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Phosphorylation of p105 PEST Sequences via a Redox-insensitive Pathway Up-regulates Processing to p50 NF-κB*

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The p105 Rel protein has dual functions; it is the precursor of the p50 subunit of NF-κB, and it acts as an IκB-like inhibitor to retain other Rel subunits in the cytoplasm. We have investigated the posttranslational regulation of p105 following activation of Jurkat T cells and find that a rapid and sustained phosphorylation of p105 is induced. The inducible phosphorylation occurs on multiple serines in the C-terminal-most 150 amino acids of the molecule, a region rich in Pro, Glu, Ser, and Thr residues. Phosphorylation of p105 in Jurkat cells treated with phorbol 12-myristate 13-acetate/ionomycin or with okadaic acid, another activator of NF-κB, is correlated with an increase in proteolytic processing to p50. Intact PEST sequences are required for the phorbol 12-myristate 13-acetate/ionomycin-induced p105 processing, as a 68-amino acid C-terminal deletion abolishes the response to stimulation. When compounds that block IκBα phosphorylation and degradation were tested, the serine protease inhibitors L-1-tosylamido-2-phenylethyl chloromethyl ketone and 1-chloro-3-tosylamido-7-amino-2-heptanone blocked inducible p105 phosphorylation, but the antioxidants pyrrolidine dithiocarbamate and butylated hydroxyanisol did not. Thus, while regulation of the p105 IκB resembles that of IκBα, involving inducible serine phosphorylation and proteolysis of the inhibitory ankyrin repeat domain, it depends on a different, redox-insensitive, signaling pathway.

The Rel proteins are a ubiquitous family of transcription factors that regulate the expression of many genes, particularly those induced during an immune response (see Refs. 1 and 2 for recent reviews). The members of this family bind to specific DNA sequences, κB sites, as homo- or heterodimers to regulate the transcription of target cellular genes and several viruses, including human immunodeficiency virus. In mammalian cells, five proteins containing the Rel homology domain characteristic of these factors have been cloned. Although in some cases Rel proteins are constitutively nuclear, notably the p50 homodimer, most κB-binding activity is in an inactive form in the cytoplasm bound to specific inhibitory proteins, known as IκBs. Upon receipt of a specific activating stimulus, Rel dimers are rapidly freed from these cytoplasmic complexes and translocated to the nucleus, mostly in the form of NF-κB, a heterodimer of p50 and p65 (also called RelA). A large variety of stimuli induce nuclear κB binding activity, including mitogens such as PHA[1] and lipopolysaccharide, cytokines like tumor necrosis factor and interleukin-1β, and stress stimuli such as double-stranded RNA or ultraviolet light (1–3). The two signals required for T cell activation, mimicked by phorbol esters and agents that increase intracellular Ca^{2+}, synergistically activate prolonged nuclear κB-binding activity (reviewed in Ref. 2).

In a given cell, several IκBs are expressed. All IκBs contain multiple copies of a motif called the ankyrin repeat, which is also found in many functionally unrelated proteins and may facilitate protein-protein interactions (see Refs. 4 and 5 for review). There are two types of IκB. Those initially described are separate protein subunits that are believed to bind as monomers to a Rel dimer. In mammalian cells these include IκBα, -β, and -γ (derived from the p105/p50 gene and present in some murine cells) (6–9). p105 and p100, the precursors of the Rel subunits p50 and p52, respectively, form the other class of IκBs. The N-terminal half of each is a Rel DNA-binding subunit, which is liberated by specific proteolysis of the C-terminal portion of the molecule (10), which contains the ankyrin repeats. These precursors most likely retain a single associated Rel subunit in the cytoplasm via interactions involving the dimerization domains of both Rel homology domains and the ankyrin repeats of the precursor (11, 12).

The mechanisms involved in the release of Rel proteins from cytoplasmic Rel-IκB complexes in response to an activating stimulus is best understood for IκBα. Activators of NF-κB typically induce rapid phosphorylation and degradation of IκBα, although the kinetics of the response depends on the activating stimulus. Two N-terminal serines were recently shown to be the targets of the inducible phosphorylation of IκBα, and the presence of constitutively phosphorylated C-terminal PEST sequences is required as well for the inducible degradation (13–17). Phosphorylation of IκBα does not cause it to dissociate from NF-κB, as was initially hypothesized; rather IκBα must be degraded from the complex to liberate the associated Rel dimer (18–23).

The regulation of cytoplasmic complexes containing either precursor protein is less well understood but clearly involves proteolysis of the ankyrin repeat domain in the C-terminal half of the molecules. Increased rates of processing of p105 have been reported in response to lipopolysaccharide (24), PMA and ionomycin (25), and double-stranded RNA (26), and phosphorylation has been implicated in up-regulation of p105 processing following stimulation with tumor necrosis factor (26, 27). However, neither the target residues nor the kinase responsible have been identified. Previous studies have reported in-

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[1] The abbreviations used are: PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; CHX, cycloheximide; PAGE, polyacrylamide gel electrophoresis; aLLnL, *N*-acetyl-Leu-Leu-norleucinal; PDTC, pyrrolidine dithiocarbamate; BHA, butylated hydroxyanisol.

creased serine and threonine phosphorylation of p105 in activated Jurkat T cells. One group also detected phosphotyrosine (28), while another did not (29).

We report here that activation of Jurkat T cells by PMA/ionomycin treatment rapidly induces phosphorylation of multiple serines in the C-terminal-most 150 amino acids of p105, a PEST-rich domain following the ankyrin repeats. We show that phosphorylation is correlated with increased processing of p105 in Jurkat cells treated with PMA/ionomycin or with okadaic acid, a serine/threonine phosphatase inhibitor and activator of NF-κB (30). p105 phosphorylation is critical for the effect of PMA/ionomycin on processing, as the latter is abolished by deletion of 68 amino acids in the inducibly phosphorylated PEST domain. Finally, we show that treatment of cells with antioxidants, which blocks phosphorylation and degradation of IκBα (21, 31, 32), does not affect PMA/ionomycin-induced p105 phosphorylation. The differential sensitivity of p105 and IκBα phosphorylation to cellular redox status indicates that, although both inhibitors undergo inducible serine phosphorylation and proteolysis following T cell activation, they are regulated by different signaling pathways.

MATERIALS AND METHODS

Cell Culture and Treatments—A Jurkat T cell line (positive for CD3 and CD2 expression by FACS) and the Tag Jurkat cell line, the generous gift of G. Crabtree (33), were grown in RPMI supplemented with 10% fetal calf serum and 1% L-glutamine. Cells were kept in culture for fewer than 15 passages and were not allowed to exceed a density of 10^6/ml. Cells were stimulated, as indicated in the text and figure legends, with 20 ng/ml PMA (Sigma) and 3.4 μg/ml ionomycin (Sigma) dissolved in Me_2SO. Control cells were treated with an equivalent volume of Me_2SO. Okadaic acid (Sigma or LC Laboratories) dissolved in Me_2SO was used at a final concentration of 0.6 μM. Cycloheximide (CHX) was used at a final concentration of 50 μg/ml. Preincubations with antioxidants and protease inhibitors were at the concentrations and times indicated in the figure legends.

^{32}P Labeling in Situ—Cells growing in log phase were incubated in phosphate-free medium (Dulbecco's modified Eagle's medium or RPMI) with 3% dialyzed fetal calf serum for 1–3 h and then with 0.2–0.33 mCi/ml [^{32}P]orthophosphate (DuPont NEN) for 1–2 h. Extract preparation and immunoprecipitation of labeled protein were as described below.

Cell Transfections—Jurkat Tag cells were transiently transfected using a Eurogentec Cellject electroporator with pulse values set at 250 V, 1500 microfarads, and infinite resistance. Approximately 10 μg of purified plasmid DNA (Quiagen) were used to transfect 10^7 cells. Typically, 3×10^7 cells were electroporated in 600 μl of RPMI. [^{32}P]orthophosphate labeling or other manipulations were performed 24–40 h later, after cells had recovered in RPMI/fetal calf serum.

Plasmids—p105 and C-terminal truncations of p105 ending at amino acid 901 or 819 were all expressed from the Rc/CMV vector (Invitrogen). The p105 and 901 plasmids have been described previously by Blank *et al.* (34); 901 corresponds to λ14 of that article. The 819 construct was subcloned into Rc/CMV from the pKC3-Pst plasmid, also originally described in Ref. 34. The ΔNp105, ΔN901, and ΔN819 plasmids, which begin at position 52 of the p105 open reading frame and encode proteins beginning at amino acid 35 of p105, were generated by digesting the above constructs in Rc/CMV with *Bam*HI and subcloning the inserts into the pcDNA3 vector (Invitrogen) at the *Bam*HI site. The 105C plasmid was created by cloning a polymerase chain reaction product encoding amino acids 367–969 of p105 into Rc/CMV.

Antisera—All antisera used are polyclonal anti-peptide rabbit antisera described previously (11). They include antisera raised against a p105 N-terminal peptide (peptide 1141), a p105 C-terminal peptide (peptide 1140), a peptide from the p105 Rel domain (peptide 1157), and a p100/p52 N-terminal peptide (peptide 1267), all the kind gifts of Nancy Rice (National Cancer Institute, Frederick, MD). Anti-MAD3 antiserum was a gift of R. T. Hay (St. Andrews, United Kingdom).

Cell Extract Preparation and Immunoprecipitation—Cytoplasmic and nuclear extracts were prepared by a modified method of Dignam. Jurkat cells (2×10^7/extract) were washed twice in cold phosphate-buffered saline with Ca^{2+} and Mg^{2+}, resuspended in Buffer A, and lysed with 0.05–0.1% Nonidet P-40. The nuclei were pelleted, the supernatant was removed, and the nuclei were washed in Buffer B and then resuspended in Buffer C with NaCl added to a final concentration of 0.4 M. After 20 min at 4 °C, the nuclei were spun out, and the nuclear extract was recovered.

Whole-cell extracts were made from $3–5 \times 10^6$ cells lysed in a modified radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 400 mM NaCl, 1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) and then briefly sonicated to shear DNA. All solutions for extract preparation were supplemented with phosphatase inhibitors (15 mM β-glycerol phosphate, 2 mM sodium pyrophosphate, 1 mM Na_3VO_4) and protease inhibitors (4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) just prior to use.

Immunoprecipitation was carried out in 500-1000 μl of radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate) with 10 μl of polyclonal rabbit antiserum for every 2×10^7 extracted cells 1–14 h at 4 °C, to which an excess of Protein A-Sepharose beads (Sigma) was added for 1–2 h at 4 °C.

Immunoblotting—SDS-PAGE gels were transferred to nitrocellulose and blocked in phosphate-buffered saline with 5% powdered milk. Blots were then incubated 14 h at 4 °C or 1 h at room temperature with antisera diluted 1:1000 in phosphate-buffered saline, 0.1% Tween-20 and milk. The blots were washed repeatedly in phosphate-buffered saline, 0.1% Tween-20 at room temperature and then incubated for 1 h with an anti-rabbit Ig biotinylated secondary antibody, washed, incubated 30 min with horseradish peroxidase coupled to streptavidin, washed, and revealed with electrochemiluminescent reagents (Amersham Corp.). Alternatively, a secondary antibody directly coupled to horseradish peroxidase was used.

Thrombin Cleavage—^{35}S-labeled 105C protein translated *in vitro* in rabbit reticulocyte lysate (Promega) or ^{32}P-labeled Jurkat cell extracts were immunoprecipitated with an antiserum against the p105 C terminus (peptide 1140), and the precipitate was washed extensively, first in radioimmune precipitation buffer and then in thrombin cleavage buffer (20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 10% glycerol). The Sepharose-bound precipitate was resuspended in 25–30 μl of thrombin cleavage buffer, 2.5 units of thrombin (Sigma) were added, and the reaction mixture was incubated at room temperature for 2–16 h.

RESULTS

Our earlier investigation of the IκB-like function of p105 showed that p50/c-Rel and p50/p65 dimers derived from complexes with p105 could be detected in the nuclear and cytoplasmic fractions of Jurkat T cells several hours after stimulation with PMA and PHA (11). Similar p105-derived dimers were difficult to detect during the same period in unstimulated cells, suggesting that the processing of p105 is up-regulated by activation. On the basis of structural and functional similarities between p105 and IκBα, we hypothesized that phosphorylation might be involved in increasing p105 processing in activated T cells.

To begin to test this hypothesis, we labeled Jurkat cells with ^{32}P-orthophosphate and treated or mock-treated them with PMA and PHA for 3 h. Cytoplasmic and nuclear extracts were prepared from the labeled cells and boiled in 1% SDS before immunoprecipitation with an antiserum directed against an N-terminal p105/p50 peptide. As shown in Fig. 1, the very low basal level of ^{32}P phospholabeling of p105 in the cytoplasm (*lane 1*) was greatly enhanced by PMA/PHA treatment (*lane 2*). (The 98-kDa phosphoprotein also seen in the immunoprecipitate may represent an alternatively spliced p105 form similar to one described in mouse, which shares N- but not C-terminal sequences with the full-length p105 (35, 36).) Precipitation of ^{32}P-labeled Jurkat extracts with a preimmune serum did not reveal a labeled band in the 110-kDa range, and two rounds of immunoprecipitation with different p105/p50 antibodies further confirmed the identity of the 110-kDa phosphorylated band as p105 (data not shown).

The increase in p105 phosphorylation could not be accounted for by an increase in protein amount, as determined by immunoblotting of fractions of the labeled extracts (Fig. 1, *lanes 3*

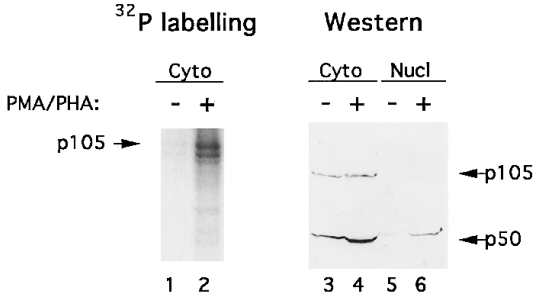

FIG. 1. **p105 is hyperphosphorylated and p50 levels are increased in Jurkat T cells 3 h postactivation.** Phosphorylation of p105 and p50 in activated and unactivated Jurkat T cells is shown. Equivalent numbers of Jurkat T cells were treated or not treated with 20 ng/ml PMA and 1 mg/ml PHA for 1 h in phosphate-free medium and then labeled with [^{32}P]orthophosphate for 2 h with or without PMA and PHA. Cytoplasmic and nuclear extracts were prepared as described under "Materials and Methods." The ^{32}P-labeled cytoplasmic extracts were boiled to dissociate complexed proteins and immunoprecipitated with an antiserum directed against the N terminus of p105/p50. The washed immunoprecipitates were analyzed by SDS-PAGE and subsequent autoradiography (*left panel*). A fraction of each extract was separated by SDS-PAGE and analyzed by immunoblotting with an anti-p105/p50 antiserum (*right panel*).

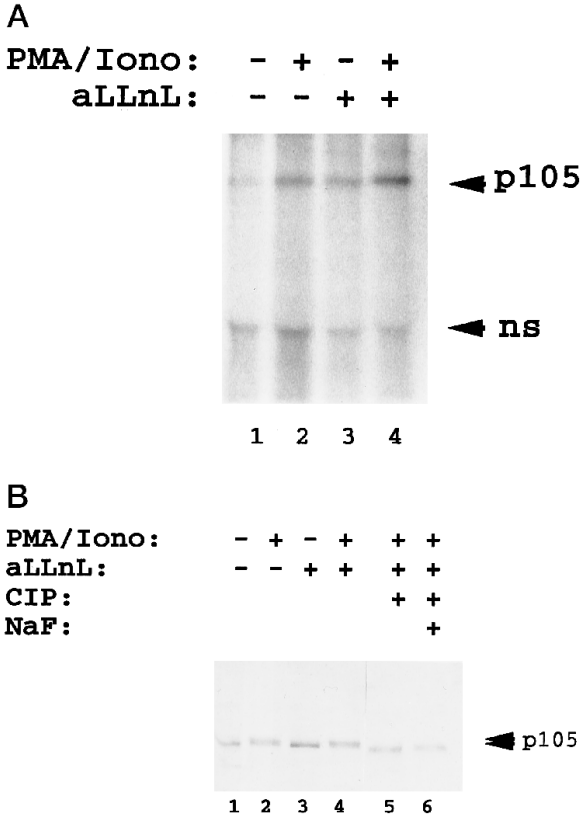

FIG. 2. **p105 is phosphorylated, and its migration in SDS-PAGE is retarded at early times following PMA/ionomycin treatment.** *A*, [^{32}P]phosphate-labeled p105, immunoprecipitated with an anti-C-terminal antiserum from extracts of ^{32}P-labeled Jurkat T cells treated with aLLnL for 1 h and/or PMA/ionomycin for 20 min, as indicated (*ns*, nonspecific band). *B*, immunoblot of p105 from cytoplasmic extracts of Jurkat T cells pretreated or not pretreated with the protease inhibitor aLLnL for 1 h prior to stimulation with PMA and ionomycin for 15 min, as indicated (*lanes 1–4*). *Lanes 5* and *6* show the effect of treatment with calf intestine alkaline phosphatase of the extract shown in *lane 4*, in the absence (*lane 5*) or presence (*lane 6*) of phosphatase inhibitors (40 mM NaF, 15 mM β-glycerol phosphate, 2 mM sodium pyrophosphate, and 1 mM sodium orthovanadate).

and *4*). Immunoblotting also revealed increased p50 in both the cytoplasmic and nuclear fractions of stimulated cells (*lanes 4* and *6*) relative to the unstimulated condition (*lanes 3* and *5*). We did not detect phospholabeled p50, in contrast to other reports (29, 37). The increased p50 seen in the PMA/PHA-treated cells must be derived from processing of the precursor, and the apparently unchanged amount of p105 observed in these cells is likely accounted for by neosynthesis following activation of the p105 promoter by NF-κB (39, 42).

The correlation between increased phospholabeling of p105 and increased p50 several hours after PMA/PHA treatment led us to examine the effects of T cell activation on p105 at earlier times. In these and subsequent experiments we used ionomycin instead of PHA to provide the Ca^{2+} signal. As shown in Fig. 2*A*, ^{32}P-phospholabeling of p105 in Jurkat T cells was increased severalfold by a 20-min PMA/ionomycin treatment (*lane 2*). In this experiment p105 was immunoprecipitated with an antiserum to the C-terminal epitope; thus, the only specific band is full-length p105. Cells were similarly labeled with [^{32}P]orthophosphate in the presence of the peptide *N*-acetyl-Leu-Leu-norleucinal (aLLnL), which inhibits the proteolytic activities of the 26 S proteasome and calpain and has been shown to inhibit both proteolysis of IκBα and processing of p105 (41). The PMA/ionomycin-induced phospholabeling of p105 was increased in cells treated with aLLnL (Fig. 2*A*, *lane 4*), and a slight increase in basal phosphorylation of p105 was also observed in the presence of aLLnL (*lane 3*).

In immunoblots of p105 in cytoplasmic extracts from Jurkat cells treated with PMA/ionomycin for 15 min, with or without aLLnL, the p105 band is upshifted (Fig. 2*B*, *lanes 2* and *4*) relative to unstimulated controls (*lanes 1* and *3*). This change in the migration of p105 in SDS-PAGE is due to phosphorylation, as determined by treatment with calf intestine alkaline phosphatase. When the extract in *lane 4* was treated with calf intestine alkaline phosphatase, the p105 band migrated more rapidly (*lane 5*), and this effect was blocked by the addition of phosphatase inhibitors to the reaction (*lane 6*).

Using the slower migration of p105 in SDS-PAGE as a marker for the addition of phosphate to the protein, we examined the kinetics of p105 phosphorylation in PMA/ionomycin-stimulated Jurkat cells. The slower migrating form was observed in anti-p105 immunoblots within a few minutes of activation, as reported by Li *et al.* (29), as well as at the latest times examined, 3–4 h poststimulation (data not shown). Immunoblots of these extracts with an antiserum against the human IκBα, MAD3, showed a short lived upshifted form within minutes of stimulation and complete degradation of the protein within 10–15 min (data not shown). In contrast, p100 migration was unaffected at early times following PMA/ionomycin treatment, although its migration on SDS-PAGE gels was slowed at later times (3 h), coincident with the appearance of nuclear p52 (data not shown).

We next sought to determine the region of p105 phosphorylated in PMA/ionomycin-stimulated Jurkat cells. Our finding that processed p50 is not phosphorylated suggested the C-terminal half of p105 as the likely target of inducible phosphorylation in the full-length molecule. To begin to identify the phosphorylated domain, we took advantage of our finding that thrombin treatment of p105 generates a 30-kDa C-terminal fragment, probably by cleaving at a site (RGS) in the sixth ankyrin repeat. Our approach was to perform the cleavage reaction on a Sepharose bead-bound immunoprecipitate and then load the supernatant and the immunoglobulin-bound fragment separately on SDS gels.

In Fig. 3*A*, an *in vitro* translated ^{35}S-labeled p105 C-terminal construct, containing amino acids 367–969 of p105 (p105C), was immunoprecipitated with an anti-C-terminal antiserum and bound to Protein A-Sepharose beads. The precipitate was

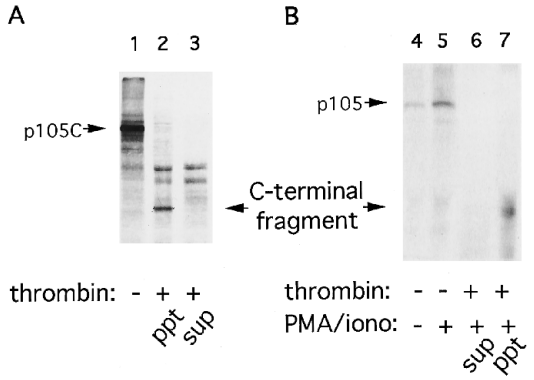

FIG. 3. **A C-terminal 30-kDa thrombin cleavage fragment of p105 contains all PMA/ionomycin-induced phosphorylation sites.** *A*, a ^{35}S-labeled *in vitro* translated p105 C-terminal protein (amino acids 367–969) (*lane 1*) was immunoprecipitated with an anti-C-terminal p105 antibody and treated with thrombin on the Protein A-Sepharose beads, as described under "Materials and Methods." Antibody-bound C-terminal fragment (*lane 2*) and half of the resulting supernatant (*lane 3*) were run separately on an SDS-PAGE gel and exposed for autoradiography. *B*, p105 was immunoprecipitated with an anti-C-terminal antiserum from ^{32}P-labeled Jurkat T cells pretreated with aLLnL (*lanes 4–7*) and stimulated or not stimulated with PMA and ionomycin for 15 min, as indicated. *Lane 7* shows the antibody-bound C-terminal fragment produced by thrombin treatment of an immunoprecipitate identical to that shown in *lane 5*. *Lane 6* contains half the supernatant of the thrombin-treated immunoprecipitate.

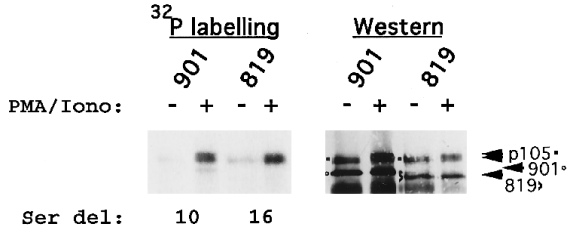

FIG. 4. **p105 constructs lacking C-terminal PEST sequences are not phosphorylated in response to PMA/ionomycin.** ^{32}P-Phosphate incorporation into p105 and transfected C-terminally truncated p105 constructs immunoprecipitated with an anti-N-terminal antiserum from cytoplasmic extracts of ^{32}P-labeled Tag Jurkat T cells treated with aLLnL (50 μM, 1 h) and with or without PMA/ionomycin for 20 min, as indicated (*left panel*), is shown. The migration of p105 (*square*) and truncated constructs ending at amino acid 901 (*open circle*) and 819 (*carat*) is indicated, and the number of serine residues deleted in each truncated construct is shown *below* the corresponding *lanes*. The *right panel* is an anti-p105/p50 immunoblot of a fraction of the labeled immunoprecipitate shown in the *left panel*.

washed and then treated with thrombin, leaving a 30-kDa C-terminal fragment bound to the beads (Fig. 3*A*, *lane 2*) and absent in the supernatant of the cleavage reaction (*lane 3*). A similar protocol was applied to ^{32}P-orthophosphate-labeled p105 immunoprecipitated with an anti-C-terminal antiserum from extracts of Jurkat cells stimulated for 15 min with PMA/ionomycin in the presence of aLLnL. Following thrombin cleavage of the bead-bound immunoprecipitate, the supernatant and bead-bound C-terminal fragment were resolved separately on an SDS-PAGE gel, and the ^{32}P-phosphate content was assessed by autoradiography. As shown in Fig. 3*B*, the bound C-terminal fragment (*lane 7*) contained all of the detectable ^{32}P-phosphate incorporated in p105 *in vivo* following PMA/ionomycin treatment (*lane 5*).

To identify the type of amino acid phosphorylated in p105 in these cells, both constitutively and following PMA/ionomycin treatment, the ^{32}P-labeled p105 and the thrombin-generated C-terminal fragment were subjected to hydrolysis and electrophoresis in two-dimensions on thin-layer cellulose plates. p105 from unstimulated cells is phosphorylated on serine and to a far lesser extent on threonine, and the phosphorylation induced by PMA/ionomycin treatment is likewise almost entirely on serine (data not shown). In contrast with the report of Neumann *et al.* (28), we did not detect phosphotyrosine in p105, either by phosphoamino acid analysis of ^{32}P-labeled p105 or by immunoblotting of p105 immunoprecipitates with an anti-phosphotyrosine antiserum (data not shown).

Immunoblotting of extracts from Jurkat cells stimulated with PMA/ionomycin in both one- and two-dimensional gels revealed at least four differently migrating p105 isoforms (data not shown), as previously reported (28). These results and those of preliminary tryptic peptide mapping experiments suggest PMA/ionomycin treatment induces phosphorylation of multiple C-terminal serines (data not shown; see results below).

As the C terminus of p105 is extremely rich in serine residues, with 20 in the presumed thrombin fragment, we chose not to attempt a mutational analysis but rather to further delimit the region of PMA/ionomycin-induced p105 phosphorylation using two C-terminally truncated constructs, ending at amino acids 901 and 819 of p105. For these experiments we transiently transfected Tag Jurkat cells, which stably express SV40 large T antigen (33), with plasmids containing the above constructs under control of the CMV promoter. Immunoblots of cytoplasmic and nuclear extracts of Tag Jurkat cells expressing either C-terminally truncated protein showed that, like the full-length (969-amino acid) protein, these molecules are almost entirely cytoplasmic.

Cells transiently transfected with plasmids encoding either the 901- or 819-amino acid construct were labeled with ^{32}P-orthophosphate in the presence of aLLnL and then treated with PMA and ionomycin for 20 min. Cell extracts were prepared as before, boiled in SDS, and immunoprecipitated with an anti-N-terminal antiserum, which recognizes both the endogenous p105 and the C-terminal truncations. As shown in Fig. 4, in the Tag Jurkat cell line, as in our normal Jurkat line, a short PMA/ionomycin treatment induced an upshift in p105 and a large increase in phospholabeling of the molecule. In contrast, the 901 construct, which lacks the last 68 amino acids of the protein, including 10 serines, shows only a slight increase in ^{32}P phospholabeling in response to the PMA/ionomycin treatment (*left panel*) and no change in migration in SDS-PAGE (*right panel*). The 819 construct, which deletes another 82 amino acids, of which six are serines, abolishes both low level constitutive and PMA/ionomycin-induced phosphorylation. Immunoblotting of a fraction of the labeled immunoprecipitate (Fig. 4, *right panel*) shows that the differences in phospholabeling cannot be accounted for by differences in the amount of protein but rather reflect the deletion of target phosphorylation sites in the C-terminal truncations.

These results localize the PMA/ionomycin-inducible and low level constitutive phosphorylation of p105 in Jurkat cells to more than one of the 16 serine residues in the C-terminal-most 150 amino acids of the protein, a PEST-rich domain, with most of the target serines in the final 68 amino acids. The phosphorylated region does not overlap the ankyrin repeats, which end at amino acid 807. This PEST domain contains multiple consensus serine phosphorylation sites, including Pro-Ser sequences and sites for protein kinase A and casein kinase II. The possible role of these kinases in p105 phosphorylation is discussed below.

We next investigated the effect of p105 phosphorylation on processing to p50 at early times following PMA/ionomycin stimulation of Jurkat T cells. To avoid any transcriptional effect due to up-regulation of p105 and IκBα via κB sites in their promoters (38–40, 42–44), we determined the levels of p105 and p50 in cells treated with CHX. We compared the ratio

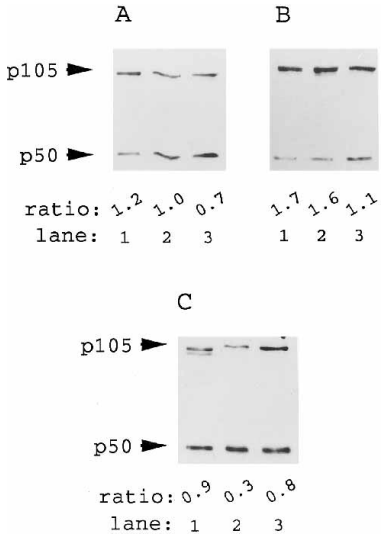

FIG. 5. **Stimulus-induced p105 phosphorylation is correlated with increased processing to p50.** *A*, immunoblot of endogenous p105 and p50 in Jurkat T cells pretreated 1 h with CHX (*lane 1*) and then stimulated with PMA and ionomycin for 20 min (*lane 2*) and 40 min (*lane 3*). The ratio of p105 to p50 is shown *below* the corresponding *lane*. *B*, immunoblot of p105 and p50 in extracts from Tag Jurkat cells overexpressing p105. The extract in *lane 1* was treated with CHX for 1 h. In *lanes 2* and *3* the cells were treated for an additional hour with CHX, in the absence (*lane 2*) or presence (*lane 3*) of PMA and ionomycin. *C*, immunoblot of p105 and p50 in extracts from Jurkat cells treated for 3 h with CHX alone (*lane 1*) or CHX and okadaic acid (0.6 μM; *lanes 2* and *3*). The cells in *lane 3* were additionally treated with aLLnL (100 μM) 3 h.

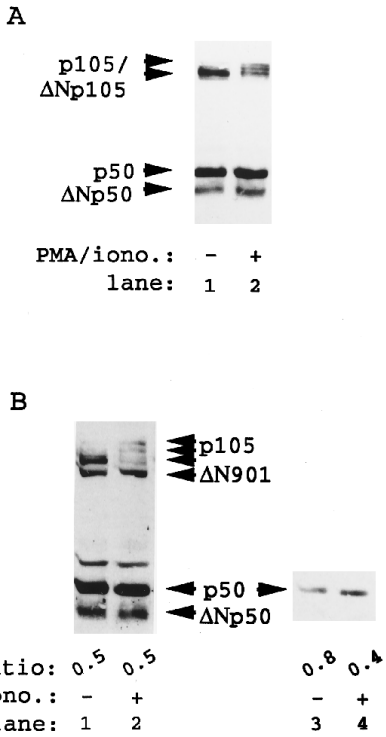

FIG. 6. **PMA/ionomycin-induced p105 processing requires intact PEST sequences.** *A*, immunoblot with antiserum against a p50 Rel homology domain peptide (peptide 1157) of extracts from Tag Jurkat cells transiently transfected with N-terminally truncated p105 (ΔNp105). Twenty-four hours posttransfection, cells were pretreated with CHX for 1 h and then treated with PMA and ionomycin 1 h (*lane 2*) or left in CHX alone (*lane 1*). *B*, immunoblot of extracts from Tag Jurkat cells transiently transfected with N-terminally truncated p901 (ΔN901). Twenty-four hours posttransfection, cells were pretreated with CHX for 1 h. The extract in *lane 2* and *4* was treated with PMA and ionomycin for an additional hour, and that in *lanes 1* and *3* was left in CHX alone. A shorter exposure of the endogenous p50 in the same blot is shown as an internal control for the effect on processing of the wild-type molecule in these cells (*lanes 3* and *4*). The ratio of ΔN901/ΔNp50 is shown in *lanes 1* and *2*, and the endogenous p105/p50 ratio is listed in *lanes 3* and *4*.

of p105 to p50 in stimulated and unstimulated cells by densitometric measurement of the relevant bands in immunoblots of SDS-PAGE separations of whole-cell extracts. We used this approach rather than pulse-chase labeling methods, as it reveals changes in the total pool of p105 rather than that of a newly synthesized subset of molecules. As the precursor-product relation of p105 and p50 is well established, any increase in p50 can be assumed to derive from processing of p105. Further, comparison of the ratios of p105 and p50 within each extract corrects for any variability in total extract amount.

Quantification of multiple experiments shows that PMA/ionomycin treatment increases p105 processing at early times (20 min to 1 h) following stimulation, extending our initial observations of increased cytoplasmic and nuclear p50 3 h poststimulation (Fig. 1). As shown in Fig. 5*A*, the ratio of p105 to p50 in whole-cell extracts from Jurkat T cells drops from 1.2 to 0.7 within 40 min of PMA/ionomycin treatment, reversing the initial excess of inhibitor over activator. Cells treated with CHX alone showed no significant change in the ratio of p105/p50 in this interval. Although the absolute ratios of p105/p50 measured using this approach varied, the decrease of p105 relative to p50 induced by PMA/ionomycin treatment was consistently observed.

Similarly, p105 transiently overexpressed in Tag Jurkat cells underwent increased processing to p50 within the first hour following PMA/ionomycin stimulation (Fig. 5*B*, *lane 1*, ratio 1.7, *versus lane 3*, ratio 1.1), whereas the p105/p50 ratio in a control treated with CHX alone reflected only low level constitutive processing (compare *lane 1*, ratio 1.7, and *lane 2*, ratio 1.6).

We also observed a slower migrating phosphorylated form of p105 in Jurkat T cells treated with okadaic acid, an inhibitor of serine/threonine phosphatases of the 1 and 2A classes and an activator of NF-κB. As shown in Fig. 5*C* (*lane 2*), okadaic acid treatment also induced processing of p105, as evidenced by a decrease in the p105/p50 ratio relative to a control treated with CHX alone (0.3 in *lane 2*, *versus* 0.9 in the control, *lane 1*). The okadaic acid-induced processing of p105 was blocked by aLLnL (ratio 0.8 in *lane 3*), suggesting that it depends on the same proteolytic machinery as other p105 processing. The kinetics of the change in relative amount of p105 induced by okadaic acid was identical to that of the accumulation of the upshifted form (3 h in Fig. 5*C*), although the exact timing of the upshift varied, perhaps due to variable drug potency.

If PMA/ionomycin-induced phosphorylation of the C-terminal PEST sequences of p105 up-regulates processing to p50, the removal of all or part of this domain would be predicted to abolish the response. To investigate this hypothesis, we used a convenient restriction enzyme site to introduce a small (36-amino acid) N-terminal deletion in full-length p105 and in the 901 and 819 C-terminal truncations described above; these N-terminally truncated forms are designated ΔNp105, ΔN901, and ΔN819. The N-terminal deletion allows us to separate the processed form of the transfected plasmid (ΔNp50) from the endogenous p50 in SDS-PAGE, and thus the influence of the C-terminal PEST domain on processing can be analyzed.

The Tag Jurkat cell line was transiently transfected with the plasmid encoding ΔNp105 (Fig. 6*A*), ΔN901 (Fig. 6*B*), or ΔN819 (not shown), and 24 h later the cells were pretreated with CHX for 1 h and then stimulated or not stimulated with PMA/ionomycin for an additional hour. As shown in Fig. 6*A*, the N-terminal deletion in ΔNp105 did not interfere with the re-

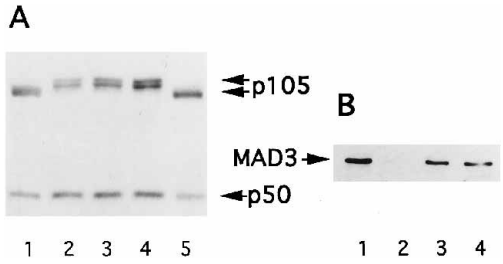

FIG. 7. **PMA/ionomycin-induced p105 phosphorylation is blocked by TPCK but not antioxidants.** *A*, immunoblot of p105 and p50 in whole-cell extracts from Jurkat T cells pretreated with CHX 1 h (*all lanes*) alone or with the antioxidants BHA and PDTC or the serine protease inhibitor TPCK. In *lanes 2–5* cells were stimulated with PMA and ionomycin for 20 min. Pretreatments were as follows: CHX only (*lanes 1* and *2*); 250 μM BHA (*lane 3*); 100 μM PDTC (*lane 4*); 50 μM TPCK (*lane 5*). *B*, immunoblot of MAD3 in whole-cell extracts of Jurkat T cells pretreated with CHX 1 h (*all lanes*). Cells in *lanes 2–4* were stimulated with PMA/ionomycin for 20 min. Pretreatments were as follows:, CHX only (*lanes 1* and *2*); 100 μM PDTC (*lane 3*); 250 μM BHA (*lane 4*).

sponse to PMA/ionomycin, as ΔNp50 increased in response to stimulation. ΔNp50 in the extract prepared from stimulated cells (*lane 2*) is 165% that in the unstimulated control (*lane 1*) by densitometry. Unfortunately, the ratio of ΔNp105 to ΔNp50 could not be determined due to comigration of ΔNp105 with endogenous p105 in stimulated cells.

In contrast to ΔNp105, processing of ΔN901 was not induced by PMA/ionomycin treatment, as levels of ΔN901 and ΔNp50 in stimulated cells were similar to those of unactivated controls (Fig. 6*B*, *lanes 1* and *2*). The effectiveness of the PMA/ionomycin treatment is reflected by the upshifting of the endogenous p105 in these extracts and the increase in p50 in stimulated cells visible in a shorter exposure of the blot (*lane 4 versus lane 3*). The densitometric analysis of this and other blots confirms that while the ratio of endogenous p105/p50 drops in response to PMA/ionomycin treatment, that of ΔN901/ΔNp50 is unaffected. In this experiment ΔN901/ΔNp50 remains unchanged at 0.5 (Fig. 6*B*, *lanes 1* and *2*), while in the same extracts the p105/p50 ratio drops from 0.8 to 0.4 in stimulated cells (*lanes 3* and *4*). Interestingly, immunoblots of extracts from cells transfected with the ΔN819 plasmid revealed ΔNp50, but unprocessed ΔN819 was barely detectable, suggesting that the absence of the PEST domain renders the protein highly susceptible to constitutive processing.

Our finding that PMA/ionomycin treatment induces phosphorylation of p105 as well as IκBα suggests that the two inhibitors might be the targets of identical signaling pathways. We used pharmacological agents known to block phosphorylation of IκBα to investigate whether the signal transduction elements leading to phosphorylation of p105 are similarly affected. These agents are of two types, antioxidants and certain serine protease inhibitors, both of which have been shown to act upstream of the serine phosphorylation that induces IκBα degradation (see Refs. 1 and 2 and references therein).

We examined the effects of antioxidants on the PMA/ionomycin-induced phosphorylation of p105 in Jurkat T cells as evidenced by altered migration on SDS-PAGE. As shown in Fig. 7*B*, pretreatment of Jurkat cells with the antioxidants PDTC (*lane 3*) or BHA (*lane 4*) prior to stimulation with PMA/ionomycin inhibited the phosphorylation and degradation of IκBα such stimulation usually induces (*lane 2*). In contrast, neither antioxidant blocked the PMA/ionomycin-induced phosphorylation of p105 (Fig. 7*A*, *lanes 3* and *4*), nor did treatment with these agents alone affect the migration of p105 (not shown).

Pretreatment of Jurkat T cells with the serine protease inhibitor TPCK (Fig. 7*A*, *lane 5*) or TLCK (not shown) blocked PMA/ionomycin-induced p105 phosphorylation, much as they block that of IκBα (20). TPCK and TLCK have previously been shown to block the degradation of IκBα (26, 45, 46) and proteolytic processing of p105 (26). However, this probably depends not on their anti-serine protease activity but rather on their alkylating properties (20).

These results show that the signaling pathway responsible for inducibly phosphorylating the p105 C terminus following PMA/ionomycin stimulation is not identical to that regulating IκBα, on the basis of their different sensitivities to the redox state of the cell. Their common sensitivity to TPCK and TLCK may indicate a shared signaling element upstream of the redox-sensitive step in the IκBα pathway, or it may simply reflect a nonspecific inhibition due to alkylation.

p105 phosphorylation induced by okadaic acid was also blocked by the serine protease inhibitor TPCK (not shown). Interestingly, it was recently reported (47, 48) that NF-κB activation induced by okadaic acid or calyculin A is insensitive to antioxidants. The similar sensitivity of okadaic acid- and PMA/ionomycin-induced p105 phosphorylation to pharmacological agents suggests they may utilize common signaling elements.

DISCUSSION

The above results show that p105 processing is not simply a constitutive process but that it can be up-regulated by PMA/ionomycin or okadaic acid, confirming similar reports with other stimuli (24–26). The up-regulation requires the presence of the 68 amino acids in the C-terminal PEST domain that contain multiple inducibly phosphorylated serines. These findings underline fundamental similarities in the regulation of p105- and IκBα-containing complexes (see Fig. 8). In both cases release of Rel dimers from the inactive complex with an IκB depends on serine phosphorylation and ensuing proteolysis of the inhibitor domain. The target serine residues are located in unique domains of each IκB outside the conserved ankyrin repeats critical for Rel protein binding. Both inhibitors also contain PEST sequences, which, in other proteins, have been correlated with rapid protein turnover (59). These regions are essential for stimulus-induced proteolysis, which probably occurs in the 26 S proteasome in the context of Rel-IκB complexes (9, 18, 19, 21–23, 41, 49). The inhibitory effect of aLLnL is not restricted to the proteasome, as this compound has been originally described as an inhibitor of calpain. However experiments carried out with more specific inhibitors suggest that the proteasome is indeed involved in p105 processing and IκBα degradation (41).

Although the basic scheme of inducible serine phosphorylation and proteolysis is conserved between the p105 precursor and IκBα, evidence presented here shows that the phosphorylation of each is regulated by a different signaling pathway. The finding that antioxidants do not block phosphorylation of p105 induced by PMA/ionomycin, as they do that of IκBα, proves the two inhibitors cannot share all upstream signaling elements, despite the similar kinetics of their induced phosphorylation. The p105 processing reportedly induced by H_2O_2 (27) likely acts via a different mechanism, as suggested by the cell specificity of this response, in contrast with the generalized effects of antioxidants (50). The insensitivity of p105 to redox status in Jurkat cells suggests that even if oxygen radicals act as second messengers in the activation of NF-κB, as has been proposed (31), they do not regulate all Rel/IκB complexes in the cell.

This difference in the sensitivity of p105 and IκBα to antioxidants suggests that the contribution of p105-containing complexes to nuclear Rel activity induced by PMA/ionomycin stimulation could be determined in their presence. In Jurkat

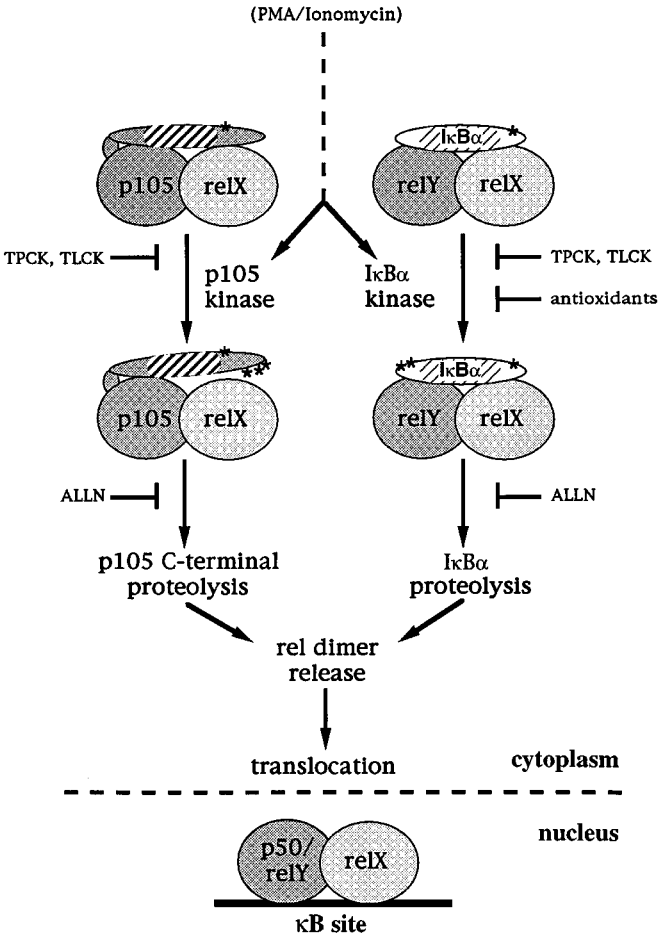

FIG. 8. **Schematic of regulation of p105-Rel and IκBα-Rel complexes.** *RelX* refers to any Rel DNA-binding subunit (p65, c-Rel, p50, or p52); *stripes* indicate ankyrin repeats; *asterisk* represents phosphoserine or phosphothreonine.

cells treated with 200 μM PDTC and activated with PMA/ionomycin, a reduced but easily detectable increase in nuclear κB binding activity, mainly in the form of p65/p50, was reproducibly observed, as was a corresponding increase in nuclear p50 in immunoblots (see, *e.g.* Ref. 51).[2] In contrast, aLLnL blocked all inducible κB binding activity and p50 translocation. However, the interpretation of these experiments is problematic for two reasons. First, antioxidants do not completely block IκBα degradation, even at high concentrations, although no upshifted phosphorylated form is observed (see Fig. 7*B* and Refs. 1 and 2). Second, when cells are activated after preincubation with antioxidants, the vast majority of IκBα is still present and could associate with dimers released by processing of p105. Under normal physiological conditions, IκBα is totally absent during the first hour following T cell activation, which would presumably allow direct translocation to the nucleus of dimers derived from processing of p105-containing complexes.

Although the effects of PMA/ionomycin treatment on p105 processing are less dramatic than the total degradation of IκBα, usually on the order of a 2-fold change in the p105/p50 ratio, this shift in the balance of inhibitor and DNA-binding forms of the protein would be expected to have a significant effect on Rel-dependent transcription. p105 processing likely contributes to κB binding activity both directly, when IκBα is absent, and indirectly, by generating dimers that associate with resynthesized IκBα. This interpretation is consistent with the results of earlier pulse-chase experiments showing dimers derived from ^{35}S-labeled p105 complexes in both the nuclear and cytoplasmic fraction of Jurkat cells several hours after activation (11). In contrast to the release of NF-κB from IκBα, which is total and transient, the hyperphosphorylation, and presumably the up-regulated processing, of p105 continues for several hours. This sustained effect on p105 may explain the continued increase in nuclear κB binding activity observed after IκBα has been resynthesized (53).[2] In contrast to the immediate effects of T cell activation on p105 and IκBα, p100 appears relatively stable (12, 25) and may be phosphorylated and processed a few hours after activation.[2] These results suggest that each IκB plays a specific role in coordinating Rel-dependent transcription.

The PEST sequences of p105 that are the target of PMA/ionomycin-induced phosphorylation contain consensus phosphorylation sites for multiple serine/threonine kinases, including one for protein kinase A, two for casein kinase II, and three for proline-directed kinases such as Cdc2, ceramide-activated and the mitogen-activated protein kinases. In *in vitro* tests using purified serine/threonine kinases, protein kinase A and casein kinase II, but neither mitogen-activated protein kinase from *Xenopus*, protein kinase C, Cdc2 kinase, nor calmodulin-dependent protein kinase II phosphorylated recombinant p105.[3]

p105 phosphorylation *in vitro* by protein kinase A has previously been reported to prevent association of the p105 C terminus or p105 with other Rel proteins (27, 54). However, these *in vitro* effects probably do not reflect events *in vivo*, as proved to be the case for similar results with *in vitro* phosphorylation of IκBα (55). Protein kinase A is unlikely to be responsible for the PMA/ionomycin-induced phosphorylation of p105, as protein kinase A is not activated by this stimulus and actually inhibits activation of NF-κB in T cells (56).

Casein kinase II has been implicated in the phosphorylation of multiple transcription factors, although there is some controversy as to whether its activity responds to signals (57). Interestingly, the inducible phosphorylation of IκBα occurs on two consensus casein kinase II sites in the N terminus, although biochemical and immunological evidence indicates casein kinase II constitutively phosphorylates only C-terminal sites in IκBα *in vivo* (58). Casein kinase II could conceivably be responsible for the inducible p105 phosphorylation described here if the increased phosphorylation of p105 is due to inactivation of a constitutive phosphatase, as discussed below.

We have evidence that a serine kinase activity that acts on the PEST domain of p105 is specifically associated with that protein,[4] and *in vitro* phosphorylation of p105 with Jurkat extracts has been reported (29). The association of a kinase and a transcription factor substrate has precedent in the mitogen-activated protein kinase family, notably the Jun kinase 1/c-Jun association (60, 61). However, unlike Jun kinases, the *in vitro* activity of the p105-associated kinase we identified is not altered by prior PMA/ionomycin treatment or other Rel-activating stimuli. If the associated kinase activity is responsible for the inducible p105 phosphorylation described here, there are at least two possibilities. Either the associated kinase is activated by PMA/ionomycin *in vivo* but deregulated in the *in vitro* kinase assays, or it phosphorylates p105 constitutively and stimulation inactivates constitutive p105 phosphatase activity. Current experimental evidence is consistent with either model, and other kinases not stably associated with p105 could phosphorylate the molecule as well.

If there is constitutive p105 kinase and phosphatase activity

[2] M. L. MacKichan, F. Logeat, and A. Israël, unpublished results.

[3] F. Logeat and C. Cochet, unpublished results.

[4] M. L. MacKichan and A. Israël, manuscript in preparation.

in cells, the balance of the two may also regulate the basal rate of processing of p105. The processing to ΔNp50 of nearly all ΔN819, the p105 construct lacking the phosphorylated C-terminal PEST domain, in the absence of stimulation suggests that this domain may also be involved in regulating constitutive processing. In this model, the PEST sequences in their unphosphorylated state protect full-length p105 from extensive processing, and phosphorylation of this domain alters the conformation of the protein, making it more accessible to the proteolytic machinery (see Fig. 8).

The results reported here not only correlate increased phosphorylation and processing of p105 in a model of T cell activation but also for the first time identify the C-terminal PEST sequences as the target of PMA/ionomycin-induced phosphorylation and show that they are required for up-regulation of processing. These findings indicate the general regulatory scheme of inducible serine phosphorylation and proteolysis is conserved between p105 and IκBα inhibitor complexes. However, the insensitivity of p105 phosphorylation to antioxidants demonstrates that each inhibitor is independently regulated, and the response induced is graded to reflect the specific role of each in determining the Rel response to changes in cell status.

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