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Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB

Kenneth H.Mellits, Ronald T.Hay[1] and Stephen Goodbourn*
Gene Expression Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX and [1]Division of Cell and Molecular Biology, Irvine Building, North Street, St Andrews, Fife KY 16 9AL, UK

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ABSTRACT

We have studied the role of protein turnover in the induction of NF-κB DNA binding activity. Treatment of cells with tumour necrosis factor (TNF), double-stranded RNA (dsRNA), or phorbol esters is shown to be associated with an increase in the rate of p105 to p50 processing, and the loss of immunologically detectable MAD3/IκBα. Phosphate-labelling experiments indicate that these events are preceded by the phosphorylation of MAD3 and p105. The protease inhibitors TLCK (Nα-p-Tosyl-L-Lysine Chloromethyl Ketone) and TPCK (Nα-p-Tosyl-L-Phenylalanine Chloromethyl Ketone) inhibit both p105 to p50 processing and MAD3 degradation, and also cause a complete block to NF-κB activation. These data suggest a model for NF-κB activation in which phosphorylation destabilises the NF-κB/MAD3 complex but that, *in vivo*, this is insufficient to lead to activation in the absence of an obligatory mechanism that degrades MAD3.

INTRODUCTION

The transcription factor NF-κB plays a central role in the regulated expression of a number of immune response genes (reviewed in 1–4). NF-κB is sequestered in the cytoplasm by association with an inhibitor called IκB and can be liberated from this complex by a variety of inducers (e.g. IL-1, TNF, dsRNA, cAMP) produced by infectious agents. Free NF-κB migrates to the nucleus and binds to the promoters of target genes in a sequence-specific manner.

The prototypical form of NF-κB consists of a heterodimer of a 50kDa subunit (p50) and a 65kDa (p65) subunit (5, 6), which are related to the products of the *c-rel* protooncogene and the *Drosophila dorsal* gene (7–15). The p50 polypeptide is produced by processing from a 105kDa precursor (p105; 16). Other members of the Rel family have been described [RelB (17), p49 (18–20)], and it is now apparent that mammalian NF-κB can consist of homodimers or heterodimers of any of the subunits. The precise DNA sequence specificity differs between dimers (21), and there are differences in the regulatory consequences of binding particular forms of NF-κB (22, 23).

Several cDNA clones have been isolated which encode proteins with IκB-like properties. These include the *MAD3* product (also called IκBα) which inhibits DNA binding by NF-κB containing p65 or c-Rel subunits (24), and the product of the *bcl-3* protooncogene which inhibits DNA binding by p50 homodimers (25–27). IκB proteins contain a tandemly reiterated element referred to as the ankyrin repeat, which is thought to mediate protein–protein interactions (reviewed in 3). Related ankyrin repeats are also present in the C-terminal regions of the p105 and p97 precursor forms of p50 and p49, and indeed it has been shown that the C-terminal half of p105 has IκB-like properties (called IκBγ; 28, 29). In addition to blocking DNA binding, MAD3 and the C-terminus of p105 can prevent nuclear uptake of NF-κB (30–33), and it has been shown that MAD3 masks the nuclear localisation sequence (NLS) of the p65 subunit (32; reviewed in 34).

A simple model for NF-κB activation is that phosphorylation of IκB by specific activated protein kinases allows dissociation of the NF-κB/IκB complex. Consistent with this, NF-κB could be activated from cytoplasmic extracts by incubation with purified protein kinases A or C (35). In addition, protein kinase C or haem-regulated kinase could cause activation of NF-κB from purified NF-κB/IκB complexes by phosphorylation of the IκB subunit (36). Phosphorylation of MAD3 has recently been reported in T-cells in response to interleukin-1 or TNF (37) and in monocytes in response to LPS (38), although the roles of these phosphorylations have yet to be determined. The observations of Rice *et al.* (39) and Neumann *et al.* (40) that p105 forms cytoplasmic complexes with other Rel-family proteins suggests an alternative mechanism for NF-κB activation, namely that NF-κB can be activated by the regulated removal of the trans-inhibitory regions of p105. Indeed, Mercurio *et al.* (41) have recently demonstrated that p105 and p97 levels decrease in response to specific stimuli. Here we provide evidence that phosphorylation of p105 and MAD3 precedes enhanced

* To whom correspondence should be addressed

processing of p105 to p50 and degradation of MAD3, and that the proteolytic events are obligatory steps in NF-κB activation.

MATERIALS AND METHODS

Cell culture and inductions

MG-63 human osteosarcoma cells (ATCC CRL 1427) were grown in Dulbecco's modification of Eagle's medium supplemented with 100μg/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, and 10% (vol/vol) foetal calf serum (Globepharm). Twenty four hours prior to NF-κB activation, the medium on confluent monolayers was replaced by serum-free medium. Inducers were added in serum-free medium to the following final concentrations: TNF, 10ng/ml (a gift from Dr Stuart Naylor, ICRF); dsRNA, 100mg/ml (Pharmacia, poly[I].poly[C]); TPA (100ng/ml, Sigma). Where applicable, cycloheximide was added to 50μg/ml. 100mM stocks of TLCK and TPCK (Sigma) were stored in aliquots at −20°C in aqueous and methanolic solution respectively.

Preparation of specific antisera

Preimmune sera were collected from female Dutch Dwarf rabbits prior to immunisation with purified, recombinant proteins produced in *E.coli* and emulsified in Freund's complete adjuvant. Animals were boosted with the same material emulsified in Freud's incomplete adjuvant until a satisfactory response had been obtained. p50 (amino acids 35−381; 9), MAD3 (amino acids 1−317; 24) and a p65-glutathione-S-transferase fusion protein (amino-acids 1−293; 12) were expressed and purified as described previously (42; J.R.Matthews, E.Watson, S.Buckley and R.T.H., submitted for publication). Antibodies used in these studies were: anti-p50, M_{FB}; anti-p65, G_3: anti-MAD3, S_{10}.

In vivo labelling and cellular extract preparation

For *in vivo* labelling experiments with ^{35}S-methionine, confluent monolayers in 6 cm dishes (approximately 5×10^5 cells) were incubated in serum- and methionine-free medium overnight. The cells were then metabolically labelled for 60 minutes with 0.35 mCi/ml of ^{35}S-methionine (>1000Ci/mmol *in vivo* labelling grade, Amersham) and the medium replaced with inducing medium. Extracts were prepared by chilling plates on ice, washing twice with ice-cold phosphate buffered saline (PBS), and lysing in 0.8 ml of ice-cold RIPA buffer (50 mM NaCl, 25 mM Tris 8.2, 0.5% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 0.1% Na azide) supplemented with pepstatin (5μg/ml), aprotinin (5μg/ml), leupeptin (30μg/ml), benzamidine (1mM), PMSF (0.5mM) and 0.1% BSA (globulin-free, Sigma). After 3 minutes, lysates were scraped off plates, sheared by 5 passages through a 25 gauge needle, transferred to a microfuge tube, centrifuged for 10 minutes at 12,000×g, and the supernatant used for immunoprecipitation. For ^{32}P labelling, confluent monolayers in 6 cm dishes (approximately 5×10^5 cells) were incubated in serum- and phosphate-free medium overnight. The cells were metabolically labelled for 60 minutes with 1mCi/ ml of ^{32}P-orthophosphate (Amersham). Inducers were added directly to this medium and samples prepared as above.

To prepare cytoplasmic and nuclear fractions, metabolically-labelled cells were harvested in 0.8ml cold PBS and transferred to a microfuge tube. Sedimented cells were resuspended in 0.2ml ice-cold Buffer A (43). Cells were swollen on ice for 5 minutes, and NP-40 added to a final concentration of 0.125%. Nuclei and cellular debris were collected by centrifugation and the supernatant removed and adjusted to 15% glycerol and stored at −70°C as the cytoplasmic fraction. The pellet was extracted at 4°C for 1 hour with 25μl Buffer C (43) prior to centrifugation at 12,000×g for 10 minutes at 4°C. The supernatant was removed and stored at −70°C as the nuclear fraction. Protease inhibitors were added to Buffers A and C to the same final concentrations as to RIPA.

Immunoprecipitations

Immunoprecipitations were performed by adding 25μl of Protein A-Sepharose CL-4B (PAS—Pharmacia, 1:1 with tris-buffered saline—TBS) and 1.5μl of pre-immune serum to lysates and incubating for 30 minutes at 4°C. Lysates were centrifuged for 1 minute at 12,000×g and 25μl of PAS and 1.5μl of specific antiserum added to the cleared supernatant. After incubation overnight at 4°C the beads were washed 3 times with 10ml ice-cold RIPA buffer and precipitated protein eluted in Laemmli sample buffer at 85°C for 10 minutes. Samples were fractionated on 7% (10% for Figure 3) polyacrylamide (29:1 mono:bis) mini-gels containing SDS (44) and visualised by flourography. To

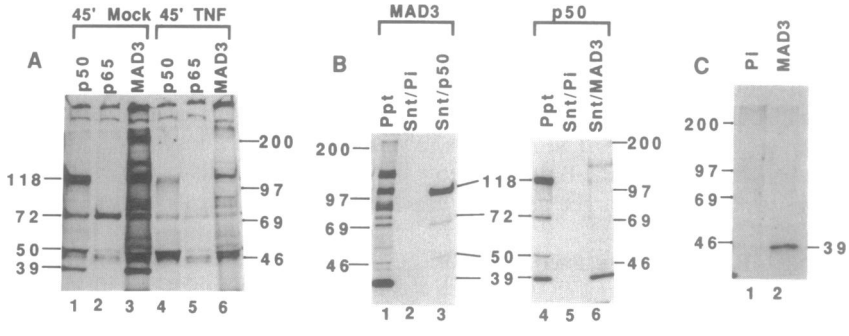

Figure 1. Identification of NF-κB and IκB components in MG-63 cells. (**A**). ^{35}S-methionine-labelled whole cell extracts from mock- (lanes 1−3) or TNF- (lanes 4−6) induced MG-63 osteosarcoma cells were immunoprecipitated with antisera raised against recombinant p50 (lanes 1 and 4), p65 (lanes 2 and 5) or MAD3 (lanes 3 and 6) as indicated. (**B**). Whole cell extracts from mock-induced cells were immunoprecipitated with MAD3 (lane 1) or p50 (lane 4) antisera. The supernatants from these samples were than re-precipitated with preimmune (lanes 2 and 5), p50 (lane 3) or MAD3 (lane 6) antisera. (**C**). Whole cell extracts from mock-induced cells were immunoprecipitated with p50 antiserum which had been covalently cross-linked to protein A-sepharose beads. The immunoprecipitate was then solubilised and reprecipitated with pre-immune (lane 1) or MAD3 (lane 2) antiserum. The number series 200, 97, 69 and 46 refer to the molecular weights of protein standards (Amersham) in kDa; the series 118, 72, 50 and 39 refer to the molecular weights of the polypeptides precipitated by p50 antiserum.

crosslink antibody to PAS beads, 1.5μl specific antiserum and 25μl beads were mixed and equilibrated in 20 volumes of 0.2M sodium borate (pH 9.0) for 30 minutes at 4°C. Unbound antibody was removed by washing 3 times in 10ml of 0.2M Na Borate, and beads were resuspended in 400μl of 0.2M Na Borate (pH 9.0) and 40mM Dimethyl Suberimidate (Pierce Ltd). After incubation at 4°C for 30 minutes beads were washed twice with 10ml of TBS, incubated for 30 minutes in 400μl of TBS, and washed twice more with 10ml of TBS. Crosslinked PAS/antibody was used in immunoprecipitations as above, and protein eluted by incubation with 40μl 1% SDS for 15 minutes before dilution in 10 volumes of RIPA buffer lacking SDS. This material was reprecipitated with a second antiserum.

Gel retardation analysis

Nuclear and cytoplasmic extracts for gel retardation analysis and Western blots were prepared as described above except that confluent 9cm dishes (10^6 cells) were used and volumes were scaled up accordingly. Protein concentrations were determined by Bradford assay (Bio-Rad Ltd). For gel retardation analysis, 10μg aliquots of extracts were assayed using a PRD II probe, as described in Visvanathan and Goodbourn (45).

Western blots

Protein samples (40μg) were heated in Laemmli buffer prior to fractionation in 10% polyacrylamide (29:1 mono:bis) mini-gels containing SDS (44). Separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell) in a semi-dry blotter (Ancos) using a buffer containing 20mM Tris, 150mM glycine (pH8.0), 20% methanol. Nitrocellulose membranes were blocked for 1 hour at 20°C in PBS containing 0.1% Tween-20 and 5% Marvel (PTM) and exposed to the primary rabbit antiserum diluted 1:3000 in PTM for 1 hour at 20°C. Membranes were washed twice with PTM buffer, then incubated for 30 minutes at 20°C with horseradish peroxidase-linked anti-rabbit immunoglobulin ($F(ab')_2$ fragment from donkey, Amersham) diluted 1:3000 in PTM. Following two washes in PTM, membranes were washed twice with PBS containing 0.1% Tween-20 and the location of bound antibody detected on X-ray film by enhanced chemiluminescence (Amersham).

RESULTS

NF-κB activation is associated with loss of MAD3 and enhanced processing of p105 to p50

To investigate changes in the levels of NF-κB and IκB components in response to extracellular stimuli, MG-63 osteosarcoma cells were serum and methionine starved overnight and then treated for 1 hour with ^{35}S-methionine to pulse-label newly synthesised proteins. Cells were then treated with NF-κB inducers in the presence of medium supplemented with unlabelled methionine. After stimulation, cell lysates were prepared and proteins were immunoprecipitated with specific antisera.

Previous experiments from other laboratories have indicated that antisera raised against different NF-κB subunits co-immunoprecipitate a variety of polypeptides, which vary in a cell type- and antisera-dependent manner (39, 40). This variability makes it important to establish the composition of immunoprecipitates in the cell-line under examination. In addition to the expected polypeptides of 50kDa and 118kDa (which we assume are p50 and its precursor p105), antiserum raised against p50 immunoprecipitated proteins of 72kDa and 39kDa from whole cell extracts of untreated MG-63 cells (Figure 1A lane 1). The minor polypeptide of 46kDa detected in this experiment was also precipitated by pre-immune rabbit serum and represents a non-specific contaminant (data not shown). The 72kDa and 39kDa polypeptides are not detected in Western blot experiments with the p50 antiserum (see Figures 4 and 5, and data not shown), suggesting that they are immunoprecipitated by virtue of association with either p50 or p105. Based upon the known interactionsof p50, we tentatively assign the 72kDa polypeptide as p65, and the 39kDa protein as IκB. These assignments were investigated by immunoprecipitating whole cell extracts with specific antisera raised against recombinant p65 or MAD3 (IκBα). The p65 antiserum precipitated a 72kDa polypeptide which co-migrated with the polypeptide precipitated by p50

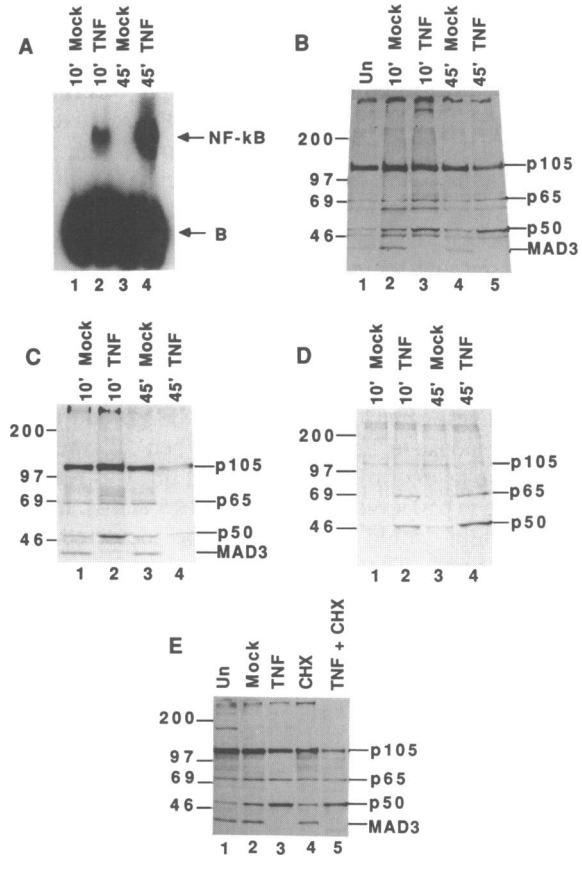

Figure 2. TNF enhances the processing of p105 to p50 and causes disruption of p50/MAD3 association. (**A**). MG-63 cells were mock- (lanes 1 and 3) or TNF- (lanes 2 and 4) induced and nuclear extracts were analysed by gel retardation analysis. The probe used comprises the human β-interferon promoter PRD II region, which detects NF-κB and a β-interferon-specific complex 'B' as indicated (45). (**B**). ^{35}S-methionine-labelled whole cell extracts from uninduced (lane 1), mock- (lanes 2 and 4) or TNF- (lanes 3 and 5) induced MG-63 osteosarcoma cells were immunoprecipitated with p50 antiserum. (**C**). Cytoplasmic fractions of mock- (lanes 1 and 3) or TNF- (lanes 2 and 4) induced cells were immunoprecipitated with p50 antiserum. (**D**). Nuclear fractions of mock- (lanes 1 and 3) or TNF- (lanes 2 and 4) induced cells were immunoprecipitated with p50 antiserum. (**E**). Whole cell extracts from uninduced (lane 1), mock- (lane 2) or TNF- (lanes 3 and 5) induced cells were immunoprecipitated with p50 antiserum. Cycloheximide (CHX) was added to cells as indicated (lanes 4 and 5). Numbers to the left of panels (B) to (E) refer to the molecular weights of protein markers in kDa, while the position of NF-κB and MAD3 components are indicated to the right of the gels.

antiserum, and which we assume is p65 (Figure 1A, lane 2). In addition, the p65 antiserum precipitated the non-specific 46kDa polypeptide, and small amounts of 50kDa (probably p50) and 39kDa polypeptides, in agreement with previously published results (39). However, in contrast to these results (39), we have failed to detect any co-precipitation of p105 with p65 antiserum, which may reflect differences between B cells and MG-63 osteosarcoma cells, or be a property of our antiserum. The MAD3 antiserum specifically precipitated a complex set of polypeptides, including an abundant 39kDa protein which co-migrated with the 39kDa species precipitated by anti-p50 and anti-p65 sera (Figure 1A, lane 3). In Western blot experiments, the MAD3 antiserum only recognises a 39kDa polypeptide (see Figures 4 and 5), suggesting that the other immunoprecipitating proteins are MAD3-associated (but see below).

To further investigate whether the 39kDa polypeptide which co-precipitated with p50 antiserum was MAD3, we undertook immunodepletion experiments. When extracts were first depleted of MAD3, p50 antiserum subsequently precipitated p105, p65 and p50, but only small amounts of the 39kDa protein (Figure 1B, lanes 1−3). In contrast, when the sample was first cleared of p50 and associated proteins, some MAD3-reactive material remained in the supernatant (Figure 1B, lanes 4−6), consistent with observations that IκB is also associated with non-p50 containing complexes (39). To confirm the identity of the p50-associated 39kDa polypeptide, the p50 immunoprecipitate was solubilised and reprecipitated with MAD3 antiserum, the 39kDa polypeptide being specifically detected (Figure 1C). These results indicate that p50 associates with p65 and MAD3 in uninduced MG-63 cells, and we refer below to the 72kDa polypeptide as p65, and the 39kDa polypeptide as MAD3.

We next investigated the changes in NF-κB and associated proteins in response to TNF induction. When cells were treated with TNF, the level of p105 declined, but this was reciprocated by an increase in p50 level (Figure 1A, lanes 1 and 4). In addition, MAD3 protein could no longer be detected in p50 immunoprecipitates. The loss of the 39kDa protein was also seen with the MAD3 antiserum (Figure 1A, lanes 3 and 6). It is striking that many of the other non-MAD3 polypeptides still precipitate in this experiment, even though MAD3 is not detectable by Western blots (see Figure 4). It is therefore unlikely that these other proteins are immunoprecipitated by virtue of their MAD3 association, and may instead be proteins (and their associated polypeptides) that are sufficiently related to MAD3 to be detectable only under non-denaturing conditions. Since it appears from the immunodepletion data that some MAD3 is not associated with p50 (Figure 1B), the loss of virtually all MAD3 upon TNF treatment suggests that activation of other NF-κB complexes may also be associated with MAD3 degradation.

The consequences of TNF treatment were then examined in more detail. Activation of NF-κB as measured by gel retardation analysis could be observed within 10 minutes of TNF treatment (Figure 2A, lanes 1 and 2), and had increased by 45 minutes (lanes 3 and 4). In a parallel experiment, the level of p50-associated MAD3 had dramatically declined by 10 minutes, and was undetectable by 45 minutes (Figure 2B, lanes 2−5). The loss of p50-associated MAD3 could also be seen in cytoplasmic extracts (Figure 2C). The conversion of p105 to p50 showed slower kinetics than the loss of MAD3, being observable at 10 minutes (Figure 2B, lanes 2 and 3), but more pronounced at 45 minutes (Figure 2B, lanes 4 and 5). Consistent with the cytoplasmic location of p105, the loss of p105 is very clear in cytoplasmic extracts (Figure 2C). The nuclear fractions in these experiments also contain some p105 prior to induction, although again the levels drop upon TNF treatment (Figure 2D). Since the existence of nuclear p105 is currently controversial (see 40, 46, 47) the p105 detected here may represent contamination of

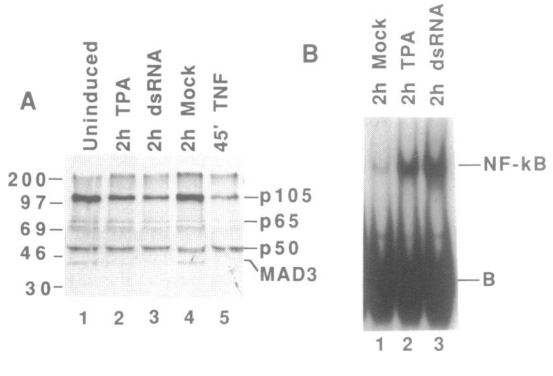

Figure 3. TPA and dsRNA enhance the processing of p105 to p50 and cause disruption of p50/MAD3 association. (**A**). Whole cell extracts from ^{35}S-methionine-labelled uninduced (lane 1), TPA- (lane 2), dsRNA- (lane 3), mock- (lane 4) or TNF- (lane 5) induced cells were immunoprecipitated with p50 antiserum. Numbers to the left of the gel refer to the molecular weights of protein markers in kDa, while the position of NF-κB and MAD3 components are indicated to the right of the gels. (**B**). Nuclear extracts corresponding to the mock- (lane 1), TPA- (lane 2) or dsRNA- (lane 3) induced samples in (A) were analysed by gel retardation analysis using PRD II probe.

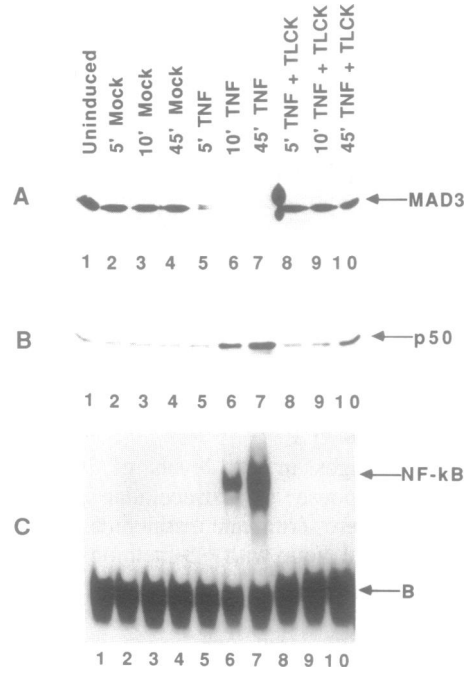

Figure 4. TLCK blocks TNF-induced NF-κB activation and MAD3 degradation. (**A**). Cytoplasmic fractions from uninduced (lane 1), mock-induced (lanes 2−4), TNF-induced (lanes 5−7), or TNF-induced in the presence of TLCK (lanes 8−10) MG-63 cells were analysed for the presence of immunoreactive MAD3 by Western blotting. (**B**). Nuclear fractions corresponding to samples in (A) were analysed for the presence of immunoreactive p50 by Western blotting. (**C**). Nuclear extracts corresponding to samples in (A) were analysed by gel retardation analysis using PRD II probe.

the nuclear fraction with cytoplasm. The p50 levels were enhanced in cytoplasmic fractions by 10 minutes after TNF treatment, but declined to pre-induced levels by 45 minutes (Figure 2C). This is presumably a consequence of nuclear uptake as a result of NF-κB activation, and as expected, increased levels of p50 (and p65) were detected in the nuclear fraction following TNF treatment (Figure 2D). To confirm that the increase in p50 seen in these experiments is not due to newly synthesised material, we repeated the pulse-chase experiment in the presence of cycloheximide and found that *de novo* protein synthesis was not necessary to cause the decrease in p105 or the increase in p50 (Figure 2E).

When NF-κB was activated by either dsRNA or the phorbol ester TPA (Figure 3B), similar induction-specific decreases in p105 and MAD3 levels, and an increase in p50 levels, were observed (Figures 3A, compare lanes 2−4). In accordance with the slower NF-κB induction kinetics for these stimuli, the acceleration in processing rate was correspondingly slower than for TNF.

In the pulse-chase experiments described above, only the fate of newly synthesised material was investigated. To determine whether the steady state levels of p105, p50 and MAD3 levels were similarly altered in response to NF-κB activation we performed Western blot analyses on TNF- or dsRNA-treated samples. TNF activation was associated with a substantial loss of MAD3 after 10 minutes (Figure 4A, lanes 3 and 6). This was preceded at 5 minutes by the appearance of a lower mobility form of MAD3 (Figure 4A, lanes 2 and 5). These results indicate that the loss of MAD3 as a p50-associated protein in immunoprecipitates is not simply due to dissociation of MAD3 from NF-κB, but is due to a complete loss of immunoreactive MAD3, presumably due to proteolytic degradation. In control experiments, there was no loss in p50, which showed the expected increase in nuclear concentration following TNF activation (Figure 4B, lanes 1−7). We have not observed a change in the levels of p105 (data not shown), suggesting that the turnover of p105 to p50 may be restricted to a pool of newly synthesised p105. When dsRNA was used as an activator the loss of MAD3 was also observed, albeit with different kinetics (Figure 5). However, we could not detect the existence of an altered mobility form of MAD3 with this inducer.

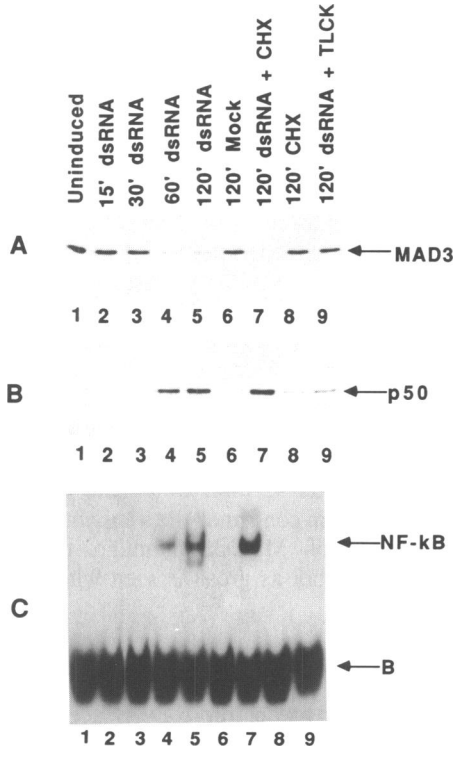

Figure 5. TLCK blocks dsRNA-induced NF-κB activation and MAD3 degradation. (**A**). Cytoplasmic fractions from uninduced (lane 1), dsRNA-induced (lanes 2−5), mock-induced (lane 6), dsRNA-induced in the presence of cycloheximide (lane 7), mock-induced in the presence of cycloheximide (lane 8), and dsRNA-induced in the presence of TLCK (lane 9) MG-63 cells were analysed for the presence of immunoreactive MAD3 by Western blotting. (**B**). Nuclear fractions corresponding to samples in (A) were analysed for the presence of immunoreactive p50 by Western blotting. (**C**). Nuclear extracts corresponding to samples in (A) were analysed by gel retardation analysis using PRD II probe.

Proteolysis of MAD3 is required for NF-κB activation

To test whether MAD3 degradation and enhanced p105 processing are prerequisites for NF-κB activation we induced cells in the presence of protease inhibitors. The serine protease inhibitor TLCK (Nα-p-Tosyl-L-Lysine Chloromethyl Ketone) was effective at blocking MAD3 degradation *in vivo* in response to both TNF (Figure 4A, lanes 8−10) and dsRNA (Figure 5A, lane 9). TLCK also blocked the activation of NF-κB as assayed by gel retardation, indicating that degradation of MAD3 is a prerequisite for NF-κB activation (Figure 4C, lanes 8−10 and Figure 5C, lane 9). This blockage to activation was associated with a substantial inhibition of p50 and p65 translocation to the nucleus (Figure 4B, 5B and data not shown), although it is notable that this was not completely blocked. The discrepancy between the presence of nuclear p50 and the absence of NF-κB DNA binding activity probably reflects the existence of nuclear MAD3 in this protease-inhibited sample (E.A.Watson and R.T.H., unpublished—see discussion).

In addition to TLCK, TPCK (Nα-p-Tosyl-L-Phenylalanine Chloromethyl Ketone) could also inhibit NF-κB activation. In the latter case complete inhibition was routinely observed at 30μM (data not shown). Although TLCK could also inhibit at 30μM,

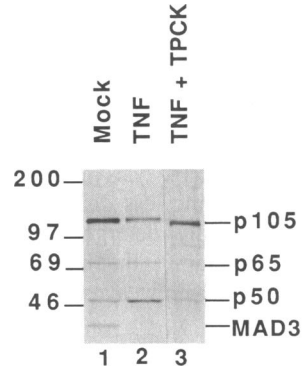

Figure 6. TPCK blocks the processing of p105 to p50, but not the disruption of p50/MAD3 association, in response to TNF. Whole cell extracts from ^{35}S-methionine-labelled MG-63 cells which had been mock-induced (lane 1), TNF-induced (lane 2), or TNF-induced in the presence of TPCK (lane 3) were immunoprecipitated with p50 antiserum. Numbers to the left of the gel refer to the molecular weights of protein markers in kDa, while the position of NF-κB and MAD3 components are indicated to the right of the gel.

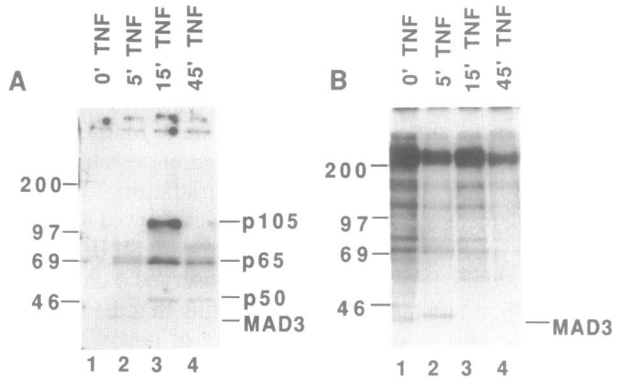

Figure 7. Activation of NF-$\varkappa$B by TNF is associated with phosphorylation of MAD3 and p105. Whole cell extracts from ^{32}P-orthophosphate-labelled cells which had been induced with TNF for the times indicated were immunoprecipitated with p50 (**A**) or MAD3 (**B**) antisera. Numbers to the left of the gels refer to the molecular weights of protein markers in kDa, while the position of NF-$\varkappa$B and MAD3 components are indicated to the right of the gels.

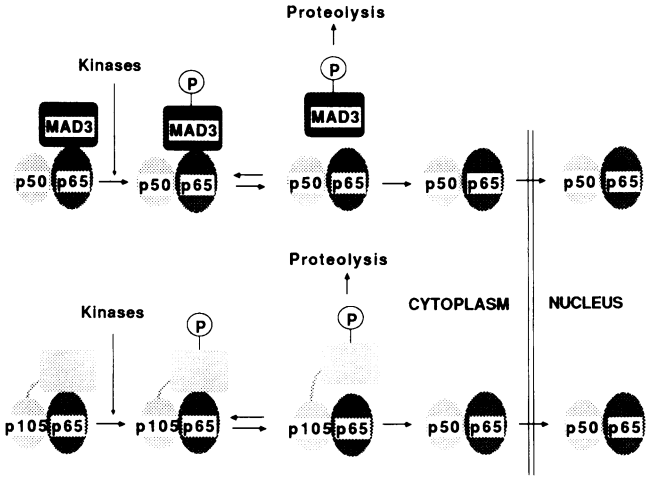

Figure 8. Model for NF-$\varkappa$B activation. See discussion for details.

we have observed some variability with this chemical, which probably reflects the extreme lability of TLCK at alkaline pH; to counter this, we routinely use TLCK at a final concentration of 300μM. Both TPCK and TLCK could be added at the same time as the inducers and could cause a complete block to activation by any of dsRNA, TNF or TPA. These inhibitors were incapable of blocking if added to cells after NF-$\varkappa$B activation, and could not prevent recombinant NF-$\varkappa$B from binding DNA at any concentration tested (data not shown). The effects of these inhibitors are not mediated through non-specific effects on DNA-binding proteins, since the level of the β-interferon promoter-specific binding protein, complex B (45) is not affected by TLCK or TPCK (Figures 4 and 5), and in other experiments we have demonstrated that CREB-1 binding activity is similarly unaffected (data not shown). The inhibitors also show specificity in their ability to block induction-specific proteolysis events since neither compound prevented the cleavage of IRF-2 (48) in response to dsRNA treatment (S.T.Whiteside and S.G., manuscript in preparation).

TPCK also blocked TNF-induced processing of p105 to p50 (Figure 6, compare lanes 2 and 3). We have obtained similar results for TLCK with any of TNF, dsRNA or TPA (data not shown). Interestingly, TPCK and TLCK did not block the TNF-induced loss of co-precipitation of MAD3 with p50 antibodies. Although these experiments use RIPA buffer (0.5% NP40, 0.5% deoxycholate, 0.1% SDS) in the lysis and washing steps, we have obtained similar results using less disruptive conditions (using 0.125% NP-40 as the only detergent component—data not shown). These results suggest that proteolysis of MAD3 is preceded by other events which weaken the association of MAD3 and p50.

MAD3 and p105 are phosphorylated in response to TNF treatment

A simple interpretation of the finding that TLCK and TPCK block the activation of NF-$\varkappa$B but do not block the dissociation of p50 and MAD3 is that a protease inhibitor-insensitive step occurs prior to activation of NF-$\varkappa$B. As noted above, a lower mobility form of MAD3 was detected by Western blot analysis at very early times in response to TNF (Figure 4A, lanes 2 and 5). In immunoprecipitation experiments, we observed a minor p50-associated polypeptide with slightly decreased electrophoretic mobility to MAD3 at 10 minutes after TNF treatment (Figure 2C, lanes 1 and 2). Using MAD3 antiserum, a similar decrease in electrophoretic mobility could be seen (data not shown), suggesting that the p50-associated protein is a modified form of MAD3. In these experiments, we also observed a decrease in mobility of a portion of the p105 material at 10 minutes after TNF treatment (Figure 2C, lane 2), although this could not be detected after 45 minutes (Figure 2C, lane 4). These mobility shifts may reflect the previously proposed ligand-induced phosphorylations of I$\varkappa$B (36). To test this directly, we labelled cells with ^{32}P-orthophosphate prior to stimulating with TNF. In uninduced cells, p50 antiserum precipitated three weakly labelled proteins which corresponded to p105, p65 and MAD3, whereas after 5 minutes, a more strongly phospholabelled protein with a mobility slightly slower than MAD3 could be detected (Figure 7A, compare lanes 1 and 2). This protein was undetectable within 15 minutes of treatment, whereas p105 became heavily labelled at this time point (Figure 7A, lane 3). The p50 and p65 proteins also increased their extent of phosphorylation during the course of induction, although unlike p105 and MAD3, these proteins remained detectable after 45 minutes (Figure 7A, lane 4). The use of anti-MAD3 serum confirmed the transient phosphorylation and mobility shift of MAD3, although the increase in phospholabelling was not as great as seen with p50 antiserum (Figure 7B).

DISCUSSION

Our results suggest a model for NF-$\varkappa$B activation which is illustrated in Figure 8. Upon induction, MAD3 and p105 become specifically phosphorylated. In the case of TNF, phosphorylated MAD3 can be detected within 5 minutes, while p105 phosphorylation can be detected within 15 minutes. These results confirm the recent observations that MAD3 can be phosphorylated in T-cells in response to TNF or interleukin-1 (37) and in monocytes in response to LPS (38), using a non-lymphoid line, and also extend them to p105. The induction-specific phosphorylations precede proteolytic degradation of both

proteins, and it is tempting to speculate that phosphorylation may induce either a dissociation of the NF-κB/MAD3 complex or conformational changes which render the MAD3 and C-terminus of p105 more prone to proteolytic degradation. When the IκB levels have declined, free NF-κB can translocate to the nucleus and activate transcription. The lability of IκB was first postulated on the basis of observations that cycloheximide could superinduce NF-κB activation (49) and it has been recently demonstrated that IκB is degraded following stimulation (37, 50). However, the observation that treatment of cells with the protease inhibitors TLCK and TPCK can completely block MAD3 degradation and induction-specific processing of p105 to p50 indicates that proteolysis of these factors is an obligatory step in NF-κB activation.

We cannot currently determine whether dissociation of the NF-κB/MAD3 complex is necessary to allow MAD3 degradation, or whether modified MAD3 can be degraded while still associated with NF-κB. In the case of p105, the presence of both the DNA binding and inhibitor activities in the same protein suggests that degradation may not require dissociation. At early times after induction, decreased mobility forms of the MAD3 are detected in association with p50. However, at later times MAD3 and p50 appear to have dissociated since they cannot be co-precipitated even when proteolysis is blocked. Taken together, these results suggest that although phosphorylated MAD3 can be detected in association with p50, a subsequent event must destabilise the p50/MAD3 complex, at least to such a degree that these proteins can no longer be co-immunoprecipitated. The fact that NF-κB is not activated in the absence of MAD3 degradation suggests that the affinity of the NF-κB/MAD3 interaction is still sufficient to prevent NF-κB release for long enough to be effectively translocated to the nucleus. However, inhibitor treatment does not completely block p50 nuclear translocation, suggesting that the NF-κB/MAD3 complex is destabilised sufficiently to release some p50. We interpret the lack of NF-κB DNA-binding activity in the nuclei of these inhibitor-treated cells as being a result of undegraded nuclear MAD3 which would complex with any translocated NF-κB. The existence of nuclear MAD3 in these cells has been detected by immunofluorescence (E.A.Watson and R.T.H., unpublished observations). A residual affinity of modified MAD3 for NF-κB may explain the requirement for proteolysis as a need to lower the concentration of MAD3 to generate free NF-κB. The observed dissociation of NF-κB and IκB in response to specific kinases *in vitro* may reflect the lack of excess IκB in these experiments. Proteolysis of MAD3 might also counter the effects of cellular phosphatases which could reactivate non-degraded IκB (but see below).

If the phosphorylation event precedes proteolysis it would be predicted that phosphorylated forms would accumulate in the presence of TLCK and TPCK. However, the more slowly migrating forms of MAD3 and p105 are not detected in the presence of the inhibitors (see Figures 4–6), and we have failed to detect TLCK/TPCK-enhanced phosphorylation of MAD3 or p105 in labelling experiments (unpublished observations). These data suggest that if not immediately degraded the phosphorylated MAD3 or p105 may be susceptible to phosphatases. However, it is striking that the undegraded, unphosphorylated MAD3 does not appear to be capable of re-associating with NF-κB, suggesting that the properties of MAD3 (or of NF-κB) have been further altered in some manner. We are also interested to note that we cannot see evidence of decreased mobility forms of MAD3 in response to dsRNA. One notable difference between dsRNA and TNF as inducers is the kinetics of NF-κB activation. In the case of TNF, IκB is modified within minutes and is then rapidly degraded. Since dsRNA requires much longer to activate NF-κB, it may be that the rate of kinase activation is much slower, and that the production and loss of phosphorylated forms of IκB is too rapid to be detected.

A surprising aspect of our results is the observation that TLCK and TPCK can block activation of NF-κB at equivalent concentrations. Although these inhibitors both block serine proteases, they have quite different specificities. This might suggest that interdependent tryptic- and chymotryptic-like pathways are involved in IκB breakdown, but this is unlikely since we are unable to detect breakdown intermediates in the presence of either TLCK or TPCK. Alternatively, IκB degradation may use a pathway which involves a cascade of proteases. One possible candidate might be the ubiquitin pathway, which targets proteins for destruction at proteosomes which contain both tryptic and chymotryptic proteases (reviewed in 51, 52). However, there is no evidence that these enzymes are linked in a cascade rather than being components of parallel pathways. We favour the hypothesis that a different class of enzyme is involved, for example cysteine proteases. It is well established that TLCK and TPCK are effective cysteine protease inhibitors, although at concentrations that are higher than that required to inhibit specific serine proteases (53–56). Consistent with this, relatively high concentrations of both inhibitor are needed to bring about blockage of NF-κB activation. In contrast to serine proteases, cysteine proteases are almost all cytoplasmic, and are often associated with complete destruction of proteins rather than a 'clipping' event (reviewed in 57). Furthermore, cysteine proteases have been associated with the destruction of unstable proteins containing PEST sequences of which the C-terminus of MAD3 and p105 are good examples. We also note that cysteine proteases can be inactivated by agents that modify thiol groups and speculate that the inhibitory effects on NF-κB induction of anti-oxidants (Schreck *et al.*, 58, 59), could be mediated by the covalent modification of cysteine in the active site of an IκB-degrading cysteine protease.

In the case of several cysteine proteases, it appears that the proteases are activated by association with 'helper' proteins. For example, the adenovirus protease requires the disulphide-linked C-terminal peptide of the pVI protein for activation (60). On the basis of these results and by analogy to the activation of another cysteine protease, propapain, these authors have suggested that protease activation is brought about by thiol-disulphide exchange between the pVI peptide and the protease leading to a conformational change in the active site. This mechanism has the advantage that protease activity requires a specific substrate for activation. It is possible that IκB (or NF-κB) contains this information as well as serving as a substrate. We are currently investigating these possibilities.

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