p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction

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The Rel/NF-κB family of transcription factors is composed of two distinct subgroups, proteins that undergo proteolytic processing and contain SWI6/ankyrin repeats in their carboxyl termini (p105, p98), and those without such repeats that do not require processing (p65, c-Rel, RelB, and Dorsal). We demonstrate that the p105 and p98 precursors share functional properties with the IκB proteins, which also contain SWI6/ankyrin repeats. Both p105 and p98 were found to form stable complexes with other Rel/NF-κB family members, including p65 and c-Rel. Association with the precursors is sufficient for cytoplasmic retention of either p65 or c-Rel, both of which are otherwise nuclear. These complexes undergo stimulus-responsive processing to produce active p50/c-Rel and p55/c-Rel complexes. These observations suggest a second pathway leading to NF-κB induction, in which processing of the precursors rather than phosphorylation of IκB plays a major role.

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Interaction of ligands with cell-surface receptors modulates transcription factor activity, thereby affecting gene expression (Karin 1992). Several mechanisms are used to transmit signals generated at the cell surface across the plasma and nuclear membranes before reaching the nucleus. Most of these mechanisms are based on post-translational activation or repression of pre-existing transcription factors and frequently involve changes in protein phosphorylation (Hunter and Karin 1992; Karin 1992). However, other mechanisms are also possible. One group of transcription factors, the Rel/NF-κB proteins, are involved in the rapid induction of genes, whose products function in protective and proliferative responses, upon exposure to a variety of external stimuli (Lenardo and Baltimore 1989; Baeuerle 1991; Baeuerle and Baltimore 1991). Members of this family also control cell differentiation (Steward 1987). Rel/NFκB proteins form dimeric complexes that are well suited for mounting rapid responses to extracellular stimuli because their activation does not require de novo protein synthesis and is dependent, in part, on translocation from the cytoplasm to the nucleus. The inactive cytoplasmic form of NF-κB can be activated to undergo nuclear translocation within minutes of an appropriate stimulus (Baeuerle and Baltimore 1991).

The major form of NF-κB is a heterodimer composed of 50-kD (p50) and 65-kD (p65) DNA-binding subunits (Baeuerle and Baltimore 1989; Urban et al. 1991). The inactive cytosolic form of NF-κB is associated with inhibitors, termed IκBs, which bind primarily through p65 (Baeuerle and Baltimore 1989; Urban and Baeuerle 1990; Beg et al. 1992). The IκBs are thought to be released in response to cell stimulation, and the NF-κB heterodimer is freed to translocate to the nucleus (Baeuerle and Baltimore 1988a,b). Release of IκB is presumed to involve phosphorylation by a yet-to-be identified protein kinase, which in vitro can be substituted by protein kinase C (Shirakawa and Mizel 1989; Ghosh and Baltimore 1990). This relatively simple picture has become progressively complex upon the discovery that p50 (Bours et al. 1990; Ghosh et al. 1990; Kieran et al. 1990; Meyer et al. 1991) and p65 (Nolan et al. 1991; Ruben et al. 1991) belong to a large family of related transcription factors, including v-Rel (Stephens et al. 1983; Wilhelmsen et al. 1984), c-Rel (Brownell et al. 1989), RelB (Ryseck et al. 1992), p98/p55 (also termed p100/p49, p50B, or LYT10) (Neri et al. 1991; Schmid et al. 1991; Bours et al. 1992; Mercurio et al. 1992), and the *Drosophila dorsal* gene product (Steward 1987). These proteins bind to similar DNA sequences, are subject to regulated subcellular localization, and contain a common structural motif, the Rel homology domain. This 300-amino-acid domain contains subregions that mediate DNA binding, dimerization and nuclear localization (Ghosh et al. 1990; Kieran et al. 1990; Logeat et al. 1991). The mammalian Rel/NF-κB proteins form homo- and heterodimers that bind with different affinities to variants of the κB motif (Ballard et

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al. 1990; Molitor et al. 1990; Radler-Pohl et al. 1990; Perkins et al. 1992). This complexity provides the cell with opportunities to fine-tune NF-κB activity and respond to a variety of activating signals.

A unique feature of p50 is that it is synthesized as a 105-kD precursor (p105) with the Rel homology domain in its amino-terminal portion. A precursor/product relationship between p105 and p50 was established (Fan and Maniatis 1991). The related protein p55 is also produced as a larger 98-kD precursor, p98 (Mercurio et al. 1992). p105 and p98 are unable to bind DNA (Bours et al. 1990; Ghosh et al. 1990; Kieran et al. 1990), and p105 was shown to be exclusively cytoplasmic (Blank et al. 1991; Henkel et al. 1992). Unlike other family members, p105 and p98 also exhibit limited sequence homology in their carboxy-terminal regions, which contain seven SWI6/ankyrin repeats (Bours et al. 1990,1992; Kieran et al. 1990; Meyer et al. 1991; Neri et al. 1991; Schmid et al. 1991; Mercurio et al. 1992). Interestingly, the IκB proteins also contain SWI6/ankyrin repeats (Ohno et al. 1990; Davis et al. 1991; Haskill et al. 1991; Hatada et al. 1992; Inoue et al. 1992). The carboxy-terminal domain of p105 is required for its cytoplasmic retention (Blank et al. 1992), and similar observations were made for p98 (F. Mercurio and C. Rosette, unpubl.). Hence, proteolytic processing of p105 and p98, leading to the loss of their carboxy-terminal portion, results in activation of their amino-terminal halves, p50 and p55, respectively. This allows for nuclear translocation and DNA binding by p50 and p55, provided they are not associated with an IκB.

An often overlooked aspect of Rel/NF-κB regulation is the potential role of the most abundant Rel/NF-κB family members, p105 and p98. Are they simply precursors, do they serve as reservoirs of p50 and p55, or do they have an unrecognized regulatory role? This study examined the functions of the two precursors, and its results lead us to propose that p105 and p98 can play a dynamic role in regulation of NF-κB activity. Both p105 and p98 are capable of forming stable complexes with other family members, including each other. p105/c-Rel and p98/c-Rel complexes were found to be processed into active p50/c-Rel and p55/c-Rel complexes. We demonstrate that cell stimulation results in an increased level of processing. Collectively, these observations suggest a mechanism according to which cytoplasmic to nuclear signaling by Rel/NF-κB complexes can occur independently of IκB.

Results

p105 and p98 form stable complexes with other family members in vitro

p105 and its homolog p98 are processed to yield mature κB DNA-binding proteins, p50 and p55, respectively (Fan and Maniatis 1991; Mercurio et al. 1992). Both p50 and p55 form stable complexes with other Rel/NF-κB proteins (Gilmore 1991; Rushlow and Warrior 1992). We examined whether p105 and p98 could form analogous complexes. This appeared reasonable in light of similar electrophoretic mobilities of p105 and p98 to previously characterized 124-kD and 115-kD v-Rel- and c-Rel-associated proteins (Simek and Rice 1988a,b; Morrison et al. 1989; Davis et al. 1990; Lim et al. 1990). Capobianco et al. (1992) demonstrated that p105 associates with v-Rel in vitro.

Rel/NF-κB proteins labeled with [^{35}S]methionine, were produced by cell-free translation using in vitro-transcribed RNA. After either separate or combined translations, the proteins were immunoprecipitated with antibodies made against unique regions of the Rel/NF-κB proteins, including anti-p105N, raised against the amino terminus of p105; anti-p105C, raised against the carboxyl terminus of p105; anti-p98N, raised against the amino terminus of p98; anti-c-Rel, raised against the carboxyl terminus of c-Rel (kindly provided by Dr. N. Rice), and anti-p65C, raised against the carboxyl terminus of p65 (kindly provided by Dr. W. Greene, Gladstone Institute of Virology, San Francisco, CA) (Fig. 1). In addition, a peptide epitope (Flag) was fused to the amino termini of some proteins to facilitate the analysis of heterocomplex formation, using monoclonal anti-Flag antibodies (Mercurio et al. 1992). Antibodies used in these studies do not cross-react with other related Rel/NF-κB proteins. These experiments, the results of which are shown in Figure 2 and summarized in Table 1, demonstrate that both p105 and p98 form stable complexes with p65 and c-Rel. In addition, both p105 and p98 form stable complexes with p50, p55, and with each other (data not shown). All of these complexes were sufficiently stable to withstand relatively stringent immunoprecipitation conditions using RIPA buffer.

Higher order complex formation among Rel/NF-κB proteins in vivo

The ability of p105 and p98 to associate with one another and other Rel/NF-κB proteins in vitro does not necessarily mean that these proteins associate in vivo. To deter-

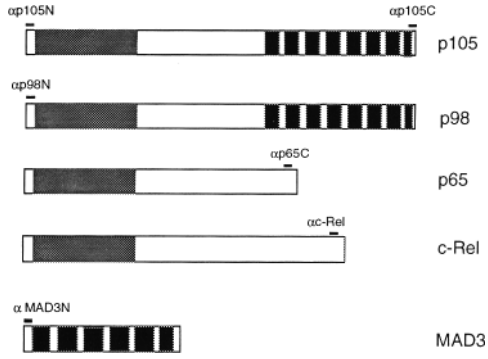

Figure 1. Schematic representation of p105, p98, p65, MAD-3, and c-Rel showing the regions against which the peptide antibodies used in this paper were directed. The stippled boxes indicate the Rel homology domain; the solid boxes indicate the SWI6/ankyrin repeats.

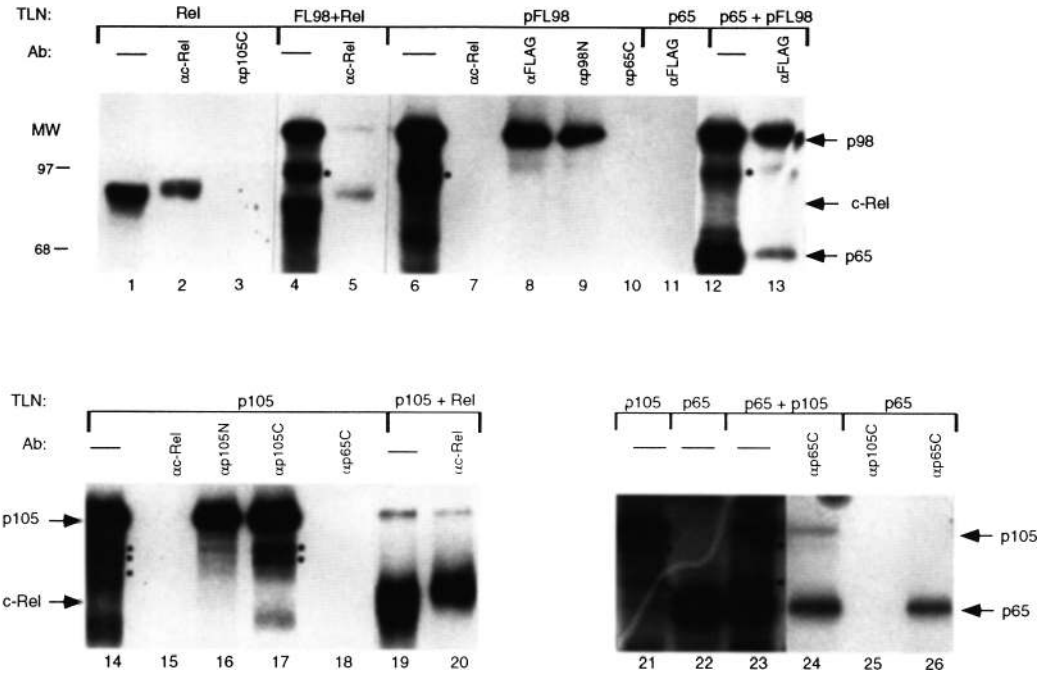

Figure 2. p105 and p98 associate with p65 or c-Rel in vitro. [^{35}S]Methionine-labeled proteins were produced either separately or in the indicated combinations using the coupled transcription/translation TNT system. Proteins were analyzed either directly or after immunoprecipitaion by fractionation on 10% SDS–polyacrylamide gels. (Lanes *1–3*) c-Rel; (lanes *4,5*) pFL98 translated with c-Rel; (lanes *6-10*) pFL98 translated alone; (lanes *11, 22, 25, 26*) p65 translated alone; (lanes *12, 13*) p65 cotranslated with pFL98; (lanes *14–18, 21*) p105 translated alone; (lanes *19, 20*) p105 cotranslated with c-Rel; (lanes *23, 24*) p65 cotranslated with p105. Lanes *1, 4, 6, 12, 14, 19,* and *21–23* represent the original translation products; the remaining lanes represent the polypeptides recovered by immunoprecipitation with the indicated antibodies. Labeled arrows denote the positions of the primary translation products; asterisks denote premature termination products or internally initiated p105 and pFL98 derivatives.

mine whether p105 and p98 associate with other family members in vivo, several immunoprecipitation protocols were employed: (Lowercase letters are used instead of numerals for protocols in Figs. 3–6.)

1. Immunoprecipitation using metabolically labeled cells lysed in RIPA buffer (see Materials and methods). This method retains strong protein–protein interactions, allowing detection of all polypeptide subunits comprising a stable complex.
2. Immunoprecipitations performed on RIPA lysates boiled in SDS to disrupt protein–protein interactions. Under these conditions only those proteins that are primarily recognized by the antiserum are precipitated. The combination of methods 1 and 2 allows the identification of proteins associated with the primary targets of each antiserum.
3. To identify the nature of the associated polypeptides, primary immune complexes isolated from RIPA lysates were disrupted by boiling in SDS and subjected to secondary immunoprecipitation with antisera directed against other Rel/NF-κB proteins.

Table 1. *Summary of Rel/NF-κB protein–protein interactions*

	p105	p50	p98	p55	p65	c-Rel
p105	N.D.	(++)*	(++)*	(++)*	++*	++*
p50	(++)	(++)	(++)	(+)	(+)*	(+)*
p98	(++)*	(++)	N.D.	(++)	++*	++*
p55	(++)*	(+)	(++)	(++)	(+)*	(+)*
p65	++*	(+)*	++*	(+)*	N.D.	(−)
c-Rel	++*	(+)*	++*	(+)*	(−)	N.D.

Summary of in vitro and in vivo interactions between Rel/NF-κB proteins. In vitro interactions were detected using ^{35}S-labeled cell-free-translated Rel/NF-κB proteins as described in the legend to Fig. 2. Those interactions that were also detected in vivo are indicated by an asterisk (*) and are inferred from the immunoprecipitation experiments shown in Figs. 3B, 4, 5, 6, and 10. The relative efficiencies of the observed interactions are indicated as follows: No detectable interaction (−); some interaction (+); efficient interaction (++). (N.D.) Those interactions that could not be determined. Parentheses indicate in vitro interactions that are not presented in Fig. 2.

4. Immune complexes isolated from RIPA lysates were eluted as intact hetero-oligomers by the addition of a large excess of the specific peptide immunogen, and the resulting supernatant was subjected to secondary immunoprecipitation as described above.

Primary immunoprecipitations of RIPA lysates of ^{35}S-labeled HeLa S3 cells with anti-p105N, using method 1, identified specifically precipitated polypeptides of 37–40, 50, 65, 80, 114, and 120 kD (the 114- and 120-kD polypeptides are not resolved in this exposure; Fig.3 A, lanes 1,2). Immunoprecipitations of the same lysates with anti-p98N identified specifically precipitated polypeptides of 50, 55, 62, 65, 114, and 120 kD (Fig. 3A, lanes 3,4). Immunoprecipitations of denatured HeLa cell lysates with anti-p98N identified a major 114-kD polypeptide and a minor 55-kD polypeptide, migrating as a doublet (Fig. 3B, lanes 5,6). These polypeptides are likely to correspond to the p98 precursor and its processed form, p55, respectively. A 62-kD polypeptide can also be detected after a longer exposure (data not shown). This polypeptide, which is clearly seen in Figures 3, A and C, and 9, B and C (below), most likely represents a protein that shares an antigenic epitope with the amino terminus of p98/p55 but is not a member of the Rel/NF-κB family, because it does not associate with other family members (see Figs. 4 and 5). The 50, 65, and 120-kD polypeptides that are precipitated by anti-p98N from native but not denatured lysates represent polypeptides associated with either p55 or p98, or both. Similar immunoprecipitations of denatured lysates with anti-p105N identified 50- and 120-kD polypeptides that are likely to correspond to the p105 precursor and its p50 product (data not shown). Therefore the 55-, 65-, and 114-kD polypeptides that are precipitated by anti-p105N, only from native lysates, are polypeptides that associate with either p50 or p105, or both. Interestingly, for both p105 and p98, the precursors give rise to signals that are considerably stronger than the signals corresponding to their products.

To identify the precise nature of the polypeptides associated with p98 and p105, we performed sequential immunoprecipitations. Immune complexes isolated with anti-p98N from RIPA lysates were disrupted in SDS

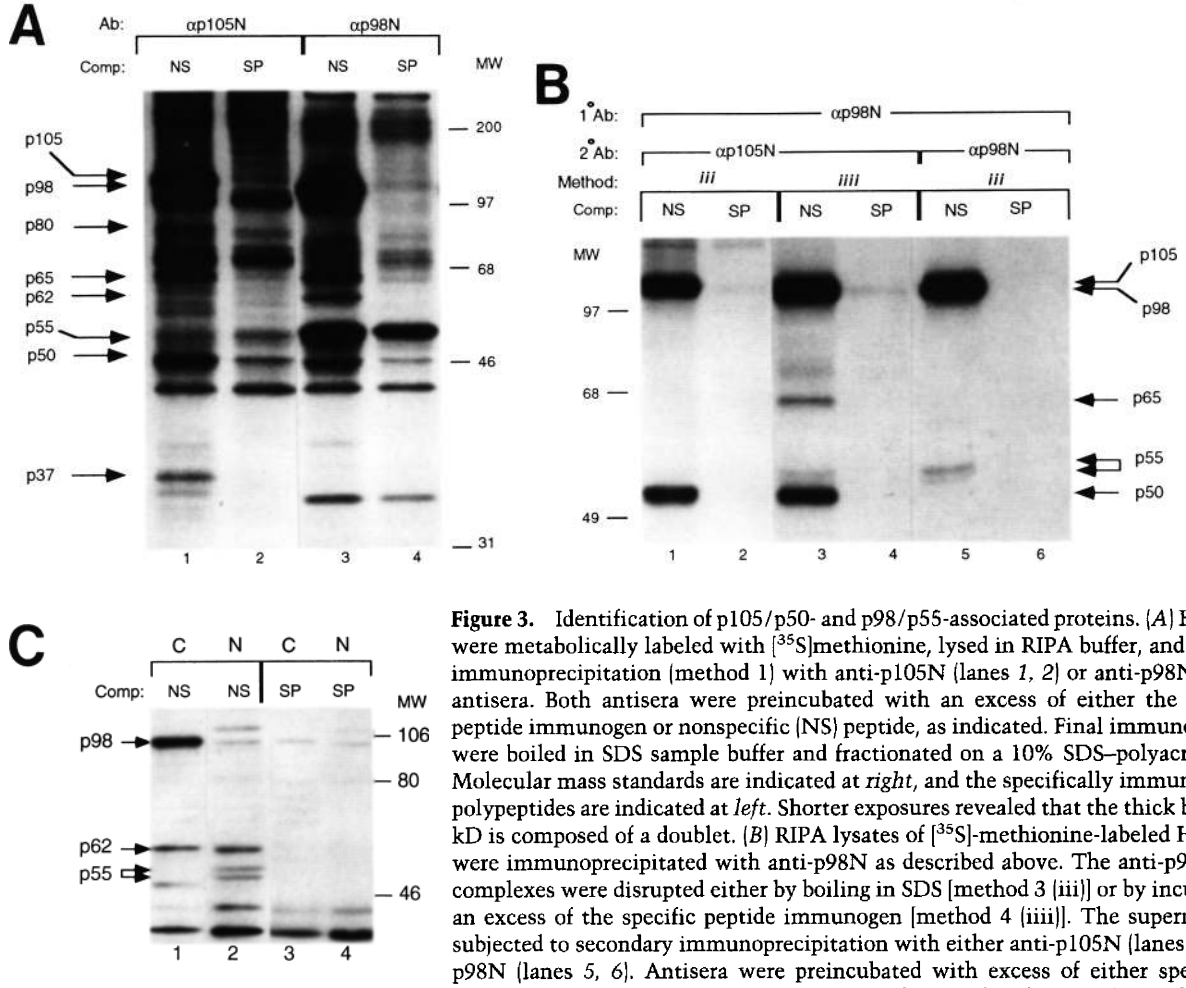

Figure 3. Identification of p105/p50- and p98/p55-associated proteins. (*A*) HeLa S3 cells were metabolically labeled with [^{35}S]methionine, lysed in RIPA buffer, and subjected to immunoprecipitation (method 1) with anti-p105N (lanes *1, 2*) or anti-p98N (lanes *3, 4*) antisera. Both antisera were preincubated with an excess of either the specific (SP) peptide immunogen or nonspecific (NS) peptide, as indicated. Final immunoprecipitates were boiled in SDS sample buffer and fractionated on a 10% SDS–polyacrylamide gel. Molecular mass standards are indicated at *right*, and the specifically immunprecipitated polypeptides are indicated at *left*. Shorter exposures revealed that the thick band at ~110 kD is composed of a doublet. (*B*) RIPA lysates of [^{35}S]-methionine-labeled HeLa S3 cells were immunoprecipitated with anti-p98N as described above. The anti-p98N immune complexes were disrupted either by boiling in SDS [method 3 (iii)] or by incubation with an excess of the specific peptide immunogen [method 4 (iiii)]. The supernatants were subjected to secondary immunoprecipitation with either anti-p105N (lanes *1–4*) or anti-p98N (lanes *5, 6*). Antisera were preincubated with excess of either specific (SP) or nonspecific (NS) peptide competitors, as indicated. (*C*) Western blot analysis was performed on nuclear (N; 25 μg) cytoplasmic (C; 50 μg) extracts prepared from TPA-stimulated HeLa cells, using anti-p98N. Before hybridization, the antiserum was preincubated with an excess of either specific (SP) or nonspecific (NS) peptide competitors. Antigen–antibody complexes were visualized by the ECL detection system (Amersham).

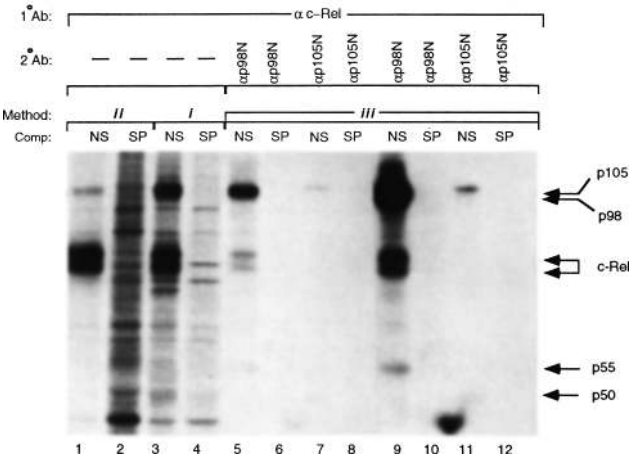

Figure 4. c-Rel forms stable complexes with p98 or p105 in vivo. Jurkat cells were stimulated with TPA (100 ng/ml) and ConA (2 μg/ml), and metabolically labeled with [^{35}S]-methionine for 5 hr. Cell lysates were prepared in either SDS-RIPA buffer and boiled [method 2 (ii); lanes *1,2*] or RIPA buffer (method 1 (i); lanes *3,4*), and immunoprecipitated with anti-c-Rel. To demonstrate complex formation between either p105 or p98 and c-Rel, primary anti-c-Rel immune complexes were disrupted by boiling in SDS-RIPA buffer and subjected to secondary immunoprecipitation [method 3 (iii)] with either anti-p98N (lanes *5,6*) or anti-p105N (lanes *7,8*). Lanes *9–12* represent a longer exposure of lanes *5–8*, respectively. The various antisera were preincubated with either specific (SP) or nonspecific (NS) peptide competitors, as indicated.

and reprecipitated with anti-p105N (Fig. 3B, lanes 1,2). The results clearly demonstrate that the 50-kD and 120-kD polypeptides associated with p98/p55 are actually p50 and p105. Thus, these results indicate that p105 associates with p55 and/or p98 in vivo. Similar results were obtained by sequential immunoprecipitation using method 4. Anti-p98N complexes were disrupted by incubation with a large excess of specific peptide competitor. The supernatant, which should contain intact hetero-oligomers, was then subjected to secondary immunoprecipitation with anti-p105N. The results show that p50, p55, p98, and p105 were all coprecipitated (Fig. 3B, lanes 3,4). Surprisingly, a 65-kD polypeptide also coprecipitated by anti-p105N. The same results were obtained using anti-p105C, and the identity of the 65-kD polypeptide was established as p65, using anti-p65C (data not shown). These results suggest that a portion of p105, p98, and p65 are engaged in a higher order complex, different from a simple heterodimer.

It was reported that p105 is exclusively cytoplasmic (Fan and Maniatis 1991; Henkel et al. 1992). To determine the subcellular localization of p98 and p55, cytoplasmic and nuclear extracts of TPA-stimulated HeLa cells were analyzed by Western blotting. Although p98 was exclusively cytoplasmic, the p55 doublet was mostly nuclear (Fig. 3C). The cytoplasmic 50-kD polypeptide was not observed in all experiments and is most likely derived from nonspecific proteolysis of p98, because it was not detected in immunoprecipitates of denatured cell lysates in which only minimal proteolysis occurs. Similar results were obtained with cytoplasmic and nuclear extracts prepared from TPA-stimulated Jurkat cells (data not shown). The 62-kD polypeptide is the cross-reactive protein described above.

Association of p105 and p98 with c-Rel and p65 in vivo

Proteins associated with v-Rel and c-Rel have been characterized extensively in reticuloendotheliosis virus T-transformed spleen cells and chicken embryo fibroblasts (Simek and Rice 1988 a,b; Tung et al. 1988; Morrison et al. 1989; Davis et al. 1990; Lim et al. 1990). These Rel-associated proteins have approximate molecular masses of 40, 70, 115, and 124 kD. The 40- and 70-kD polypeptides represent avian IκBα (pp40) and Hsp70, respectively (Lim et al. 1990; Davis et al. 1991), whereas the 124-kD polypeptide corresponds to p105 (Capobianco et al. 1992). It is therefore reasonable to assume that the 115-kD protein corresponds to p98. To examine interactions of c-Rel with other Rel/NF-κB proteins in mammalian cells, lysates of TPA/concanavalin (ConA)-stimulated Jurkat cells, metabolically labeled with [^{35}S]methionine, were immunoprecipitated with anti-c-Rel antibodies. Immunoprecipitation under denaturing conditions (method 2) identified c-Rel as an 80- to 85-kD doublet (Fig. 4, lanes 1,2). Immunoprecipitation of RIPA lysates with anti-c-Rel (method 1) revealed several c-Rel-associated polypeptides, of which a poorly resolved doublet (seen in a shorter exposure) of 114 and 120 kD was most intense (Fig. 4, lanes 3,4). The nature of the c-Rel-associated proteins was determined by sequential

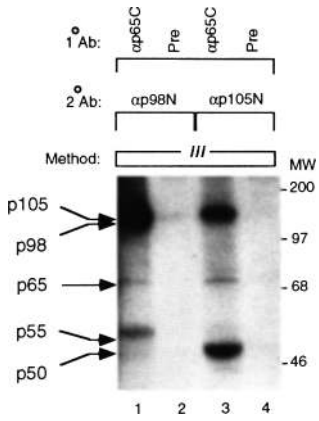

Figure 5. p65 associates with p105 or p98 in vivo. Jurkat cells were stimulated with TPA (100 ng/ml) and ConA (2 μg/ml), metabolically labeled with [^{35}S]methionine for 5 hr, and lysed in RIPA buffer. Immune complexes were isolated using either anti-p65C or pre-immune serum (Pre) and boiled in SDS-RIPA buffer, and the supernatants were subjected to secondary immunoprecipitation [method 3 (iii)] with either anti-p98N (lanes *1,2*) or anti-p105N (lanes *3,4*) antisera. The final immune complexes were analyzed as indicated above.

immunoprecipitation (method 3). Secondary immunoprecipitation of disrupted anti-c-Rel immune complexes with anti-p98N revealed that the 114-kD polypeptide corresponds to p98 (Fig. 4 lanes 5,6). A longer exposure (Fig. 4, lanes 9,10) revealed a small amount of p55. Interestingly, a small amount of the c-Rel doublet is reprecipitated by anti-p98N even from denatured supernatants (lane 5), and a small amount of p98 is coprecipitated with c-Rel from the denatured lysate (lane 1). These results suggest either incomplete dissociation of the initial complex or, more likely, rapid reassociation of the dissociated subunits upon dilution of the denatured lysate. Secondary immunoprecipitation with anti-p105N revealed that the c-Rel-associated 120-kD polypeptide corresponds to p105 (Fig. 4, lanes 7,8). A longer exposure (Fig. 4, lanes 11,12) revealed a very small amount of p50. We were unable, however, to detect either p50 or p55 in c-Rel complexes isolated from lysates of unstimulated Jurkat cells (data not shown). These experiments demonstrate that c-Rel associates with both p105 and p98 in vivo.

To determine whether p65 associates with p105 and p98 in vivo, immunoprecipitations were performed using anti-p65C (kindly provided by Dr. W. Greene), directed against the carboxyl terminus of p65. Immunoprecipitation with anti-p65C of RIPA lysates of metabolically labeled Jurkat cells identified p65 accompanied by several other polypeptides, with masses of 50, 55, and 115–120 kD (data not shown). These primary anti-p65C immune complexes were disrupted by boiling in SDS-RIPA buffer and reprecipitated with anti-p98N or anti-p105N. These experiments identified the 115-kD polypeptide as p98 and the 55-kD polypeptide as p55 (Fig. 5, lanes 1,2); whereas the 120- and 50-kD polypeptides correspond to p105 and p50, respectively (Fig. 5, lanes 3,4). Thus, like c-Rel, p65 also associates with p105 and p98 in vivo. As observed above for c-Rel (Fig. 4), a small amount of p65 reassociated with both p105 and p98. This reassociation most likely occurred upon dilution of the renatured lysate before immunoprecipitation. A summary of the in vitro and in vivo interactions identified between Rel/NF-κB proteins is shown in Table 1.

MAD-3 is not associated with p105 complexes in vivo

Cytoplasmic localization of NF-κB is mediated by association with inhibitor proteins of approximate molecular mass 37–40 kD—IκBα and IκBβ (Baeuerle and Baltimore 1988a,b; Ghosh and Baltimore 1990; Zabel and Baeuerle 1990). Recently Haskill et al. (1991) have cloned an immediate–early gene from human monocytes, termed MAD-3, encoding a 37-kD protein thought to correspond to IκBα. To determine whether MAD-3 associates with precursor-containing complexes in vivo, sequential immunoprecipitations (method 3) of disrupted anti-p105N and anti-p105C immune complexes were performed with either anti-p65C or anti-MAD-3 antisera (Fig. 6). Although p65 was found in both the anti-p105N and anti-p105C immune complexes, MAD-3 was present only in the p105N immune complex. Because anti-p105C reacts only with the precursor and not the mature p50 product, it appears that MAD-3 is not associated with p105 in vivo. Although it is formally possible that anti-p105C is incapable of reacting with MAD-3-containing complexes, it is important to note that MAD-3 is only a minor component of the complexes isolated by anti-p105N (seen only after a longer exposure of Fig. 6). Therefore, even if MAD-3 associates with some p105-containing complexes, it is not present in the majority of these complexes.

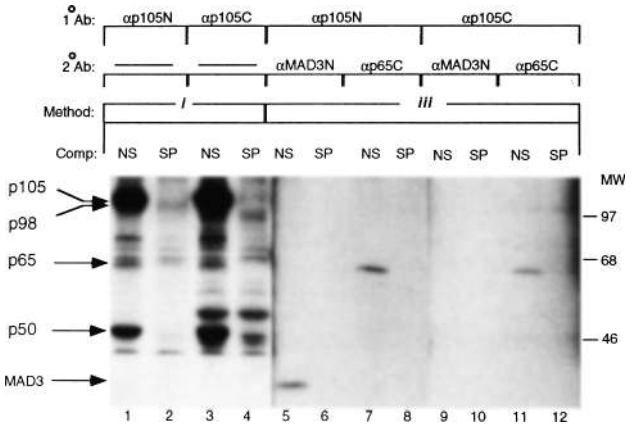

Figure 6. MAD-3 is not associated with p105-containing complexes. TPA/ConA-stimulated Jurkat cells were metabolically labeled with [^{35}S] methionine for 5hr and lysed in TNT buffer, which retains NF-κB/IκB associations, and immunoprecipitated with either anti-p105N (lanes *1,2*) or anti-p105C (lanes *3,4*) antisera. To determine whether MAD-3 or p65 is present within the primary immune complexes, these complexes were disrupted in SDS-RIPA buffer and reprecipitated [method 3 (iii)] with either anti-MAD-3 (lanes *5,6,9,10*) or anti-p65C (lanes *7,8,11,12*) antisera. All antisera were preincubated with either specific (SP) or nonspecific (NS) peptide competitor, as indicated.

Association with either p105 or p98 leads to cytoplasmic retention of p65 and c-Rel

NF-κB-mediated transcriptional activation is regulated in part by subcellular localization (Baeuerle 1991). Because both p105 and p98 can associate with either c-Rel or p65 in vitro and in vivo, and both of these proteins contain SWI6/ankyrin repeats similar to IκB, we explored the possibility that this association could result in cytoplasmic retention of c-Rel and p65. To this end, a sequence encoding a peptide epitope (Flag) was fused to the initiation codons of the cDNAs encoding c-Rel and p65 to enable their detection by immunofluorescence using a monoclonal anti-Flag antibody. The FL–c-Rel and FL–p65 chimeras were inserted into a mammalian expression vector and transiently transfected into CV-1 cells alone or cotransfected with increasing levels of expression vectors encoding either p105 or p98. When transfected alone, both FL–c-Rel and FL–65 were confined to the nucleus (Fig. 7a,b, panels A,B). However,

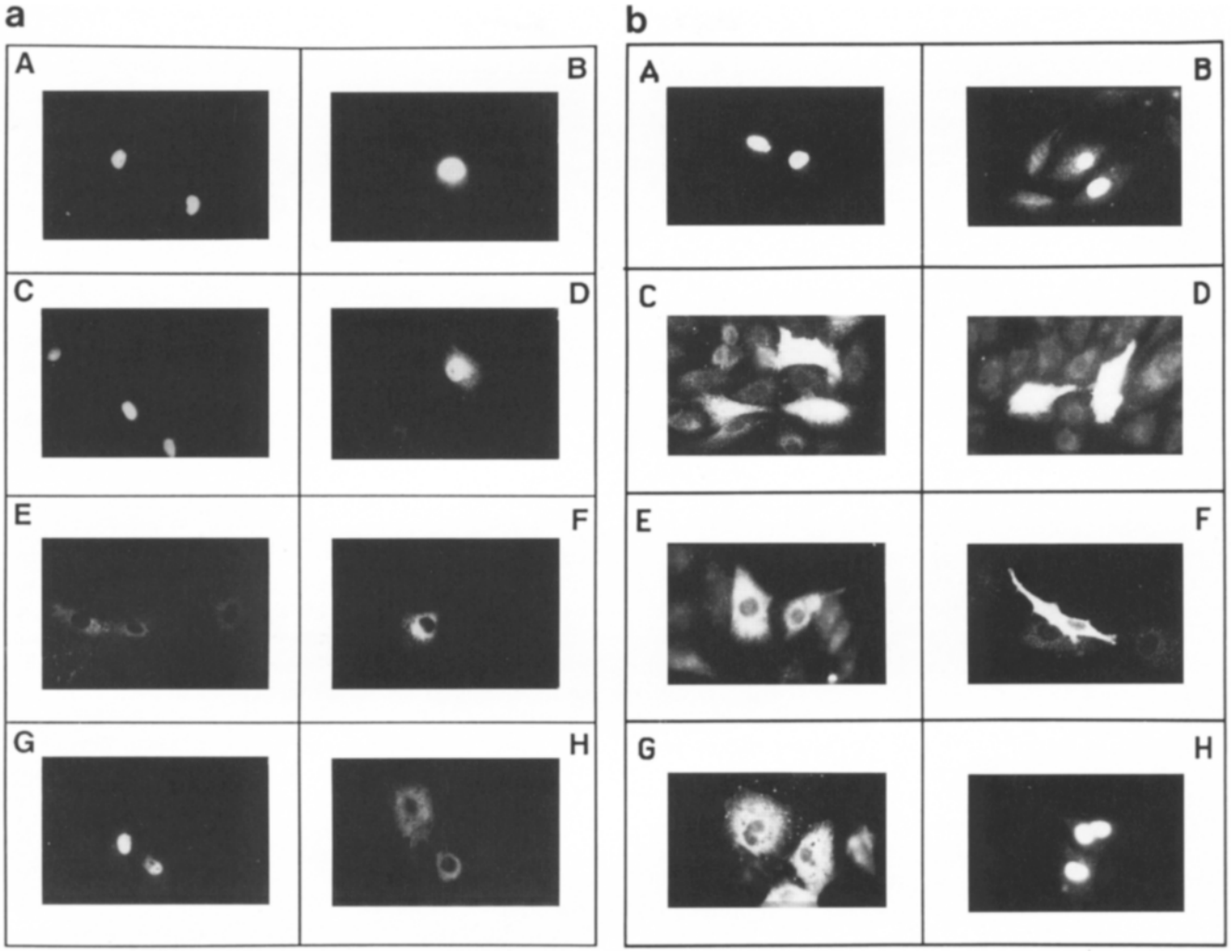

Figure 7. p105 and p98 retain p65 and c-Rel in the cytoplasm. (*a*) CV-1 cells were transfected with expression vectors encoding either FL–65 (*A, C, E*) or FL–c-Rel (*B, D, F*), either alone (panels *A,B*) or in the presence of equimolar amounts (panels *C,D*) or a fourfold molar excess (panels *E,F*) of an expression vector encoding p105. As controls, the FL–65 expression vector was cotransfected with a threefold molar excess of an expression vector encoding either p50 (*G*) or MAD-3 (*H*). (*b*) For panels *A–F* CV-1 cells were transfected exactly as described above (*a*) for *A–F*, with the single exception that an expression vector encoding p98 was used instead of the p105 vector. As controls, FL–c-Rel was cotransfected with a threefold molar excess of p55 (*H*) or threefold molar excess of both p55 and MAD-3 (*G*). At 16–24 hr post-transfection the cells were fixed and analyzed by indirect immunofluoresence, as described in Materials and methods, using anti-Flag antibodies.

when cotransfected with equimolar amounts of p98, FL–c-Rel and FL–65 were partially retained in the cytoplasm (Fig. 7b, panels C,D). While an equimolar amount of the p105 plasmid caused partial cytoplasmic retention of FL–c-Rel, it had little effect on FL–65, which remained nuclear (Fig. 7a, panels C,D). However, cotransfection with a fourfold molar excess of either p105 or p98 led to almost complete cytoplasmic retention of both FL–c-Rel and FL–65 (Fig. 7a,b, panels E,F). Cotransfection of p50 with FL–65 (Fig. 7a, panel G) or p55 with FL–c-Rel (Fig. 7b, panel H) had no affect on the nuclear localization of the tagged proteins. In contrast, cotransfection of a threefold molar excess of MAD-3 with either FL–65 (Fig. 7a, panel H) or p55 and FL–c-Rel (Fig. 7b, panel G) resulted in the cytoplasmic retention of both tagged proteins. These findings suggest that both p105 and p98 can function in an "IκB-like" capacity, and this process is dependent on the presence of the carboxy-terminal halves of the precursors.

Pulse-chase analysis of p105 and p98 processing

Because both p105 and p98 can provide an IκB-like function, it is possible that the subcellular localization of complexes involving either precursor could be regulated, at least in part, by proteolytic processing. We therefore examined the effect of cell stimulation on the rate of p105 and p98 processing. HeLa S3 cells were pulse-la-

beled with [^{35}S]methionine for 60 min and chased with cold methionine either in the absence or presence of TPA stimulation. The levels of ^{35}S-labeled p105 and p98 were determined by immunoprecipitation of denatured cell lysates with either anti-p105N or anti-p98N. TPA stimulation led to an increased rate of proteolytic processing for both p105 (data not shown) and p98, as indicated by the appearance of higher amounts of mature products in TPA-stimulated cells (Fig. 8).

The relative rates of processing of p105 and p98 were compared. Cells were stimulated with either TPA (HeLa) or TPA/Con A (Jurkat) for 1.5 hr, pulse-labeled for 45 min with [^{35}S]methionine, and chased with cold methionine in the presence of continued stimulation. In stimulated HeLa cells all labeled p105 disappeared within 5 hr (Fig. 9A), whereas only 30% of the labeled p98 disappeared within the same period (Fig. 9B). In both cases, decreased levels of the precursors were associated with increased levels of their products. Interestingly, the level of proteolytic processing was much lower in Jurkat cells for both p105 (data not shown) and p98 (Fig. 9C).

The results described in Figure 4 indicated that the majority of c-Rel in Jurkat cells was associated with p98. If the p98/c-Rel complexes are precursors for functional p55/c-Rel complexes, they must be processed before nuclear translocation. We therefore followed the fate of these complexes to determine whether they are processed into potentially active p55/c-Rel complexes. Jurkat cells were induced with TPA and ConA for 2 hr, metabolically labeled for 1 hr with [^{35}S]methionine, and chased with cold methionine. Cells were harvested at various time points, lysed in RIPA buffer, and immunoprecipitated with anti-c-Rel antibodies. After dissociation of the immune complexes, secondary immunoprecipitations were performed with anti-p98N. The results

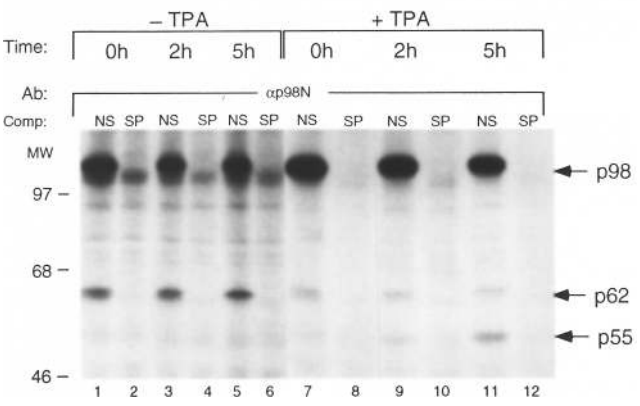

Figure 8. TPA stimulation increases the rate of p98 processing. HeLa cells were serum starved for 24 hr, pulse-labeled for 60 min with [^{35}S]methionine, and chased with cold methionine either in the absence (lanes *1–6*) or presence (lanes *7–12*) of TPA (100 ng/ml). p98 was immunoprecipitated from SDS-RIPA lysates prepared at the indicated time with anti-p98N. The antibodies were preincubated with either specific (SP) or nonspecific (NS) peptide competitors. Immunoprecipitates were analyzed as described above.

indicated that p98 associated with c-Rel is processed to yield mature p55 (Fig. 10). This should result in formation of potentially active p55/c-Rel complexes. In other experiments in which TPA-treated HeLa cells were fractionated into cytosolic and nuclear fractions, we found that all of the newly processed p55 was nuclear, whereas p98 remained in the cytoplasm (data not shown). These results are consistent with the Western blot analysis shown above (Fig. 3C).

Discussion

NF-κB and related complexes formed by Rel/NF-κB family members are involved in rapid gene activation in response to extracellular signals. Transcriptional activation by these complexes is regulated in part by their subcellular localization. Inactive Rel/NF-κB complexes are present in the cytoplasm before cell stimulation, where they are efficiently removed from their nuclear targets. In response to extracellular stimuli these complexes translocate to the nucleus, where they activate transcription of Rel/NF-κB-responsive genes (Baeuerle 1991; Baeuerle and Baltimore 1991). One group of proteins responsible for cytoplasmic retention of Rel/NF-κB complexes are the IκB inhibitors. Release of Rel/NF-κB complexes from binding to IκB is a signal-dependent event, thought to involve the direct phosphorylation of IκB (Shirakawa and Mizel 1989; Ghosh and Baltimore 1990; Kerr et al. 1991). However, a direct proof that the association of any IκB molecule with Rel/NF-κB complexes is regulated by phosphorylation in vivo is yet to be provided. The work presented above demonstrates the existence of a second pathway whereby extracellular stimuli can affect the intracellular location of Rel/NF-κB complexes in the absence of IκB (Fig. 11). We show that both p105 and p98 precursor proteins can associate with other Rel/NF-κB family members in vitro and in vivo. This association can result in cytoplasmic retention of either c-Rel or p65 and possibly other family members. Cell stimulation with TPA results in increased processing of both p105 and p98 leading to formation of their p50 and p55 products, respectively. Because these proteins can no longer retain p65 or c-Rel in the cytoplasm, this should result in the formation of active Rel/NF-κB complexes free to undergo nuclear translocation. While this manuscript was being reviewed, Rice et al. (1992) reported similar findings with p105.

In vitro both p105 and p98 can form stable complexes with various Rel/NF-κB proteins, analogously to their products p50 and p55 (Gilmore 1991; Blank et al. 1992). In particular, p105 and p98 can associate with p50, p55, p65, c-Rel, and each other (Table 1). Formation of similar complexes in vivo was demonstrated by sequential immunoprecipitations using subunit-specific antibodies. All of these complexes are very stable, surviving multiple immunoprecipitations done under relatively stringent conditions. The possibility of subunit exchange was examined by challenging the p98/FL–65 heterocomplex with a 10-fold excess of p55, and no subunit exchange was found (J. DiDonato, unpubl.). As reported previously

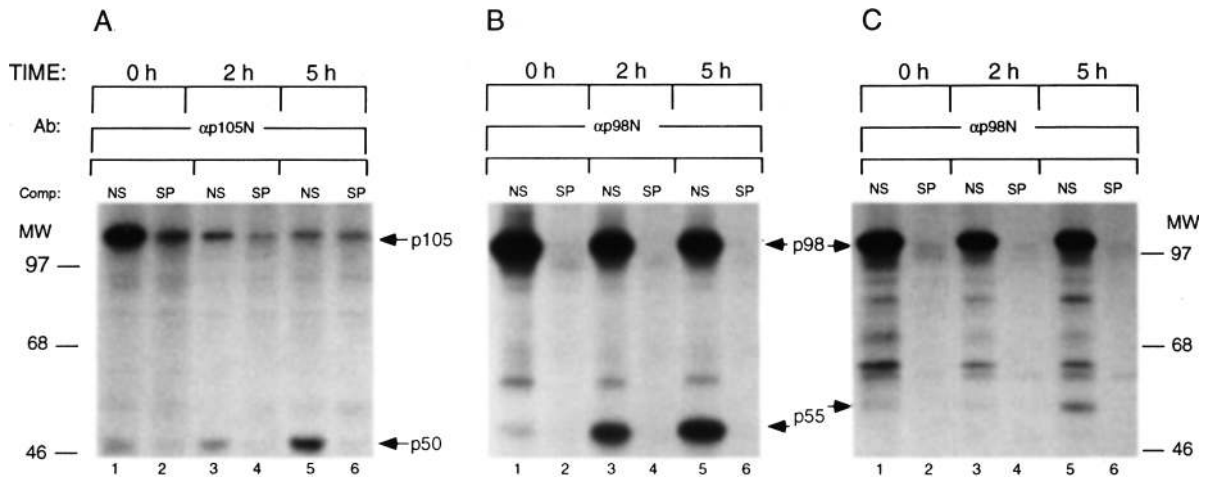

Figure 9. Processing of p105 and p98 in HeLa and Jurkat cells. HeLa cells (panels *A*,*B*) were stimulated with TPA (100 ng/ml) for 1.5 hr, pulse-labeled for 45 min with [^{35}S]methionine, and subsequently chased with cold methionine. Cell lysates were prepared in SDS-RIPA buffer and boiled at the indicated time points and analyzed by immunoprecipitation with either anti-p105N (*A*) or anti-p98N (*B*). Jurkat cells (*C*) were stimulated with TPA (100 ng/ml) and ConA (2 μg/ml) for 1.5 hr, pulse-labeled with [^{35}S]methionine, and subsequently chased with cold methionine. Cell lysates were prepared in SDS-RIPA buffer at the indicated time points and analyzed by immunoprecipitation using anti-p98N. The immune complexes were analyzed by 10% SDS-PAGE.

(Blank et al. 1991; Henkel et al. 1992), p105 is an exclusively cytoplasmic protein (F. Mercurio and C. Rosette, unpubl.), and by indirect immunofluorescence and cell fractionation studies we found that p98 is also restricted to the cytoplasm (Fig. 3C; F. Mercurio and C. Rosette, unpubl.). This implies that any complexes containing either p105 or p98 must also be cytoplasmic. By virtue of their cytoplasmic localization, p105 and p98 can provide an IκB-like function, leading to the cytoplasmic retention of otherwise nuclear proteins, such as p50, p65, or c-Rel. This proposal was substantiated by transfection experiments using constructs encoding p65, c-Rel, p98, and p105. Immunofluorescence analysis of epitope-tagged p65 and c-Rel indicated that in the absence of cotransfected p105 or p98, both tagged proteins were predominately nuclear. However, expression of increasing amounts of either p98 or p105 led to cytoplasmic retention of both p65 and c-Rel. Cytoplasmic retention of c-Rel or p65 by p98 or p105 appeared to be as complete as the retention achieved upon cotransfection of a MAD-3 expression vector. Cotransfection with expression vectors encoding the processed forms of p105 or p98 did not affect the nuclear localization of either c-Rel or p65. Therefore, the carboxy terminal domains of either p105 or p98 are required for their cytoplasmic retention function.

IκB proteins are composed primarily of SWI6/ankyrin repeats (Blank et al. 1992). Recent studies suggest that IκB proteins retain Rel/NF-κB complexes in the cytoplasm by masking the nuclear transfer signals (NTS) within the Rel homology domains (Beg et al. 1992). This interaction is likely to be mediated by the SWI6/ankyrin repeats (Blank et al. 1992). A similar function was proposed for the IκBγ protein, which is produced by alternative splicing of the primary p105 transcript (Inoue et al. 1992). IκBγ consists mostly of the carboxy terminal domain of p105, containing its SWI6/ankyrin repeats, and can inhibit p50/p65 DNA binding in vitro and pre-

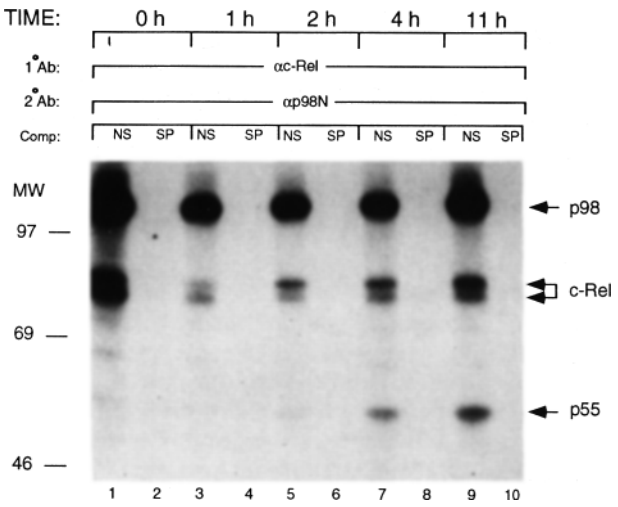

Figure 10. Pulse-chase analysis of c-Rel-associated p98. Jurkat cells were serum starved for 22 hr, induced with TPA (100 ng/ml) and ConA (2 μg/ml) for 2 hr, pulse-labeled for 45 min with [^{35}S]methionine, and chased with cold methionine. Cells were lysed in RIPA buffer at the indicated time points and subjected to primary immunoprecipitation with anti-c-Rel antiserum. The primary immune complexes were disrupted by boiling in SDS-RIPA buffer, and the supernatant was reprecipitated with anti-p98N antiserum, which was preincubated with either specific (SP) or nonspecific (NS) peptide competitors, as indicated. Final immunoprecipitates were analyzed as described above. As noticed earlier, a small amount of c-Rel reprecipitated with p98/p55 most likely due to reassociation after dilution of the denatured lysate before immunoprecipitation.

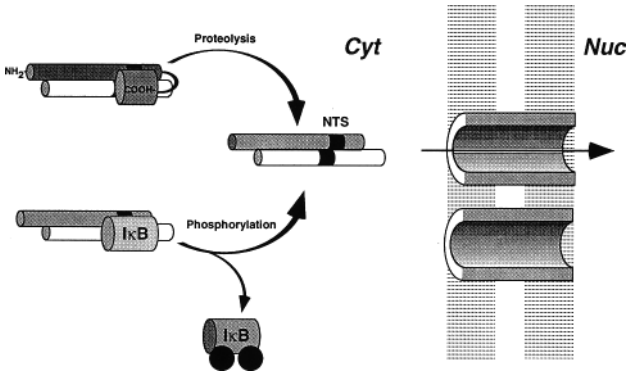

Figure 11. Alternative pathways for activation of cytoplasmic NF-κB/Rel complexes. One pathway involves the proteolysis of a p98 or p105 precursor whose carboxy-terminal domain containing IκB-like repeats prevents the recognition of the NTS of both subunits (The interaction with the NTS is based on the recent work of Beg et al. 1992.) The other, more conventional, pathway is thought to involve the phosphorylation of IκB, which prevents the recognition of the NTS while bound to the NF-κB/Rel complex (phosphorylation of IκB in vivo is yet to be demonstrated). The liberated complexes display their NTS and can undergo nuclear transfer.

vent the nuclear translocation in vivo of the heterocomplex. The cytoplasmic retention of p65 and c-Rel by p105 and p98, described here, is unlikely to be the result of the production of IκBγ or a similiar derivative of p98 because such proteins were not detected under our experimental conditions (see Figs. 3 and 9; F. Mercurio, unpubl.). Recently, Henkel et. al. (1992) proposed an intramolecular masking model, whereby the SWI6/ankyrin repeats of p105 act indirectly to prevent its nuclear transfer by providing an extension that allows a remote carboxy-terminal domain to fold back and mask the NTS and dimerization motifs within the Rel homology domain. In part, that model was based on the failure of a particular anti-p105 carboxy-terminal antiserum to coprecipitate p65 with p105. It is possible that this particular antiserum is incapable of recognizing p105/p65 complexes because of steric hinderance between p65 and the antigenic epitope. Our results using two different anti-p105 antisera, directed against either amino- or carboxy-terminal sequences clearly demonstrate that this protein is associated with p65 and other Rel/NF-κB family members, such as c-Rel, p55/p98, and p50, in vivo. Similar experiments demonstrated the association of p98 with c-Rel, p65, and p50/p105 (Table 1). Thus, it is unlikely that the carboxy-terminal region of either precursor folds back on its Rel domains and inhibits its interactions with other family members. It is more likely that the carboxy-terminal halves of either p105 and p98, which are both similar in their repeat structure to IκB, provide an IκB-like function and prevent recognition of the NTS of both subunits (see Fig. 11). The SWI6/ankyrin repeats may also anchor the complex to a cytoplasmic structure, providing an additional mechanism for cytoplasmic retention.

Having demonstrated that p105 and p98 are capable of associating with Rel/NF-κB proteins leading to their cytoplasmic retention, we investigated whether the state of these complexes is affected by extracellular stimuli. In theory, proteolytic processing of the precursors, which removes their carboxy-terminal IκB-like domains, should result in either the liberation of an active complex from its restricted cytoplasmic localization or reveal its NTS. Either way, nuclear localization should ensue. Regulated proteolytic processing could control the level and composition of Rel/NF-κB complexes undergoing nuclear translocation, as well as affect the kinetics of this process. Alternatively, the liberated complexes may encounter IκB molecules and remain cytoplasmic until a proper extracellular signal leads to their dissociation from IκB. Riviere et al. (1991) have shown that the human immunodeficiency virus 1 (HIV-1) protease can process p105, leading to increased levels of active nuclear NF-κB complex. We found that TPA stimulation of both HeLa and Jurkat cells increases the rate of processing of both precursors. At this point, we do not know whether this is the result of increased activity of the processing protease or a post-translational modification of the precursors, which increases their susceptibility to processing. Alternatively, the efficacy of the processing reaction could increase in response to the elevated concentrations of the precursors, whose synthesis is TPA inducible (Bours et al. 1990,1992; Mercurio et al. 1992). Fan and Maniatis (1991) demonstrated that an ATP-dependent protease is responsible for p105 processing. It remains to be determined whether the same activity is responsible for the processing of p98.

We found that the kinetics of total p98 processing were not significantly different from those of the population of p98 associated with c-Rel. This suggests that the processing protease does not discriminate between uncomplexed p98, if it exists, c-Rel-complexed p98, and p98 complexed with other family members. The rate of p98 processing was sufficiently slow to cause a prolonged release of supposedly active p55/c-Rel complexes. These slow processing kinetics fit the recently described kinetics of nuclear translocation of c-Rel (Tan et al. 1992) or the appearance of c-Rel-containing-NF-κB complexes (Ballard et al. 1990). It is likely that the delayed nuclear translocation of c-Rel-containing complexes is regulated at the level of proteolytic processing of p98, as the latter appears to be an abundant c-Rel-associated protein and the p98/c-Rel complexes are particularly stable. In addition, cell fractionation experiments suggest that once p55 is generated by the processing of p98, it enters the nucleus rather rapidly, because all of the detectable p55 was found to be confined to the nuclear fraction (Fig. 3C; data not shown).

The rate and extent of processing of both precursors was considerably different between Jurkat and HeLa cells. Both p105 and p98 displayed lower relative levels of processing in Jurkat cells compared with HeLa S3 cells. Differential cell-type levels of proteolytic processing in response to extracellular stimuli should determine the relative contribution of the proteolytic pathway versus the IκB-mediated pathway to induction of NF-κB ac-

tivity (Fig. 11). We propose that the cell exploits a regulated proteolytic processing event to further modulate the nuclear translocation of Rel/NF-κB complexes. Neri et. al. (1991) have recently identified a B-cell chromosomal translocation involving *p98* (*lyt-10*), generating a *lyt-10/Cα-1* fusion gene that retains the Rel homology domain but lacks the IκB-like carboxy-terminal regulatory domain. This fusion protein displays similar characteristics to the normal p55 protein. The inability of this protein to retain either itself or other family members, such as c-Rel, in the cytoplasm may be responsible for its oncogenic potential.

Materials and methods

In vitro translations

In vitro translations were performed by adding plasmid DNAs encoding the cDNAs for p105 (Kieran et al. 1990; kindly provided by Dr. A. Israel, Institute Pasteur, Paris, France), pFL98 (Mercurio et al. 1992), hc-Rel (Brownell et al. 1989; kindly provided by Dr. N. Rice, National Institutes of Health, Bethesda, MD), and p65 (Nolan et al. 1991; Mercurio et al. 1992) to the TNT-coupled transcription/translation rabbit reticulocyte lysate system (Promega), using either the T3 or T7 RNA polymerase and incubation temperatures recommended by the manufacturer. The translation reactions contained either 1 μg of plasmid DNA for single gene expression or 1.5 μg of total plasmid DNA for reactions requiring the expression of two genes. Translation products were radioactively labeled by including 20 μCi of [^{35}S]methionine (Amersham) per reaction. Labeled proteins were analyzed on 10% SDS–polyacrylamide gels. Immunoprecipitations of in vitro-translated polypeptides were carried out as described (Mercurio et al. 1992).

Plasmids

Plasmids encoding p65 (pCMV65) and FL–p98 were described previously (Mercurio et al. 1992). pCMV969 and CMV399 contain the cDNAs encoding p105 and a truncated p105 (Kieran et al. 1990; a gift from Dr. A. Israel). pBShc-Rel was made by ligating the *Hin*dIII–*Eco*RV and *Eco*RV– *Eco*RI fragments of pUC clone 1 and pUC clone 2, respectively (generously provided by Dr. N. Rice; Brownell et al. 1989) and contains the *hc-rel* cDNA into Bluescript SK+ (Stratagene). RSV–FL–c-Rel contains the Flag epitope (IBI–Kodak) fused in-frame to the amino terminus of hc-Rel under the control of the Rous sarcoma virus long terminal repeat (RSV LTR). pCMV–MAD-3 contains the cDNA encoding MAD-3, which was generated by polymerase chain reaction (PCR; sequenced to verify its identity), and inserted between the *Xba*I and *Apa*I sites of Rc/CMV (Invitrogen). pCMV–FL–65 contains the Flag epitope fused in-frame to the amino terminus of p65. pCMV98 and pCMV55 contain the cDNAs encoding p98 and p55, respectively, which were cloned into the *Hin*dIII and *Xba*I site of Rc/CMV (Invitrogen).

Cells and transfections

HeLa S3 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. Jurkat cells were grown in RPMI 1640 supplemented with 10% FCS, 50 μM β-mercaptoethanol, and 2 mM glutamine. CV-1 cells were grown in DMEM supplemented with 5% FCS and 2 mM glutamine. As described by Angel et al. (1988), each 60-mm culture dish was incubated with a calcium phosphate/DNA coprecipitate containing RSV–FL–c-Rel (2 μg) or pCMV–FL–65 (1.5 μg) either alone or in combination with additional expression constructs as designated in the text. Coprecipitates were removed after 8 hr, and cells were prepared for immunofluorescence 16 hr after transfection.

In vivo immunoprecipitation

Immunoprecipitation of endogenous proteins was performed as described (Mercurio et al. 1992). Subsequent to metabolic labeling with [^{35}S]methionine, cells ($5 \times 10_6$, method 1; 1×10^7, method 2; and 3×10^7, methods 3 and 4) were pelleted and lysed in either native RIPA buffer or RIPA buffer containing 1% SDS. Cell lysates were precleared by incubation with nonimmune sera (3–15 μl) and protein A–Sepharose (Pharmacia). The cleared lysates were incubated further with the appropriate primary antiserum, which was preincubated with either specific or nonspecific peptide competitors (1 μg of peptide/1 μl of antisera) for 30 min at room temperature. Primary immunoprecipitates were analyzed as described (Mercurio et al. 1992) (methods 1 and 2) or disrupted either by resuspending in 1% SDS–RIPA buffer and boiling for 5–10 min — supernatants were made 0.1% SDS before immunoprecipitation (method 3) — or by resuspending in RIPA buffer and incubating with a large excess (10 μg of peptide/μl of antisera) of specific peptide competitor for 8–12 hr at 4°C. The resulting supernatants were subjected to secondary immunoprecipitation with the appropriate antiserum as described above. The final immune pellets were analyzed on 10% SDS–polyacrylamide gels.

For pulse-chase analysis, HeLa S3 and Jurkat cells were pulse-labeled for the period of time indicated in the figure legends with [^{35}S]methionine (100 μCi/ml) and chased with 100-fold excess of cold methionine (25 mM). Immunoprecipitations were performed as described above.

Antibodies

The anti-p105N and anti-p98N antisera are directed against the amino-terminal sequences of p105 and p98, respectively (Mercurio et al. 1992). Peptide KB-2 (NH_2-CPHDYGQEGPLEGKI-COOH) containing the carboxy-terminal sequence of p105 was coupled to keyhole limpet hemocyanin (KLH) with maleimidobenzoyl-*N*-hydroxysuccinimide ester as described (Mercurio et al. 1986). Generation of anti-p105C from the KLH–KB2 immunogen was performed as described (Mercurio et al. 1992). Anti-MAD-3 is prepared against amino acids 6–20 of MAD-3, anti-p65N is directed against a peptide corresponding amino acids 1–15 of human p65 (kindly provided by Dr. A. Baldwin, University of North Carolina, Chapel Hill, NC), anti-p65C is a peptide antibody directed against the carboxy-terminal amino acids of human p65 (kindly provided by Drs. W. Greene and D. Ballard, Gladstone Institute), and anti-c-Rel is a peptide antibody (no. 265) prepared against hc-Rel amino acids 604–619 (kindly provided by Dr. N. Rice; Brownell et al. 1989).

Preparation of cell extracts and Western blot analysis

HeLa cell nuclear and cytoplasmic extracts were prepared essentially as described by Dignam et al. (1983), with the following modifications: Isolated nuclei were gently washed in hypotonic buffer made 0.02% NP-40, and the nuclear pellet was then resuspended in the same buffer and pelleted through a 50% sucrose cushion made in hypotonic buffer. Cytoplasmic (50 μg) and nuclear (25 μg) extracts were fractionated on a 10% SDS–polyacrylamide gel and transferred to Immobilon paper for Western blot analysis. The blot was incubated with a 1 : 400

dilution of anti-p98N antiserum, which was preincubated with either a specific or nonspecific peptide competitor. The blot was then developed with the ECL Western blotting detection system (Amersham) according to the manufacturer's instructions.

Immunofluorescence

All manipulations were carried out at room temperature according to standard procedures (Harlow and Lane 1988). Fixed cells (in 4% paraformaldehyde) were washed in PBS − 5 mM glycine, permeabilized in PBS + 0.3% Triton X-100 (8 min), and saturated in PBS + 1% gelatin and 1% BSA (15 min). Binding of the primary antibody was for 1 hr using 1.0 μg/ml of anti-Flag antibodies (M2 antibodies, IBI). After four washes with PBS, either rhodamine- or fluorescein-conjugated secondary antibody (Cappel) was applied for 30 min followed by several washes with PBS and nuclear staining with 0.0001% DAPI (10 min). The slides were mounted in Mowiol mounting solution, viewed on a fluorescent light microscope, and photographed.

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Note added in proof

At a recent meeting sponsored by the Howard Hughes Medical Institute, a new nomenclature for the NF-κB/Rel family of proteins was suggested. Our study was completed and submitted before the new nomenclature system was decided on. However, consistent with the suggestions at the meeting, the designations of the NF-κB/Rel proteins should be as follows: p105/p50 is NF-κB1; p100/p52 (p49), also known as p98/p55, is NF-κB2; v-Rel, c-Rel, RelB, and Dorsal remain the same; p65 is RelA. In the IκB molecules, MAD-3, pp40, and RL-IF1 are to be referred to as IκBα; IκBγ, Bcl-3, and IκBβ remain the same.

References

Angel, P., E.A. Allegretto, S. Okino, K. Hattori, W.J. Boyle, T. Hunter, and M. Karin. 1988. Oncogene jun encodes a sequence–specific activator similar to AP-1. *Nature* **332:** 166–171.

Baeuerle, P.A. 1991. The inducible transcriptional activator NF–κB: Regulation by distinct protein subunits. *Biochim. Biophys. Acta* **1072:** 63–80.

Baeuerke, P.A. and D. Baltimore 1988a. IκB: A specific inhibitor of the NF-κB transcription factor. *Science* **242:** 540–546.

———. 1988b. Activation of DNA–binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. *Cell* **53:** 211–217.

———. 1989. A 65 kD subunit of active NF–κB is required for inhibition of NF-κB by IκB. *Genes & Dev.* **3:** 1689–1698.

———. 1991. The physiology of the NF–κB transcription factor. In *The horomonal control regulation of gene transcription* (ed. P. Cohen and J.G. Foulkes), pp. 423–446. Elsevier Science Publishers, Amsterdam.

Ballard, D.W., W.H. Walker, S. Doerre, P. Sista, J.A. Molitor, E.P. Dixon, N.J. Peffer, M. Hannink, and W.C. Green. 1990. The v–rel oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. *Cell* **63:** 803–814.

Beg, A.A., S.M. Rubin, R.I. Scheinman, S. Haskill, C.A. Rosen, and A.S. Baldwin Jr. 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: A mechanism for cytoplasmic retention. *Genes & Dev* **6**:1899–1913.

Blank, V., P. Kourilsky, and A. Israel. 1991. Cytoplasmic retention, DNA-binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. *EMBO J.* **10:** 4159–4167.

———. 1992. NF-κB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. *Trends Biol. Sci.***3:** 135–140.

Bours, V., J. Villalobos, P.R. Burd, K. Kelly, and U. Siebenlist. 1990. Cloning of a mitogen-inducible gene encoding a κB-binding protein with homology to the Rel oncogene and to cell-cycle motifs. *Nature* **348:** 76–80.

Bours, V., P.R. Burd, K. Brown, J. Villalobos, S. Park, R.-P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist. 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. *Mol. Cell. Biol.* **12:** 685–695.

Brownell, E., N. Mittereder, and N.R. Rice 1989. A human *rel* proto-oncogene cDNA containing an Alu fragment as a potential coding exon. *Oncogene* **4:** 935–942.

Capobianco, A.J., D. Chang, G. Mosialos, and T. Gilmore. 1992. p105, the NF-κB p50 precursor protein, is one of the cellular proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. *J. Virol.* **66:** 3758–3767.

Davis, N., W. Bargmann, M.-Y. Lim, and H. Bose. 1990. Avian reticuloendotheliosis virus-transformed lymphoid cells contain multiple pp59$^{v\text{-}rel}$ complexes. *Oncogene* **5:** 1109–1115.

Davis, N., S. Ghosh, D.L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H.R. Bose Jr. 1991. Rel-associated pp40: An inhibitor of the Rel family of transcription factors. *Science* **253:** 1268–1271.

Dignam, J.D, R.M. Lobovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract isolated from isolated mammalian nuclei. *Nucleic Acids Res.* **11:** 1475–1489.

Fan, C.-M. and T. Maniatis. 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. *Nature* **354:** 395–398.

Ghosh, S. and D. Baltimore. 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. *Nature* **344:** 678–683.

Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan, and D. Baltimore. 1990. Cloning of the p50 DNA binding subunit of NF-κB: Homology to *dorsal* and *rel*. *Cell* **62:** 1019–1029.

Gilmore, T.D. 1991. Malignant transformation by mutant Rel proteins. *Trends Genet.* **7:** 318–322.

Harlow, E. and D. Lane. 1988. Cell staining. In *Antibodies: A*

laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Haskill, D., A.A. Beg, S.M. Tompkins, J.S. Morris, A.D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A.S. Baldwin Jr. 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. *Cell* **65:** 1281–1289.

Hatada, E.N., A. Nieters, G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T.W. McKeithan, and C. Scheidereit. 1992. The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene *bcl-3* act as specific inhibitors of NF-κB DNA binding. *Proc. Natl. Acad. Sci.* **89:** 2489–2493.

Henkel, T., U. Zabel, K. Van Zee, J.M. Muller, G.J. Arnold, E. Fanning, and P. Baeuerle. 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. *Cell* **68:** 1121–1133.

Hunter, T. and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell* **70:** 375–387.

Inoue, J.I., L.D. Kerr, A. Kakizuka, and I.M. Verma. 1992. IκBg, a 70kd protein identical to the C-terminal half of p110 NF-κB: A new member of the IκB family. *Cell* **68:** 1109–1120.

Karin, M. 1992. Signal transduction from cell surface to nucleus in development and disease. *FASEB J.* **6:** 2581–2590.

Kerr, L.D., J. Inoue, N. Davis, E. Link, P.A. Baeuerle, H.R. Bose Jr., and I.M. Verma. 1991. The rel-associated pp40 protein prevents DNA-binding of rel and NF-κB: Relationship with IκBβ and regulation by phosphorylation. *Genes & Dev* **5:** 1464–1476.

Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Ball, M.B. Urban, P. Kourilsky, P.A. Baeuerle, and I. Israel. 1990. The DNA binding subunit of NF-KB is identical to factor κBF1 and homologous to the rel oncogene product. *Cell* **62:**1007–1018.

Lenardo, M.J. and D. Baltimore. 1989. NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58:** 227–229.

Lim, M.Y., N. Davis, J. Zhang, and H.R. Bose. 1990. The v-Rel protooncogene is complexed with cellular proteins including its protooncogene product and heat shock protein 70. *Virology* **175:** 149–160.

Logeat, F., N. Israel, R. Ten, V. Blank, P. Kourilsky, and A. Israel. 1991. Inhibition of transcription factors belonging to the rel/NF-κB family by a transdominant negative mutant. *EMBO J.* **10:** 1827–1832.

Mercurio, F., R.A. Houghten, and J.C. Lagarias. 1986. Site-directed antisera to the chromophore binding site of phytochrome: Characterization and cross-reactivity. *Arch. Biochem. Biophys.* **248:** 35–42.

Mercurio, F., J. DiDonato, C. Rosette, and M. Karin. 1992. Molecular cloning and characterization of a novel Rel/NF-κB family member displaying structural and functional homology to NF-κB p50/p105. *DNA Cell Biol.* **11:** 523–537.

Meyer, R., E.N. Hatada, H.P. Hohmann, M. Haiher, C. Bartsch, V. Rothlisberger, H.-W. Lahm, E.J. Schlaeger, A.P.G.M. Van Loon, and C. Scheidereit. 1991. Cloning of the DNA-binding subunit of human nuclear factor κB: The level of its mRNA is strongy regulated by phorbol ester of tumor necrosis factor a. *Proc. Natl. Acad. Sci.* **88:** 966–970.

Molitor, J.A., W.H. Walker, S. Doerre, D.W. Ballard, and W.C. Greene. 1990. NF-κB: A family of inducible and differentially expressed enhancer-binding proteins in human T cells. *Proc. Natl. Acad. Sci.* **87:** 10028–10032.

Morrison, L.E., N. Kabrum, S. Mudri, M.J. Hayman, and P.J. Enrietto. 1989. Viral rel and cellular rel associate with cellular proteins in transformed and normal cells. *Oncogene* **4:** 677–683.

Neri, A., C.-C. Chang, L. Lombardi, M. Salina, P. Corradini, A.T. Maiolo, R.S.K. Chaganti, and R. Dalla-Favera. 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene, *lyt-10,* homologous to NF-kappa B p50. *Cell* **67:** 1075–1087.

Nolan, G.P., S. Ghosh, H.-C. Liou, P. Tempst, and D. Baltimore. 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a rel-related polypeptide. *Cell* **64:** 961–969.

Ohno, H., G. Takimoto, and T.W. McKeithan. 1990. The candidate proto-oncogene *bcl-3* is related to genes implicated in cell lineage determination and cell cycle control. *Cell* **60:** 991–997.

Perkins, N.D., R.M. Schmid, C.S. Duckett, K. Leung, N.R. Rice, and G.J. Nabel. 1992. Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation. *Proc. Natl. Acad. Sci.* **89:** 1529–1533.

Radler-Pohl, A., I. Pfeuffer, M. Karin, and E. Serfling 1990. A novel T-cell trans-activator that recognizes a phorbol-ester-inducibille element of the interleukin-2 promotor. *New Biol.* **2:** 566–573.

Rice, N.R., M.L. MacKichan, and A. Israel. 1992. The precursor of NF-κB has IκB-like functions. *Cell* **71:** 243–253.

Riviere, Y., V. Blank, P. Kourilsky, and A. Israel. 1991. Processing of the precursor of NF-κB by the HIV-1 protease during acute infection. *Nature* **350:** 625–626.

Ruben, S.M., P.J. Dillon, R. Schreck, T. Henkel, C.-H. Chen, M. Maher, P. Baeuerle, and C.A. Rosen. 1991. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. *Science* **251:** 1490–1493.

Rushlow, C. and R. Warrior. 1992. The rel family of proteins. *BioEssays* **14:** 89–95.

Ryseck, R.-P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo. 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. *Mol. Cell Biol.* **12:** 674–684.

Schmid, R.M., N.D. Perkins, C.S. Duckett, P.C. Andrews, and G.J. Nabel. 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. *Nature* **352:** 733–736.

Shirakawa, F. and S.B. Mizel. 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol. Cell. Biol.* **9:** 2424–2430.

Simek, S. and N.R. Rice. 1988a. p59v-Rel, the transforming protein of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed chicken lymphoid cells. *J. Virol.* **2:** 4730–4736.

———. 1988b. Detection and characterization of the protein encoded by the chicken *c-Rel* protooncogene. *Oncogene Res.* **2:** 103–119.

Stephens, R.M., N.R. Rice, R.R. Hiebsch, H.R. Bose Jr., and R.V. Gilden. 1983. Nucleotide sequence of v-*rel:* The oncogene of reticuloendotheliosis virus. *Proc. Natl. Acad. Sci.* **80:** 6229–6233.

Steward, R. 1987. Dorsal, an embryonic polarity gene in Drosophila, is homologous to the vertebrate proto-oncogene, *c-rel. Science* **238:** 692–694.

Tan, T.-H., G.P. Huang, A. Sica, P. Ghosh, H.A. Young, D.L. Longo, and N.R. Rice. 1992. κB site-dependent activation of the interleukin-2 receptor α-chain gene promotor by human c-Rel. *Mol. Cell Biol.* **12:** 4067–4075.

Tung, H.Y.L., W.J. Bargmann, M.Y. Lim, and H.R. Bose, Jr. 1988. The v-rel oncogene product is complexed to a 40kD phosphoprotein in transformed lymphoid cells. *Proc. Natl. Acad. Sci.* **85:** 2479–2483.

Urban, M.B. and P.A. Baeuerle. 1990. The 65kD subunit of NF-κB is a receptor of IκB and a modulator of DNA-binding specificity. *Genes & Dev.* **4:** 1975–1984.

Urban, M.B., R. Schreck, and P.A. Baeuerle. 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* **10:** 1817–1825.

Wilhelmsen, K.C., K. Eggleton, and H.M. Temin. 1984. Nucleic acid sequences of the oncogene v-*rel* in reticulendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. *J. Virol.* **52:** 172–182.

Zabel, U. and P.A. Baeuerle. 1990. Purified human IκB can rapidily dissociate the complex of the NF-κB transcription factor with its cognate DNA. *Cell* **61:** 255–265.

p105 and p98 precursor proteins play an active role in NF-kappa B-mediated signal transduction.

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