Interleukin 1-induced phosphorylation of MAD3, the major inhibitor of nuclear factor κ B of HeLa cells

Interference in signalling by the proteinase inhibitors 2,4-dichloroisocoumarin and tosylphenylalanyl chloromethylketone

François GUESDON,*|| Tetsuro IKEBE,†|| Eleni STYLIANOU,‡|| Jan WARWICK-DAVIES,* Stephen HASKILL§ and Jeremy SAKLATVALA*

*Cytokine Laboratory, Department of Development and Signalling, The Babraham Institute, Cambridge CB2 4AT, U.K., †Department of Biochemistry, Kyushu University School of Dentistry, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812, Japan, ‡Department of Medicine, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K., and §Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, U.S.A.

The regulation of the inhibitor of nuclear factor κ B (I κ B) by interleukin 1 (IL1) was investigated in HeLa cells. Two forms of I κ B were resolved by ion-exchange chromatography. The major form (75%) was identified as MAD3 by specific antisera. IL1 generated rapidly (6 min) an electrophoretically retarded form of MAD3 that was stable in acid and was converted into the unmodified form by phosphatase 2A. It thus corresponded to a phosphorylation of the protein on serine or threonine. IL1 also caused the disappearance of MAD3 from the cells, which was complete 15 min after stimulation and coincided with a 46% reduction of cellular I κ B activity. Newly-synthesized MAD3 accumulated to pre-stimulation levels between 60 and 90 min after stimulation and this coincided with the down-regulation of the phosphorylating activity. The serine proteinase inhibitors 3,4-dichloroisocoumarin (DCI) and tosylphenylalanyl chloro-

methylketone (TPCK) prevented phosphorylation and disappearance of MAD3. At the same concentrations (10–100 μ M), they also increased basal phosphorylation of the small heat shock protein (hsp27) and prevented the IL1- and phorbol 12-myristate 13-acetate-induced increases of its phosphorylation. The inhibitors were thus interfering with protein kinases when blocking degradation of MAD3. Recombinant MAD3 phosphorylated *in vitro* by protein kinase C was not electrophoretically retarded, suggesting that MAD3 was phosphorylated by another kinase in IL1-stimulated cells. Our results suggest that the IL1-induced phosphorylation of MAD3 on serine or threonine leads to its degradation. DCI and TPCK blocked phosphorylation mechanisms and it could not be concluded that serine proteinases were involved in the breakdown of MAD3.

INTRODUCTION

A rapid cellular effect of the inflammatory cytokine interleukin 1 (IL1) is to activate the transcriptional regulator, nuclear factor (NF) κ B [1]. This response is mediated through the type I but not the type II, receptor [2]. The mechanism of the activation, and the post-receptor signalling events which cause it, are obscure.

NF- κ B proteins are members of the *rel* family [3]. The best characterized form is the activatable heterodimer of the DNA-binding subunit p50 with p65. In resting cells, this heterodimer resides in the cytoplasm, complexed with an inhibitory protein, I κ B [4]. Upon appropriate stimulation of the cells, for instance by bacterial lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) or cytokines, active heterodimer appears in the nucleus where it is presumably bound to the κ B DNA motif. To date, the evidence points to the inhibitor, rather than the nuclear factor itself, being modified during activation. For example, activation is accompanied by down-regulation of cellular I κ B [4–9] and purified I κ B proteins inhibit binding of NF- κ B to DNA *in vitro* [4,10].

I κ B was originally characterized as a cytoplasmic factor that could be dissociated from NF- κ B by sodium deoxycholate (DOC) [4]. Two forms, I κ B α and I κ B β , were purified from human

placenta [10]. They differed in size, sensitivity to phosphatase treatment and reactivity with NF- κ B/*rel* proteins. The major one, I κ B α (37 kDa), inhibited only p65, whereas I κ B β (43 kDa) also prevented the binding of the *c-rel* gene product to the κ B site [5,11]. I κ B α is presumed to be identical to a protein called MAD3, whose cDNA was cloned from monocytes [12,13]. Several other I κ Bs have also been described. They include pp40 [12], Bcl-3 [14], the p105 NF- κ B precursor [15] and a 70 kDa protein identical to its C-terminal portion, I κ B γ [16].

Since PMA is an activator of protein kinase C, phosphorylation of I κ B has been proposed as a major mechanism of NF- κ B activation [17]. Cell stimulants, such as LPS, IL1 and tumour necrosis factor (TNF), that activate NF- κ B, cause phosphorylation and a concomitant loss of MAD3/I κ B α [6–8,18–20], but whether phosphorylation of I κ B is sufficient to release the nuclear factor or whether degradation is also needed is unclear. Recent reports have suggested that proteolysis is necessary for activation since blocking degradation of MAD3 with serine proteinase inhibitors prevents release of nuclear factor [6,19].

In order to understand how IL1 impinges upon the NF- κ B system, we have characterized the forms of I κ B in an IL1-responsive cell. We have found that MAD3 was responsible for most of the I κ B activity of HeLa cells and that IL1 induced its

Abbreviations used: DCI, 3,4-dichloroisocoumarin; DOC, sodium deoxycholate; DMEM, Dulbecco's modification of Eagle's medium; DTT, dithiothreitol; E64, 1-*trans*-epoxysuccinyl-leucylamide(4-guanidino)butane; FCS, fetal calf serum; hsp27, small heat shock protein; I κ B, inhibitor of NF- κ B; IL1, interleukin 1; LPS, bacterial lipopolysaccharide; MAP kinase, mitogen activated protein kinase; MAPKAP kinase, MAP kinase activated protein kinase; NF- κ B, nuclear factor κ B; NP40, Nonidet P40; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; PP, phosphoprotein phosphatase; PTP, phosphotyrosine phosphatase; TPCK, tosylphenylalanyl chloromethylketone; TNF, tumour necrosis factor.

||These authors contributed equally to this investigation.

¶To whom correspondence should be addressed.

phosphorylation on serine or threonine and its degradation. Inhibitors of serine proteinases were used to try to cause accumulation of the phosphorylated form, but they prevented the IL1-induced breakdown of MAD3 by interfering with intracellular signalling by IL1. Our evidence nevertheless suggests that the IL1-induced phosphorylation labels MAD3 for degradation.

MATERIALS AND METHODS

Materials

HeLa cells and Dulbecco's modified Eagle's medium (DMEM) were from Flow Laboratories (Rickmansworth, U.K.). Fetal calf serum (FCS) was from GIBCO (Uxbridge, U.K.). Recombinant human IL1 α was purified as described [21]. Recombinant human phosphotyrosine phosphatase (PTP) 1B was given by Dr. D. Barford (Department of Molecular Biophysics, University of Oxford, U.K.). The catalytic subunit of phosphoprotein phosphatase 2A (PP2A) was purified from rabbit muscle as described [22]. Its specific activity was 3300 units/mg, one unit being the amount of enzyme releasing 1 nmol of phosphate per minute from phosphorylase α . Okadaic acid was from Calbiochem-Novabiochem (U.K.). PMA, phenylmethanesulphonyl fluoride (PMSF), Hepes buffer, 3,4-dichloroisocoumarin (DCI), 1-*trans*-epoxysuccinyl-leucylamide(4-guanidino)butane (E64), NaF, Na₂VO₄, β -glycerophosphate, tosylphenylalanyl chloromethylketone (TPCK), BSA, calf thymus double-stranded DNA-cellulose, DOC, Nonidet P40 (NP40), TPCK-treated trypsin, soybean trypsin inhibitor and protein A-coupled agarose were supplied by Sigma. [γ -³²P]ATP for labelling of oligonucleotides (7000 Ci/mmol) was from ICN Biomedicals Ltd. (Bucks., U.K.) and [γ -³²P]ATP for phosphorylation of proteins *in vitro* (3200 Ci/mmol) was from Amersham plc (Little Chalfont, U.K.). ¹²⁵I-labelled donkey antibody against rabbit immunoglobulin (750–3000 Ci/mmol) and [³²P]orthophosphoric acid (9000 Ci/mmol) were from Amersham plc. T4 polynucleotide kinase was from Stratagene (Cambridge, U.K.) and poly(dI-dC) was from Pharmacia (Milton Keynes, U.K.). A synthetic peptide with the sequence of residues 6–20 of MAD3 (peptide 6–20, ERPQEW-AMEGPRDGL) and antigen affinity-purified rabbit antibodies to it (Ab 6–20) were from NBS Biological, Hatfield, U.K. Baculovirus-expressed recombinant MAD3 was a gift from Dr Martha Ladner (Chiron Corporation, Emeryville, CA, U.S.A.). The rabbit antisera to it (AS10) and to the synthetic peptide (AS9) were made by standard methods. Rabbit antiserum to the human small heat shock protein (hsp27) was made against homogenous antigen purified from ME180 cells as described elsewhere [23] by N. Freshney in this laboratory. Protein kinase C (α , β , γ) purified from bovine brain [24] was a gift from Dr P. Parker (ICRF Laboratories, London, U.K.).

Cell culture

HeLa cells were maintained in DMEM containing 10% (v/v) heat-inactivated FCS at 37 °C in a humidified 95% air–5% CO₂ atmosphere. Confluent cells grown in 10 cm² wells or 175 cm² flasks were used for experiments.

Preparation of nuclear extracts and cytosolic fractions

Cell extracts were prepared according to published methods [1,25]. HeLa cells grown in flasks were detached, either by treatment with trypsin/EDTA in PBS, or by placing the flasks on ice and scraping the cells in ice-cold PBS: both methods gave similar results (not shown). Detached cells were placed on ice and washed firstly in ice-cold PBS and then in ice-cold hypotonic

buffer [buffer A: 10 mM Hepes, pH 7/1.5 mM MgCl₂/10 mM KCl/0.05 mM dithiothreitol (DTT)/0.3 mM DCI/0.1 mM E64/1 μ g/ml pepstatin/0.1 mM phenanthroline/10 mM NaF/25 mM β -glycerophosphate/0.1 mM Na₃VO₄]. They were then lysed for 10 min on ice in buffer A containing 0.1% NP-40. Lysates were centrifuged for 10 min at 10000 *g* at 4 °C and the supernatants were kept as cytosolic fractions.

The pelleted nuclei were resuspended in extraction buffer [buffer C: 20 mM Hepes, pH 7.9/420 mM NaCl/1.5 mM MgCl₂/0.2 mM EDTA/25% (v/v) glycerol/0.5 mM PMSF/0.5 mM DTT] and incubated at 4 °C for 15 min. Nuclei were then centrifuged for 10 min at 10000 *g* at 4 °C and the supernatants, referred to as the nuclear extracts, were supplemented with a 5-fold volume of storage buffer [buffer D: 20 mM Hepes, pH 7.9/50 mM KCl/0.2 mM EDTA/20% (v/v) glycerol/0.5 mM PMSF/0.5 mM DTT] [1]. These extracts were snap frozen and stored at –70 °C.

Electrophoretic mobility shift assay

NF- κ B binding activity was detected with synthetic oligonucleotide probes. The wild-type κ B (5'-AGCTTGGGGACTTC-CGAG-3') or mutant κ B (5'-AGCTTGCTCACTTCCGAG-3') oligonucleotides were radiolabelled with [γ -³²P]ATP by T4 polynucleotide kinase. They were then isolated by precipitation with ammonium acetate/ethanol and purified by precipitation with 80% ethanol.

DNA-binding reactions were performed in a total volume of 20 μ l containing the binding buffer [10 mM Tris/HCl, pH 7.5/100 mM NaCl/1 mM EDTA/4% (v/v) glycerol/5 mM DTT/100 μ g/ml BSA], the sample, an aliquot of ³²P-labelled oligonucleotide (10⁵ c.p.m.) and 3 μ g of poly (dI-dC). After incubation for 15 min at room temperature, samples were electrophoresed in a 5% polyacrylamide gel in a 22.5 mM Tris-borate buffer containing 0.5 mM EDTA [26]. After electrophoresis gels were dried and then autoradiographed by exposure to medical X-ray film. For quantification, the DNA–protein complex bands were located by autoradiography, excised and radioactivity was measured by Cerenkov counting.

Measurement of I κ B activity

Samples to be assayed for I κ B activity (4–10 μ g of protein) were preincubated for 5 min with standard amounts (6 μ g of protein) of nuclear extract preparations from IL-1-stimulated HeLa cells containing NF- κ B. Control incubations of the nuclear extract without samples were also carried out. DNA-binding reactions and the electrophoretic mobility shift assay were then performed. In the case of DOC-treated cytosolic samples, NP40 was added to a final concentration of 1.2% before electrophoresis. Trials using varying amounts of recombinant MAD3 or flow-through fractions of cytosols showed that inhibition of NF- κ B activity was proportional to the amount of sample (results not shown).

Preparation of I κ B from HeLa cells

Crude I κ B was prepared essentially as described previously [4]. Briefly, HeLa cytosolic proteins (6 mg) were adjusted to 0.6% DOC in a final volume of 1 ml and applied to a 0.5 ml calf thymus double-stranded DNA–cellulose column. The column was first equilibrated with buffer G [10 mM Tris/HCl, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM DTT/5% (v/v) glycerol/0.2% DOC/0.2% NP40/0.5 mM PMSF]. Material that failed to bind to the column was collected and NP40 was added to a final concentration of 1.2%. This was saved for assay of I κ B activity. The DNA–cellulose was finally eluted with buffer G

containing 0.5 M NaCl. The eluate was dialysed against buffer G and then tested for NF- κ B by the electromobility shift assay.

For further purification of I κ B, the flow-through fraction from a sample of 12 mg of cytosolic protein was dialysed against buffer D [20 mM Hepes, pH 7.9/50 mM KCl/0.2 mM EDTA/10% (v/v) glycerol/0.5 mM PMSF/0.5 mM DTT/0.25% NP40], and subjected to anion-exchange chromatography on a HR 5/5 Mono Q column (Pharmacia) equilibrated with buffer D. The column was eluted at a flow rate of 1 ml/min with a linear salt gradient (0.05–1 M KCl) in an FPLC system (Pharmacia).

Metabolic labelling of cells

Cells grown in 10 cm² wells were washed twice for 15 min in phosphate-free DMEM (ICN) supplemented with 20 mM Hepes/NaOH (pH 7.5) and 1% heat-inactivated FCS that had been dialysed against 150 mM NaCl (labelling medium). They were then incubated for 6 h in 2 ml/well of labelling medium supplemented with 50 μ Ci/ml [³²P]orthophosphoric acid [27].

Preparation of lysates

Plates or flasks were put on ice and the cells were washed for 1 min in ice-cold PBS. Next, they were taken into chilled immunoprecipitation buffer [40 mM Tris/HCl, pH 7.5/100 mM NaCl/1% (w/v) NP40/1% (w/v) DOC/0.1% (w/v) SDS/50 mM β -glycerophosphate/50 mM NaF/200 μ M sodium orthovanadate/1 mM EDTA/1 mM EGTA/0.02% Na₃N₃] freshly supplemented with 1 mM PMSF, 1 mM orthophenanthroline, 10 μ M E64 and 1 μ M pepstatin A (500 μ l/well or 5 ml/flask). The resulting lysates were stirred thoroughly (30 s on whirlmix, maximum speed), left on ice for 20 min, cleared by centrifugation (5 min, 11 000 g) and kept at –20 °C.

Immunoprecipitation of MAD3 and hsp27

The immunoprecipitation procedure was carried out at 4 °C. Lysates were precleared by incubation for 30 min with 40 μ l of a 1:1 (v/v) suspension of protein A-agarose in immunoprecipitation buffer. The protein A-agarose was then left to settle. Immunoprecipitations were carried out by incubating the supernatants for 3 h with serum (0.5%, v/v) or purified antibodies (1 μ g/ml). Protein A-agarose (10 μ l of packed beads) was then added and the incubation was continued for a further 30 min with occasional mixing. The beads were then washed five times with immunoprecipitation buffer (1 ml per wash).

SDS/PAGE

Cell lysates were mixed directly with twice-concentrated SDS/PAGE sample buffer. For Mono Q fractions, proteins were precipitated by addition of carrier BSA (50 μ g/ml) and 0.4 vol. of 20% trichloroacetic acid (10 min, 4 °C), the precipitates were collected by centrifugation (10 min, 10 000 g), washed with diethyl ether, dried, and dissolved in SDS/PAGE sample buffer. Immunoprecipitates were taken into SDS/PAGE sample buffer. The samples were heated (100 °C, 3 min) and electrophoresed on gels containing 11.5% acrylamide and 0.31% bis-acrylamide for MAD3, or 12.5% acrylamide and 0.33% bis-acrylamide for hsp27 [28]. Gels were then either stained with Coomassie Brilliant Blue and dried or Western blotted.

Western blotting

The gels were electrotransferred to nitrocellulose (250 mA, 6 h) in a buffer containing 25 mM Tris base, 192 mM glycine and

20% methanol (v/v). The membrane was incubated for 1 h at 37 °C in blocking buffer [10 mM Tris/HCl, pH 7.4, 154 mM NaCl, 1% (w/v) BSA and 1% (w/v) milk powder], then in blocking buffer supplemented with antiserum (1:500, v/v) or Ab 6–20 (0.67 μ g/ml) for 16 h at 4 °C. After ten washes in Tris-buffered saline (10 mM Tris/HCl, pH 7.4/154 mM NaCl), the membrane was incubated at room temperature for 30 min in blocking buffer followed by 4 h in blocking buffer supplemented with ¹²⁵I-labelled antibody to rabbit immunoglobulin (0.25–0.5 μ Ci/ml). The blots were finally washed ten times in Tris-buffered saline, air dried and exposed to medical X-ray film. They were quantified by counting bands excised from nitrocellulose in a Packard Auto Gamma 500 counter.

Activation of protein kinase C

Bovine protein kinase C (α , β and γ) was incubated at 75 μ g/ml in 10 mM Tris, pH 8.0, buffer containing 10 mM 2-mercaptoethanol, 0.5 mg/ml BSA and 5 μ g/ml trypsin for 2.5 min at 30 °C. The reaction was stopped by addition of soybean trypsin inhibitor to a final concentration of 150 μ g/ml. The activity of the enzyme was measured on histone H1 substrate (1.25 mg/ml) as described [24], except that CaCl₂, PMA, phosphatidyl serine and Triton X-100 were omitted; 1 unit transfers 1 nmol of phosphate per min.

RESULTS

MAD3 is the major form of I κ B in HeLa cells

Antisera to a recombinant MAD3 containing a tag of seven extra amino acids at its C-terminus [13], AS10, or to a synthetic peptide corresponding to residues 6–20 of the protein, AS9, and affinity-purified antibodies to the same peptide, Ab 6–20, were used to check whether or not the protein was expressed in HeLa cells. AS9 has been used in previous studies of MAD3 and its specificity is well documented [18,20].

Several antigens were detected when cell lysates were subjected to Western blot with either Ab 6–20 (Figure 1, lane 1), or with AS10 (Figure 1, lane 5), but only one antigen, of about 38 kDa, was detected equally well by the serum and the antibodies

Figure 1 Identification of MAD3-related antigens in lysates of HeLa cells by Western blotting

HeLa cells grown in flasks were lysed in immunoprecipitation buffer as described in the Materials and Methods section. Immunoprecipitations were carried out on 1 ml aliquots of the lysate with a non-immune rabbit serum (lane 2), antisera raised against the recombinant protein (AS10, lane 3) or a synthetic peptide of MAD3 (AS9, lane 4) and affinity-purified antibodies to the same peptide, Ab 6–20, that had been either preincubated for 2 h with excess control peptide (lane 6) or not (lane 7). Samples of the lysate (100 μ l, lanes 1 and 5), immunoprecipitates (lanes 2–4, 6 and 7) and 2.5 ng of baculovirally-expressed recombinant MAD3 (lane 8) were subjected to SDS/PAGE and Western blotting with either Ab 6–20, (lanes 1–4) or AS10 (lanes 5–8). Strips of autoradiographs containing proteins in the 50–25 kDa range are shown. The molecular mass markers ovalbumin (45 kDa) and carbonic anhydrase (29 kDa) are indicated. The positions of the 38 kDa antigen and of the recombinant MAD3 are indicated by arrows and an arrowhead respectively.

Figure 2 Mono Q anion-exchange chromatography of HeLa cell I κ B

Cytoplasmic extracts of resting HeLa cells were treated with 0.6% DOC, chromatographed on DNA-cellulose and applied to a Mono Q column as described in the Materials and Methods section. The column was eluted with a gradient of 0.05–1 M KCl and 1 ml fractions were collected. (a) I κ B activity assay: samples of Mono Q fractions were incubated for 5 min with a standard amount of nuclear extract containing NF- κ B. The mixtures were subjected to electrophoretic mobility shift assay as described in the Materials and Methods section and the gels were then autoradiographed. Strips of the autoradiographs containing the probe–NF- κ B complexes are shown. Fraction numbers are indicated on top. (b) Fractions 14–18 were Western blotted with AS9 as described in the Materials and Methods section. The positions of molecular mass markers (kDa) are shown on the left and the arrow indicates that of the 38 kDa antigen. Fraction numbers are indicated. (c) As for (b) except that AS10 was used.

Figure 3 Neutralization of HeLa cell I κ B activity by the antiserum to MAD3, AS10

Recombinant MAD3 (1 ng, lanes 2 and 3), DNA-cellulose flow-through fraction of cytosol (5 μ l, lanes 4 and 5) or Mono Q fractions 16 (lanes 7–10) and 27 (lanes 11–14) of the chromatogram shown in Figure 2 were incubated for 16 h at 4 $^{\circ}$ C with 5 μ l of either antiserum AS10 or pre-immune serum, as indicated. Controls without serum and I κ B-containing samples were also carried out (lanes 1 and 6). The mixtures were then assayed for I κ B activity by incubation with a standard amount of NF- κ B-containing nuclear extract followed by electrophoresis as in Figure 2a. The probe–NF- κ B complexes are indicated by arrows.

(arrow). The 38 kDa antigen was precipitated by AS10, AS9 and Ab 6–20 (lanes 3, 4 and 7), but not by pre-immune serum to AS10 (lane 2) or by Ab 6–20 that had been pre-incubated with excess peptide antigen (lane 6). Its molecular mass corresponded to that reported for MAD3 [13] and Western blots of two-dimensional electrophoresis gels showed that it had the same isoelectric point as the recombinant protein (results not shown). The difference in molecular mass between the 38 kDa antigen and the recombinant MAD3 (Figure 1, lanes 7 and 8) was that

expected from the extra seven amino acids of the latter [13]. The 38 kDa protein was thus identified as MAD3. Quantifications of Western blots in which recombinant protein was used as a standard showed that about 10 ng of MAD3 was immunoprecipitated under our conditions (results not shown).

In order to assess the contribution of MAD3 to the cellular I κ B activity, the distributions of activity and antigen in anion-exchange chromatography fractions of NF- κ B-depleted cytosols were compared (Figure 2). Cytosols of unstimulated cells were

Table 1 Reduction of cytosolic I κ B activity of HeLa cells by IL1 or PMA

Cells were left resting or were stimulated with IL1 (10 ng/ml, 15 min) or PMA (100 ng/ml, 1 h). DNA-cellulose flow-through fractions of DOC-treated cytosols were then prepared and samples were incubated with a standard amount of NF- κ B as in Figure 2(a). Control incubations without samples were also carried out. The mixtures were then subjected to electrophoretic mobility shift assays. The probe–NF- κ B complexes were located by autoradiography and their amounts were quantified by Cerenkov counting. The experiments were done with probes of different specific activities. I κ B activity (column 5) was calculated as the percentage reduction in the amount of probe–NF- κ B complex formed in the presence of sample (column 4), relative to the amount formed without sample (column 2). The reduction in I κ B activity in fractions from stimulated cells was then expressed as the percentage reduction relative to the activity in fractions from resting cells (column 6).

Experiment	NF- κ B activity without sample (c.p.m.)	Stimulant	NF- κ B activity with sample (c.p.m.)	Inhibition of NF- κ B by cytosolic fraction (%)	Reduction in I κ B activity by stimulant (%)
1	2320	IL1	1759	24	47
		None	1270	45	
2	919	IL1	632	31	48
		None	366	60	
3	4630	IL1	3634	22	58
		None	2201	52	
4	1846	IL1	913	50	27
		None	572	69	
5	1549	PMA	882	43	30
		None	592	62	
6	4630	PMA	3694	20	68
		None	1694	63	
7	1846	PMA	880	52	32
		None	439	76	

treated with 0.6 % DOC to dissociate I κ B from cytosolic NF- κ B and applied to a DNA cellulose column to retain the transcription factor. The NF- κ B-depleted flow-through was then chromatographed on Mono Q. Fractions were screened for I κ B activity by incubating them with a standard nuclear extract containing NF- κ B and subjecting the mixtures to electrophoretic mobility shift assay. The resulting autoradiograph (Figure 2a) shows that two regions of the chromatogram contained factors that interfered with the formation of the probe–NF- κ B complex. The first component eluted at around 0.4 M KCl (Figure 2a, fractions 16–18), the second at 0.85 M KCl (Figure 2a, fraction 27 and 28). Quantifications showed that they accounted for about 75 and 25 % of the total I κ B activity of the chromatogram respectively. Western blotting with antisera AS9 and AS10 showed that MAD3 co-eluted with the major I κ B detected by the electrophoretic mobility shift assay (Figures 2b and 2c, fraction 16). No antigen was detected by Western blotting with either antiserum in the minor I κ B peak, fractions 27 and 28 (results not shown).

Incubation of recombinant MAD3 with the antiserum to it, AS10, prevented the protein from inhibiting formation of the probe–NF- κ B complex, whereas the corresponding pre-immune serum had no effect (Figure 3, lanes 1–3). This indicated that AS10 was specifically neutralizing MAD3, and we used this property to check whether or not the 38 kDa antigen had I κ B activity (Figure 3). AS10, but not the pre-immune serum, strongly inhibited the I κ B activities of the flow-through fraction from the DNA column (lane 5) and of Mono Q fractions 16 (lanes 8 and 10), thus increasing binding of NF- κ B to the probe. By contrast, the minor I κ B peak (Mono Q fraction 27) was only partially inhibited by AS10 (lanes 12 and 14) relative to controls with the non-immune serum (lanes 11 and 13). MAD3 was thus responsible for the major form of I κ B of the Mono Q chromatogram (fraction 16) and the later-eluting, minor form of I κ B

(fraction 27) was also immunologically related to it. It was concluded that most (75 %) of the I κ B activity in the cell extracts was due to MAD3.

Reduction of I κ B activity in the cytosol of HeLa cells after stimulation by IL1 or PMA

The I κ B activity of the flow-through fractions of unstimulated and stimulated cells was measured in seven different experiments (Table 1), four with IL1 and three with PMA. Stimulation times of 15 min for IL1 and 60 min for PMA were chosen because in time-course experiments they were found to coincide with maximal activation of NF- κ B (results not shown). Table 1 shows that IL1 or PMA partially reduced the I κ B activity of the extracts in all experiments, the reduction ranging from 27 to 68 % and being on average 46 % for IL1 and 43 % for PMA. The two stimulants thus caused quantitatively similar down-regulation of I κ B activity.

IL1-induced phosphorylation and degradation of MAD3

Stimulation of HeLa cells by IL1 caused disappearance of MAD3, which started 6 min after stimulation and was complete between 15 and 45 min after stimulation (Figure 4a). The antigen then gradually reappeared and returned to pre-stimulation amounts between 60 and 90 min after stimulation. When the antiserum to the protein AS10 was used instead of AS9, the same loss of antigen was observed and no truncated form of the protein was detected (results not shown). This indicated that MAD3 was being degraded rather than undergoing a modification preventing its recognition by antibodies to its N-terminus.

Interestingly, the onset of the degradation of MAD3 coincided with the generation of a form of the antigen with reduced electrophoretic mobility (Figure 4a, 6 and 10 min). This modified form was also detected when the antigen reappeared in cells 60 min after stimulation, but was no longer detectable when it had returned to pre-stimulation levels (90 min). The reduction of mobility indicated that IL1 was causing a modification of MAD3, possibly a phosphorylation, over a time-course that matched that of the degradation of the protein.

Attempts to detect the modified form of MAD3 by immunoprecipitation from cells metabolically labelled with 2 mCi/well of [32 P]orthophosphoric acid were unsuccessful. No labelled protein was specifically precipitated by AS9 or AS10 from resting or stimulated cells (results not shown). However, the slower migrating modified form of MAD3, detected by Western blot, was converted into the unmodified form of the protein by the phosphatase PP2A (Figure 4b, lane 2). This could be prevented by okadaic acid (lane 3), showing that the reduction in mobility corresponded to a phosphorylation. Incubations with the phosphotyrosine phosphatase, PTP-1B (lane 4), or with 0.1 M HCl (Figure 4c, lane 2) had no effect. PP2A is known generally as a phosphoserine/phosphothreonine phosphatase, but also dephosphorylates the acid-labile phosphoamino acid, phosphohistidine [29]. The stability of the phosphorylation of MAD3 in acid indicated that it was on either a serine or a threonine residue. Failure to detect it as a 32 P-labelled protein was thus not attributable to the possible degradation of an acid-labile phosphorylation during gel staining.

Isocoumarin and chloromethylketone proteinase inhibitors prevent cytokine and PMA signalling: effects on hsp27 phosphorylation

Certain inhibitors of serine proteinases have recently been reported to inhibit the activation of NF- κ B by TNF or PMA by

Figure 4 IL1-induced phosphorylation and breakdown of MAD3

(a) Cells grown in wells were stimulated with 20 ng/ml IL1 for the indicated times, then lysed. Immunoprecipitation and Western blotting were both carried out with Ab 6–20 as in Figure 1. Strips of autoradiographs containing the antigen are shown. (b) HeLa cells grown in two flasks were stimulated for 6 min with 20 ng/ml IL1 and lysed. MAD3 was immunoprecipitated with Ab 6–20 as before and the immunoprecipitate was then washed a further four times in phosphatase buffer [20 mM Tris/HCl, pH 7.5/0.1 mM EGTA/20% (v/v) glycerol] at 4 °C. It was finally resuspended in phosphatase buffer supplemented with 0.1% 2-mercaptoethanol and split into five aliquots. One was left on ice (lane 1), the others were incubated at 30 °C for 30 min with 40 units/ml PP2A (lane 2), 40 units/ml PP2A and 50 μM okadaic acid (lane 3), 100 μg/ml PTP-1B (lane 4) or 100 μg/ml PTP-1B and 2 mM sodium orthovanadate (lane 5) with occasional resuspension of the protein A–agarose beads (every 5 min). Electrophoresis and Western blotting were then carried out as in (a). (c) An immunoprecipitate was prepared from one flask of stimulated cells as in (a), then further washed in water once and split into two aliquots. One sample was resuspended in 30 μl of water (lane 1), the other in the same volume of a solution of 0.1 M HCl (lane 2). The samples were then incubated at room temperature for 4 h. The HCl-treated sample was neutralized by the addition of 100 mM NaOH prior to electrophoresis.

Figure 5 Effects of serine proteinase inhibitors on the IL1-induced phosphorylation and loss of MAD3

(a) HeLa cells grown in wells were treated for 30 min with DCI added as 100-fold concentrated solutions in dimethyl sulphoxide, or with vehicle. The cells were then left resting or stimulated with 20 ng/ml IL1 for 8 or 15 min. Lysis, immunoprecipitation and Western blotting were carried out as in Figure 4(a). A strip of the autoradiograph containing the antigen is shown. The final concentrations of inhibitor and stimulation times are indicated. (b) As for (a) except that TPCK was used.

preventing proteolysis of IκB [6,19]. It was possible that they might cause accumulation of the phosphorylated MAD3 and facilitate its labelling with ^{32}P . Figure 5a shows that DCI inhibited the loss of MAD3 induced by IL1 in HeLa cells. Partial inhibition was observed in the presence of 10 and 30 μM DCI, complete inhibition required 100 μM. This concentration also prevented IL1 inducing NF-κB (results not shown). Interestingly, the inhibitor did not cause accumulation of the phosphorylated form of MAD3, which might have been expected if it were specifically blocking breakdown of the protein. Instead, DCI prevented the IL1-induced phosphorylation of the antigen at doses that blocked its breakdown (Figure 5a). TPCK had similar inhibitory effects on IL1-induced phosphorylation and disappearance of MAD3 (Figure 5b); 20 μM caused partial inhibition of the effect of IL1

and 50 μM caused complete inhibition. These results indicate that the serine proteinase inhibitors were preventing phosphorylation of MAD3. It was possible that they were specifically blocking a proteolytic process that was part of the signalling mechanism leading to MAD3 phosphorylation, or that they were interfering non-specifically with protein kinases.

IL1, TNF and PMA are known to induce rapid changes in phosphorylation of other cellular proteins, one of which is hsp27. In order to check the integrity of signalling mechanisms, the effect of the proteinase inhibitors upon changes in hsp27 phosphorylation was investigated. HeLa cells were metabolically labelled with [^{32}P]orthophosphoric acid, then pretreated with increasing concentrations of DCI or TPCK before stimulation with IL1 or PMA. Hsp27 was then immunoprecipitated.

Figure 6 Effects of DCI and TPCK on IL1- and PMA-induced phosphorylation of hsp27

(a) HeLa cells were labelled with [32 P]orthophosphoric acid as described in the Materials and Methods section and DCI or vehicle were then added to the labelling medium, as in Figure 5(a), 45 min before cell lysis. Stimulations by PMA (100 ng/ml) were started immediately after adding the inhibitor or vehicle, those with IL1 (20 ng/ml) 30 min later. Cell lysis, immunoprecipitation of hsp27 and electrophoresis were carried out as described in the Materials and Methods section. Strips of autoradiographs of the gels containing hsp27 are shown. The final concentrations of inhibitors and the stimulations by IL1 (i) and PMA (p), or controls without stimulants (c), are indicated. (b) As for (a) except that TPCK was used.

IL1 strongly increased the phosphorylation of hsp27 in untreated cells (Figure 6a and b, lanes 1 and 2). Pretreatment with DCI for 30 min perturbed this effect. At 10 μ M DCI the basal phosphorylation was slightly increased but the response to IL1 was not impaired (this concentration partially inhibited the IL1-induced phosphorylation of MAD3 and slowed down its breakdown, Figure 5a). At 100 μ M DCI, the unstimulated cells showed strongly increased hsp27 phosphorylation and there was no increase in this with IL1 stimulation (this concentration blocked phosphorylation and breakdown of MAD3; Figure 5a). At 200 μ M DCI, hsp27 phosphorylation was similar to that seen in untreated, unstimulated cells, and stimulation with IL1 caused no increase. TPCK also perturbed the IL1-induced hsp27 phosphorylation over a similar concentration range (Figure 6b).

PMA increased hsp27 phosphorylation, although to a lesser extent than IL1, and this too was perturbed by DCI (Figure 6a) or TPCK (Figure 6b). Thus, both proteinase inhibitors interfered with the basal, IL1- and PMA-induced phosphorylations of hsp27 over precisely the concentration range at which they inhibited the IL1-induced breakdown of MAD3. Moreover, when extracts of labelled cells were directly loaded on gels, the inhibitors were found to cause changes in the pattern of basal phosphorylation of several proteins of resting cells (results not shown).

The prevention of MAD3 phosphorylation by the proteinase inhibitors, together with their interference with protein kinases involved in constitutive phosphorylations and signal transduction, strongly suggested that they were preventing MAD3 breakdown by interfering with IL1 signalling rather than blocking proteolysis.

MAD3 is not a substrate for protein kinase C

Protein kinase C has been reported to phosphorylate and inactivate I κ B *in vitro* [5,11,17], and there is evidence that some forms of the kinase are translocated to the plasma membrane upon stimulation by IL1 [30]. We therefore studied the ability of this enzyme to phosphorylate the recombinant MAD3. The protein was incubated with pure, proteolytically activated, protein kinase C (α , β and γ) at a high enzyme:substrate ratio (1:7

Figure 7 Phosphorylation *in vitro* of recombinant MAD3 by protein kinase C

(a) Baculovirus-expressed recombinant MAD3 (5.1 μ g, 130 pmol) was incubated with 0.7 μ g (0.07 units) of proteolytically activated protein kinase C at room temperature for up to 2.5 h in 60 μ l of buffer containing 20 mM Tris (pH 7.5), 8 mM MgCl₂, 0.8 mM ATP and tracer [γ - 32 P]ATP. The specific activity of the ATP was 180 c.p.m./pmol. Aliquots of the reaction mixture containing 22 pmol of MAD3 (10 μ l) were taken at the indicated times and subjected to SDS/PAGE. The gel was dried and autoradiographed. A strip of the autoradiograph showing the phosphorylated substrate is shown. The migration position of Coomassie-Blue-stained unphosphorylated recombinant MAD3 is indicated. (b) The phosphorylation of MAD3 was quantified by counting Cerenkov radiation of bands excised from the gels. Counts were corrected for background. A line fitted visually to the experimental points is shown.

by weight). Samples of the reaction mixtures were resolved by gel electrophoresis. Autoradiographs showed that protein kinase C phosphorylated MAD3 but did not cause any electrophoretic retardation of the protein (Figure 7a). Quantification of the 32 P incorporated in the protein showed that the reaction was very slow. MAD3 phosphorylation increased linearly during the 2.5 h incubation, and no reduction in the rate of this reaction was observed, suggesting that it was not approaching completion. After 2.5 h, 1.5 pmol of phosphate (262 c.p.m.) had been incorporated onto 22 pmol of substrate, which corresponded to phosphorylation of only 7% of MAD3 molecules (Figure 7b).

These results strongly suggested that MAD3 was not a physiological substrate of protein kinase C.

DISCUSSION

Before investigating how IL1 regulates the NF- κ B/I κ B system, it was necessary to characterize the types of I κ B in the IL1-responsive cell line being studied.

We found two components with I κ B activity in HeLa cells which could be separated by anion-exchange chromatography. The major one was detected by immunoblotting with two different antisera to MAD3, had a very similar molecular mass to this protein and was strongly inhibited by the antiserum, AS10. The same antiserum largely neutralized the I κ B activity of DOC-treated HeLa cell cytosol. These findings indicated that MAD3/I κ B α is the major I κ B of HeLa cells. The second and minor form of I κ B found in HeLa cells was immunologically related to MAD3. It was much more acidic than MAD3 and is thus unlikely to correspond to the second I κ B isolated from human placenta, I κ B β , which was less acidic than I κ B α [10,11].

IL1 stimulation of HeLa cells caused the appearance of an electrophoretically retarded form of MAD3 which was detected by Western blot. The retarded MAD3 was converted into the native form by the phosphatase PP2A, but not by PTP-1B or low pH. This showed that the change in mobility was due to its phosphorylation on a serine or a threonine residue.

Appearance of the phosphorylated form of MAD3 coincided with the onset of its degradation. Loss of antigen was complete between 15 and 45 min after stimulation by IL1 and this was associated with a 46 % decrease in the I κ B activity detectable in cell extracts. Phosphorylated MAD3 was still detectable when the antigen reappeared 60 min after stimulation, but had disappeared when pre-stimulation amounts of the protein were restored, after 90 min. This strongly suggested that the IL1-induced phosphorylation of MAD3 was a signal for its degradation and that the later accumulation of newly synthesized MAD3 required down-regulation of the phosphorylating activity before the normal resting level could be restored. The signalling mechanisms of IL1 and TNF involve the activation of several protein kinases; three distinct types of MAP kinase and β casein kinase have been shown to be regulated by the cytokines [23,31–34]. The phosphorylation and degradation of MAD3 in IL1-stimulated HeLa cells followed a time-course similar to the IL1-induced activation of kinases in fibroblasts [31,32].

Extensive efforts were made to detect the retarded form of MAD3 as a 32 P-labelled protein by immunoprecipitating it from lysates of labelled cells. Labelling was done with large amounts of [32 P]orthophosphate long enough to ensure equilibration of the ATP pool. However, we were unable to detect any 32 P-labelled MAD3. When hsp27 was used as a basis for comparison, it was calculated that the amount of retarded MAD3 in the immunoprecipitates should have been detectable as a 32 P-labelled protein. A possible explanation of this paradox may be that MAD3 phosphorylation uses a specific pool of phosphate donor whose turn-over rate is less than the labelling times that we used: this could be the case, for instance, if some ATP was complexed to the latent form of the putative IL1-activated MAD3 kinase.

Others have also reported phosphorylation and breakdown of MAD3/I κ B α in cells stimulated by LPS [20], TNF [6,7,9,18,35] or IL1 [18]. The phosphorylated MAD3 was detected in most cases as an electrophoretically retarded form of the protein by Western blotting or by immunoprecipitation from [35 S]methionine-labelled cells, and treatment with calf intestinal phosphatase was needed to demonstrate that it corresponded to a phosphorylated form of the protein [7,9,18,20,35]. There is only one

report showing stimulant-induced incorporation of 32 P into MAD3/I κ B α [6]. This suggests that labelling the phosphorylated MAD3 with 32 P was generally not possible. It may be the reason why the nature of the phosphorylated residues has not been determined before and may hinder identification of the phosphorylation site(s) of MAD3.

In agreement with the work of Henkel *et al.* [19] and Mellits *et al.* [6], we found that the disappearance of MAD3 from stimulated cells was blocked by the proteinase inhibitors DCI or TPCK. However, we also found that the inhibitors prevented the IL1-induced phosphorylation of MAD3, and they interfered with the basal, IL1- and PMA-induced phosphorylations of hsp27 over the same concentration range at which they inhibited MAD3 breakdown. The phosphorylation state of hsp27 is regulated by a wide range of extracellular stimuli [27], and it is a simple indicator both of cytokine signal transduction and of PMA action. The serine proteinase inhibitors probably affected different kinases (and possibly phosphatases) depending upon the concentration at which they were used. Because of their effects on kinases and signalling, it was not possible to conclude that DCI and TPCK were inhibiting MAD3 breakdown by blocking the proteinase(s) involved. However, recently published experiments with new proteinase inhibitors suggest that the proteasome is responsible for the degradation of MAD3 in TNF-stimulated cells [35].

Some forms of I κ B may be phosphorylated and activated by protein kinase C [5,17]. We were unable to phosphorylate more than 7 % of MAD3 with a large amount of this kinase, and the electrophoretic mobility of the resulting phospho-form was unaltered. We concluded that it is not a physiological substrate for the enzyme. This is consistent with experiments in which activation of NF- κ B by IL-1, TNF or LPS was not impaired in cells either pretreated with inhibitors of protein kinase C [2,36,37] or depleted of the kinase by long-term exposure to PMA [20,37]. Raf, a kinase which can be activated by protein kinase C [38], has been proposed as a regulator of I κ B [39,40]. It is not known whether IL1 activates Raf. IL1 could cause phosphorylation of MAD3 either by activating a kinase or by inhibiting a phosphatase.

In conclusion, IL1 induces phosphorylation of MAD3 on serine or threonine residues and this may label the protein for rapid breakdown.

This work was supported by grants from the Arthritis and Rheumatism Council and the Medical Research Council of the United Kingdom. We are grateful to Dr. N. Freshney, of this laboratory, for the antiserum to hsp27, Dr M. Ladner (Chiron Corporation, Emeryville, CA, U.S.A.) for the recombinant MAD3, Dr. D. Barford (Department of Molecular Biophysics, University of Oxford, U.K.) for PTP-1B and Dr. P. Parker (ICRF, London, U.K.) for bovine brain protein kinase C. We also thank Christopher Green and Judith Webdell (Strangeways Research Laboratory, Cambridge, U.K.) for the photographic work.

REFERENCES

- Osborn, L., Kunkel, S. and Nabel, G. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2336–2340.
- Stylianou, E., O'Neill, L. A. J., Rawlinson, L., Edbrooke, M. R., Woo, P. and Saklatvala, J. (1992) *J. Biol. Chem.* **267**, 15836–15841.
- Baeuerle, P. A. (1991) *Biochem. Biophys. Acta* **1072**, 63–80.
- Baeuerle, P. A. and Baltimore, D. (1988) *Science* **242**, 540–546.
- Kerr, L. D., Inoue, J.-i., Davis, N., Link, E., Baeuerle, P. A., Bose, H. R. and Verma, I. M. (1991) *Genes Dev.* **5**, 1464–1476.
- Mellits, K. H., Hay, R. T. and Goodbourn, S. (1993) *Nucleic Acids Res.* **21**, 5059–5066.
- Brown, K., Park, S., Kanno, T., Franzoso, G. and Siebenlist, U. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2532–2536.
- Sun, S.-C., Ganchi, A., Ballard, D. W. and Greene, W. C. (1993) *Science* **259**, 1912–1915.

- 9 Sun, S.-C., Ganchi, P. A., Béraud, C., Ballard, D. W. and Greene, W. C. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1346–1350
- 10 Zabel, U. and Baeuerle, P. A. (1990) *Cell* **61**, 255–265
- 11 Link, E., Kerr, L. D., Schreck, R., Zabel, U., Verma, I. and Baeuerle, P. A. (1992) *J. Biol. Chem.* **267**, 239–246
- 12 Davis, N., Ghosh, S., Simmons, D. L., Tempst, P., Liou, H.-C., Baltimore, D. and Bose, H. R. (1991) *Science* **253**, 1268–1271
- 13 Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P. and Baldwin, A. S. (1991) *Cell* **65**, 1281–1289
- 14 Franzoso, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K. and Siebenlist, U. (1992) *Nature (London)* **359**, 339–342
- 15 Rice, N. R., MacKichan, M. L. and Israel, A. (1992) *Cell* **71**, 243–253
- 16 Inoue, J.-I., Kerr, L. D., Kakizuka, A. and Verma, I. M. (1992) *Cell* **68**, 1109–1120
- 17 Ghosh, S. and Baltimore, D. (1990) *Nature (London)* **344**, 678–682
- 18 Beg, A., Finco, T. S., Nantermet, P. V. and Baldwin, A. S. (1993) *Mol. Cell Biol.* **13**, 3301–3310
- 19 Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y. and Baeuerle, P. A. (1993) *Nature (London)* **365**, 182–185
- 20 Cordle, S. R., Donald, R., Read, M. A. and Hawiger, J. (1993) *J. Biol. Chem.* **268**, 11803–11810
- 21 Bird, T. A. and Saklatvala, J. (1990) *J. Biol. Chem.* **265**, 235–240
- 22 Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Stralfors, P. and Tung, H. Y. L. (1988) *Methods Enzymol.* **159**, 390–408
- 23 Guesdon, F., Freshney, N., Waller, R. J., Rawlinson, L. and Saklatvala, J. (1993) *J. Biol. Chem.* **268**, 4236–4243
- 24 Marais, R. M. and Parker, P. J. (1989) *Eur. J. Biochem.* **183**, 129–137
- 25 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
- 26 Smeal, T., Angel, P., Meek, J. and Karin, M. (1989) *Genes Dev.* **3**, 2091–2100
- 27 Saklatvala, J., Kaur, P. and Guesdon, F. (1991) *Biochem. J.* **277**, 635–642
- 28 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 29 Kim, Y., Huang, J., Cohen, P. and Matthews, H. R. (1993) *J. Biol. Chem.* **268**, 18513–18518
- 30 Kracht, M., Heiner, A., Resch, K. and Szamel, M. (1993) *J. Biol. Chem.* **268**, 21066–21072
- 31 Guesdon, F., Waller, R. J. and Saklatvala, J. (1994) *Biochem. J.* **304**, 761–768
- 32 Saklatvala, J., Rawlinson, L., Marshall, C. J. and Kracht, M. (1993) *FEBS Lett.* **334**, 189–192
- 33 Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J. and Saklatvala, J. (1994) *Cell* **78**, 1039–1049
- 34 Kracht, M., Truong, O., Totty, N. F., Shiroo, M. and Saklatvala, J. (1994) *J. Exp. Med.* **180**, 2017–2025
- 35 Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T. (1994) *Cell* **78**, 773–775
- 36 Montgomery, K. F., Osborn, L., Hession, C., Tizard, R., Goff, D., Vassalla, C., Tarr, P. I., Bomsztyk, K., Lobb, R., Harlan, J. M. and Pohlman, T. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6523–6527
- 37 Bomsztyk, K., Rooney, J. W., Iwasaki, T., Rachie, N. A., Dower, S. K. and Sibley, C. H. (1991) *Cell Regul.* **2**, 329–335
- 38 Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U. R. (1993) *Nature (London)* **364**, 249–25.
- 39 Li, S. and Sedivy, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9247–9251
- 40 Finco, T. S. and Baldwin, A. S. (1993) *J. Biol. Chem.* **268**, 17676–17679