CTR (deAR−GST). When lysates prepared from 230−238 (pre-B cells) and WEHI 231 (mature B cells) were tested, the anti-CTR antiserum specifically detected three major bands (110, 70 and 57 kDa) and one minor band (36 kDa), which could be competed by including pure deAR−GST protein (the immunizing antigen) in the anti-CTR antiserum solution during the immunoblotting process (Figure 5). The 110 kDa

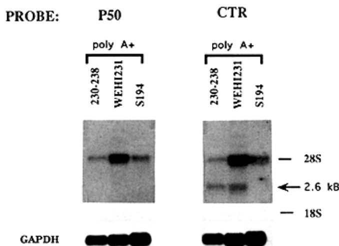

Fig. 4. CTR is expressed as an independent 2.6 kb RNA. Northern blot analyses of p50 and CTR poly(A)$^+$ RNAs in B lineage cells were performed. 230−238, WEHI 231 and S194 are pre-B, B and plasmacytoma cell lines respectively. Filters were probed with DNA probes representing either the rel-homologous region of p50 (p50, left panel) or the CTR of p105 (CTR, right panel). Arrow indicates the 2.6 kb RNA. For quantification of the amount of RNA, the filters were further hybridized with GAPDH probe after stripping of the previous probes.

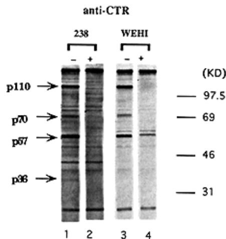

Fig. 5. CTR protein exists in the cells. Cell lysates prepared from 230−238 and WEHI 231 cells were resolved by SDS−PAGE and incubated with solution containing the following reagents: anti-CTR antiserum (lanes 1 and 3), anti-CTR antiserum plus the immunizing antigen (deAR−GST protein) (10 μg/ml) (lanes 2 and 4). The specific bands were indicated.

band corresponded to the p105 precursor because anti-p50 antiserum also detected this band (data not shown). The 70 kDa band was recognized by the anti-CTR antiserum and blocked by the immunogen and thus presumably corresponds to the product of the 2.6 kb mRNA, as was also concluded recently by Inoue *et al.* (1992) who called this protein IκBγ. The other bands are discussed below.

Discussion

Our studies on the CTR of the p50 precursor, p105, suggest that the CTR has an IκB-like activity that is distinct from IκBα. The initial observation came from the striking homology among the ankyrin repeats found in the CTR, MAD-3 and pp40. Because the latter two have been shown to be IκBs, we tested the CTR of p105 for IκB activity using EMSA and found that it was active against p50 but much less so against p65 or NF-κB. This was in striking contrast to the product of the MAD-3 clone which was inactive against p50 but very effectively inhibited p65 or NF-κB. We could show that the inhibition of DNA binding by the IκBs correlated with a direct physical association between these two proteins as measured by *in vitro* precipitation assays. CTR has a much higher affinity for p50 than does MAD-3, whereas at high protein concentration, both MAD-3 and CTR interact with p65. The association of CTR with p50 *in vivo* suggests that the interaction between these two molecules occurs in cells. Furthermore, immature B cells predominantly express a 2.6 kb mRNA of CTR, which may encode a protein independently of its occurrence in p105. Proteins containing CTR epitope can also be detected by specific antiserum for CTR in lymphoid cell lines tested.

A recent study reported the cloning of the 2.6 kb cDNA for CTR and showed that it encodes a protein of 70 kDa, which they termed IκBγ (Inoue *et al.*, 1992). The 70 kDa protein initiates from the methionine at position 365 (immediately following the nuclear localization signal), contains all seven ankyrin repeats and ends at the same stop codon as p105. Therefore it contains the glycine-rich region which is thought to be the target of p105 processing. The artificial CTR protein used in the biochemical work reported here initiated from the Kozak-rule methionine at amino acid 482 and ended at the same stop codon, giving it an estimated molecular weight of 55 kDa. Our immunoblotting experiments revealed four polypeptides which could be recognized by antiserum against the CTR. The 'p110' protein is the p105 precursor. The 70 and 36 kDa proteins detected by our anti-CTR antiserum may correspond to the 70 kDa IκBγ and a 36 kDa peptide immunoprecipitated by the anti-IκBγ serum (Inoue *et al.*, 1992). The 57 kDa protein, which was not observed by Inoue *et al.* (1992), could result from a cross-reaction or could be derived from initiation from a Kozak-rule methionine at position 482 (22 acids upstream of the first ankyrin repeat), which would give rise to a protein of 55 kDa. Alternatively, it could be a degradation product during the processing of p105 precursor. The detection of CTR mRNA and protein(s) synthesized independently from the precursor p105, suggests that CTR may play a regulatory role *in trans*.

Protein purification studies revealed the existence of at least two IκBs (α and β) that specifically inactivate the DNA binding of NF-κB or c-*rel* (Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990; Urban and Baeuerle, 1990; Zabel and Baeuerle, 1990; Kerr *et al.*, 1991). The inhibitors characterized up to now (purified IκBα, IκB-β, cloned MAD-3 and pp40) have had p65 or c-*rel* as their targets. Inhibitors of the other subunit of NF-κB, p50, had not been previously identified. Therefore, the CTR of p105 represents the first identified inhibitor that shows preferential inhibitory specificity for p50.

CTR is apparently not the only IκB with inhibitory specificity for p50. Our independent studies on the proto-oncogene *bcl*-3, which also contains seven ankyrin-like repeats with homology to CTR, MAD-3 and pp40, suggest that similarly to CTR, *bcl*-3 has a higher inhibitory activity for p50 than for p65 or NF-κB (G.P.Nolan and D.Baltimore, manuscript in preparation). We thus propose that there are at least two groups of inhibitors for the NF-κB/*rel* gene family. One is represented by MAD-3 and pp40 which are specific inhibitors for p65 or *rel*. The other group, as represented by CTR and *bcl*-3, has specificity for p50 or a related member of that subfamily of *rel*-related proteins. We may thus predict that the p100(p49) or *Lyt*10 gene (Neri, 1991; Schmid *et al.*, 1991), a *rel*-related gene with a structural arrangement closely resembling that of p105, may very well encode an inhibitory domain at its carboxy-terminal portion that has an affinity for either its amino-terminal, p49, or other closely related members of the NF-κB family, like p50.

Dual roles of the CTR

How might CTR protein function in the cell? It is likely that it affects p50 both *in cis*, as a part of p105, and *in trans*, as an independent IκB. *In cis*, its IκB activity might be responsible for inhibiting the potential binding of the p105 precursor protein to DNA (Ghosh *et al.*, 1990; Kieran *et al.*, 1990), maintaining the p105 protein in the cytoplasm (Blank *et al.*, 1991; Henkel *et al.*, 1992) and modulating the processing of p105 to p50 (Blank *et al.*, 1991; Fan and Maniatis, 1991). When separately expressed from an independent mRNA, the carboxyl region of p105 could display its IκB activities *in trans*, allowing it to inhibit the binding of p50 to DNA and it may also prevent the nuclear translocation of p50 (Hatada *et. al.*, 1992; Henkel *et al.*, 1992; Inoue *et al.*, 1992). Such a p50-targeted inhibitory

activity has not been postulated previously but might not have been evident.

The interaction between CTR and p50 may have important biological significance. In cells, p50 is thought to function independently of NF-κB as a homodimeric transcription factor, KBF-1, which is important for the expression of the class I MHC genes (Kieran *et al.*, 1990; Fujita *et al.*, 1992). Thus, it is conceivable that CTR and related p50-targeted IκBs regulate the p50 (KBF-1) activity and subsequently influence the expression of the cellular genes that are predominantly regulated by p50 homodimer.

Alternatively, the interaction of CTR and p50 may deplete p50 from the NF-κB/*rel* protein pool. Differential combinations of dimer formation among *rel* family members could generate molecules with distinct binding or transcriptional specificity (Ballard *et al.*, 1990; Molitor *et al.*, 1990; Schmid *et al.*, 1991; Schmitz and Baeuerle, 1991; Fujita *et al.*, 1992; Ryseck *et al.*, 1992). Depletion of p50 by CTR might allow preferential interaction among other members of NF-κB/*rel* family which in turn could skew the balance among *rel* family members and subsequently affect the regulation of their target genes.

Thus, multiple IκB activities may participate in the regulation of NF-κB/*rel* family of proteins. Disruption of the balance of activators and inhibitors could lead to tumorigenesis as suggested by numerous observations linking the *rel*/anykrin families of proteins to oncogenesis in birds and humans (Nolan and Baltimore, 1992).

Materials and methods

Plasmid constructs

The DNA fragment of the CTR of p105 was generated by amplifying a fragment corresponding to amino acids 482–971 (a methionine of good Kozak consensus sequence to the stop codon) of the p105 cDNA clone (Ghosh *et al.*, 1990). For immunization of rabbits, the deAR fragment corresponding to amino acids 785–971 was generated. The PCR fragments with appropriate cloning sites were cloned either into the *Bam*HI and *Eco*RI sites of pGEX2T vector (Pharmacia) to generate the CTR–GST construct and deAR–GST, or into the *Hin*dIII and *Xba*I sites of a CDM8–neo expression construct to generate the CTR–CDM8–neo construct. The MAD-3 fragment was obtained by PCR amplification of the MAD-3 cDNA from human RNA using oligonucleotides derived from the cloned sequence (Haskill *et al.*, 1991); it contains the entire coding sequence from the first methionine to the stop codon. This MAD-3 DNA fragment was also cloned into the *Bam*HI and *Eco*RI sites of the pGEX2T vector to generate the MAD-3–GST construct for bacterial expression. For *in vitro* translation experiments, p50-pBS+ and p65-pSK+ plasmids were used. p50-BS+ was constructed by subcloning the 1.3 kb *Nco*I fragment of the p105 cDNA (corresponding to amino acids 1–430) into a modified pBS+ vector (Stratagene). p65-pSK+ has been previously described (Nolan *et al.*, 1991). p50-HA-CDM8-neo contains the coding sequence of the murine p50 cDNA (amino acids 1–430) (Ghosh *et al.*, 1990) and a tagging sequence derived from the influenza virus hemagglutinin antigen (HA) epitope.

Purification of bacterial GST fusion proteins

Bacterial cultures containing the GST fusion proteins were cultured at 30°C and induced with 100 μM IPTG for 1 h before harvest. The bacterial pellets were then dissolved and sonicated in a 1% PBS–Triton X-100 and 50 mM EDTA solution. The soluble fractions were incubated with glutathione–agarose beads for 1 h at 4°C before washing three times with PBS. GST fusion proteins were further competed off from the beads with 5 mM glutathione–50 mM Tris (pH 7.5) and dialyzed against Buffer D.

Electrophoretic mobility shift assay (EMSA) and quantitative analysis

GST and fusion proteins (CTR–GST and MAD-3–GST) were purified to near homogeneity by using glutathionine–agarose beads. Recombinant p50, p65 and p65-Δ proteins produced in a baculoviral expression system were used in all of the experiments (Fujita *et al.*, 1992). The NF-κB was reconstituted by co-incubation of equimolar p50 and p65 at 37°C for 30 min. Since p65 binds to DNA non-specifically, we used a carboxy-terminal truncated p65, termed p65-Δ, which binds to DNA more specifically and still retains its ability to interact with IκB (Nolan *et al.*, 1991; Fujita *et al.*, 1992). Electrophoretic mobility shift assays for NF-κB–IκB interaction were described previously (Sen and Baltimore, 1986). Constant amounts (10–40 fmol) of baculoviral-expressed and purified p50, p65-Δ and reconstituted NF-κB were incubated with 0.1- to 15-fold molar excess of either CTR–GST or MAD-3–GST protein followed by the addition of the radiolabeled κB probe (κB site from the κ immunoglobulin gene. The resulting complexes were resolved on 4% native polyacrylamide gels and quantified by the Phosphoimage analyzer.

In vitro co-precipitation assay

In vitro transcription and translation were undertaken following the manufacturers' protocols (Promega and Stratagene) using linearized p50-pBS+ or p65-pSK+ construct with T7 (for p50-pBS+) or T3 (for p65-pSK+) polymerase. The translated p50 or p65 lysates were incubated with either GST, MAD-3–GST or CTR–GST protein in ELB buffer [250 mM NaCl, 0.01% NP-40, 50 mM HEPES (pH 7.9), 50 mM EDTA] for 1 h at 4°C. Glutathione–agarose beads were then added to the protein mixture and incubated further for 30 min at 4°C before washing the beads three times with PBS buffer. The components of the resulting complexes were resolved by electrophoresis through SDS–polyacrylamide gels.

In vivo co-immunoprecipitation experiments

Calcium phosphate transfection method was used for transfection of 293 cells. About 5 μg of each construct DNA (CTR–CDM8-neo or p50–HA-CDM8 or both) was used in the transfection in a 150 mm plate. Two days after the transfection, cells were labeled with 250 μCi/ml of [^{35}S]methionine for 4–6 h. Cells were lysed in 2 ml of lysis buffer [50 mM HEPES (pH 7.9), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate and 100 mM NaF]. Immunoprecipitation was performed according to standard methods using either control mouse ascites or anti-HA ascites (derived from 12CA5 monoclonal hybridoma line).

Immunoblotting analysis

The anti-CTR (90–213) and anti-p50 (90–112) antisera were obtained by immunization of rabbits with deAR–GST protein (corresponding to amino acids 785–971 of p105) or p50–GST protein (corresponding to the *rel* domain of p105). The sera were tested positive using purified proteins and were used in the immunoblotting analyses at 1:200 dilution. Both 230–238 and WEHI 231 cell lysates were resolved by SDS–PAGE with large wells and transferred to a nitrocellulose filter. The blot was then cut into small strips for incubation with different antisera. In the antigen competition experiments, 10 μg/ml of purified deAR–GST protein was included with anti-CTR antiserum. Incubation with the first antisera proceed for 2 h at room temperature followed by washing and incubation with second antibodies. Chemiluminescence method (Amersham International, ECL Western detection kit) was utilized for detection of the antibody-reactive bands.

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