

Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs

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Lipopolysaccharide (LPS) stimulates production of inflammatory mediators, partly by stabilizing [interleukin-6 (IL-6), cyclooxygenase 2 (COX-2)] and/or stimulating translation [tumour necrosis factor- α (TNF- α)] of their mRNAs. Such regulation depends on AU-rich elements (AREs) within the 3'-untranslated regions and is partially suppressed by SB 203580 (which inhibits SAPK2a/p38). The LPS-induced production of TNF- α and IL-6 is suppressed in MAPKAP-K2-deficient mice (a kinase activated by SAPK2a/p38). Here, we identify 18 macrophage proteins that bind to AREs and show that hnRNP A0 is a major substrate for MAPKAP-K2 in this fraction. MAPKAP-K2 phosphorylated hnRNP A0 at Ser84 *in vitro* and this residue became phosphorylated in LPS-stimulated cells. Phosphorylation was prevented by SB 203580 and suppressed in macrophages derived from MAPKAP-K2-deficient mice. The mRNAs encoding TNF- α , COX-2 and macrophage inflammatory protein-2 (MIP-2) bound to hnRNP A0 in LPS-stimulated macrophages, an interaction prevented by SB 203580. The LPS-induced stabilization of MIP-2 mRNA and production of MIP-2 protein were abolished when macrophages were incubated with SB 203580 plus PD 184352 (which inhibits the classical MAP kinase cascade). Our data suggest that LPS-induced binding of hnRNP A0 to AREs may contribute to the post-transcriptional regulation of specific mRNAs.

Keywords: AU-rich element/hnRNP/LPS/MAPKAP-K2/
p38 MAP kinase

Introduction

Inflammation, a key process in the host defence system, is highly regulated in order to confine its action to the time and place where it is needed. Loss of control can lead to a number of diseases, including rheumatoid arthritis, neurodegenerative disorders and septic shock syndrome. Important mediators of inflammation are pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- α , interleukin-6 (IL-6) and IL-8. The expression of these genes is regulated at both the transcriptional and post-

transcriptional level. Thus, understanding how the expression of these proteins is controlled could be of great importance in overcoming the pathological effects of uncontrolled inflammation.

The presence of AU-rich sequences in the 3'-untranslated region (3'-UTR) of a number of inflammatory mediators and immediate early genes is critical for their post-transcriptional regulation (Grzybowska *et al.*, 2001). These sequences are formed by the presence of one or more copies of the pentamer AUUUA (Xu *et al.*, 1997), and bind to proteins involved in stabilizing or destabilizing these mRNAs. In the case of TNF- α , the AU-rich element (ARE) is known to be involved in repressing the translation of its mRNA (Kontoyiannis *et al.*, 1999), while in the case of IL-6 and cyclooxygenase-2 (COX-2), it is critical for mRNA stability (Ridley *et al.*, 1998; Winzen *et al.*, 1999).

A number of proteins have been identified that bind to the TNF- α ARE. These include TIA-1 (Gueydan *et al.*, 1999; Piecyk *et al.*, 2000) and tristetraprolin (TTP) (Lai *et al.*, 1999), which are thought to be negative regulators of TNF- α synthesis. Thus, in response to inflammatory stimuli that activate macrophages, such as lipopolysaccharide (LPS), intracellular signalling pathways are activated that carry the signal needed to activate inflammatory mediator production. LPS, acting via the toll-like receptor 4 (TLR4), stimulates several mitogen-activated protein (MAP) kinase cascades, as well as the pathway leading to activation of the transcription factor NF- κ B (Lee *et al.*, 2000; Zhu *et al.*, 2000; Perera *et al.*, 2001). A role for some of these pathways in the post-transcriptional regulation of TNF- α has already been reported. For example, the classical MAP kinase cascade appears to be involved in the nucleo-cytoplasmic transport of TNF- α mRNA via its ARE (Dumitru *et al.*, 2000). The stress-activated protein kinase 2a (SAPK2a, also called p38) pathway also plays a key role in stimulating the translation of TNF- α , because SB 203580 and closely related pyridinyl imidazoles, which inhibit this protein kinase, suppress the synthesis of this cytokine (Lee *et al.*, 1994). The effect of SAPK2a/p38 on TNF- α synthesis seems to be mediated via the activation of its downstream target MAPKAP-K2, because the LPS-induced production of TNF- α *in vivo* is decreased by 90% in mice that do not express MAPKAP-K2 (Kotlyarov *et al.*, 1999). The production of IL-6 and interferon- γ is reduced similarly in MAPKAP-K2-deficient mice, although the effect on IL-6 appears to result from an increase in mRNA stability (Neininger *et al.*, 2002). The stability of the mRNA encoding COX-2 is also decreased by SB 203580 (Ridley *et al.*, 1998) and may involve MAPKAP-K2 (Lasa *et al.*, 2000).

The mechanism by which MAPKAP-K2 controls the translation of TNF- α mRNA and the stability of the mRNA encoding IL-6 is unknown, but is dependent on the

Table I. Identification of ARE-binding proteins isolated from murine RAW macrophages

Name of protein matched	Protein accession No.	Mass of protein (kDa)	% of tryptic peptides matched	Species matched to	Sequence coverage of peptides (%)
hnRNP M	1710636	77.5	33	Human	43
FUSE BP2	4504865	73.1	59	Human	32
p68 RNA helicase	6681157	69.3	13	Mouse	16
FUSE BP	4503801	67.5	45	Human	30
FUSE BP3	1575609	63.9	43	Human	27
Hypothetical protein 55 kDa	7657015	55.2	43	Human	32
Nuclear matrix protein 55	1895081	54.2	45	Human	42
DAZAPI	8895708	43.1	15	Mouse	17
TIAR	6678349	43.3	56	Mouse	42
TIA-1	14714709	41.5	27	Mouse	26
RBM7	12837596	30.1	16	Mouse	29
hnRNP D	870749	38.4	25	Human	26
AU-rich RNA-binding protein 1	6671602	29.9	25	Mouse	32
hnRNPA3	1710627	39.7	20	Human	25
hnRNPA2/B1	7949053	36.0	37	Mouse	56
hnRNPA1	4504445	34.2	32	Mouse	54
CBF-A (hnRNP A/B)	6754222	30.9	18	Mouse	35
hnRNP A0	12859567	30.5	36	Mouse	24

The masses of tryptic peptides were scanned against the Swiss-Prot, Genpep and NCBItr databases as described in Materials and methods.

presence of the ARE (Kontoyiannis *et al.*, 1999; Neining *et al.*, 2002) and on the catalytic activity of MAPKAP-K2 (Kotlyarov *et al.*, 2002). A working hypothesis is that the mechanism involves the phosphorylation of one or more proteins that bind to the ARE. For this reason, we decided to identify the major proteins that bind to the TNF- α ARE and to investigate whether they were substrates for MAPKAP-K2. This study led us to focus on the heterogeneous nuclear ribonucleoproteins (hnRNPs). Here, we identify hnRNP A0 as a novel physiological substrate for MAPKAP-K2 and implicate this protein in the LPS-induced post-transcriptional regulation of TNF- α , COX-2 and macrophage inflammatory protein 2 (MIP-2).

Results

Identification of proteins that bind to the AU-rich element of the TNF- α mRNA

Extracts from RAW cells, a murine macrophage-like cell line, were subjected to affinity chromatography on magnetic beads coated with streptavidin to which biotinylated RNA oligonucleotides corresponding to the longer ARE of the TNF- α mRNA had been bound (see Materials and methods). Proteins that bound specifically were resolved on two-dimensional gels and stained with Sypro-Ruby (Figure 1). Each of the spots with pI values between 6 and 11 were excised, digested with trypsin and subjected to tryptic mass fingerprinting. Those proteins that could be identified unequivocally are indicated (Table I; Figure 1). Proteins that could not be identified are unmarked. Many of the spots appearing as clusters with the same apparent mass but different net charge turned out to be different electrophoretic forms of the same protein and probably arise by post-translational modifications. Similarly, several spots with different molecular masses were identified as the same protein, presumably arising by alternative splicing of the same gene. Thus, the 60 major spots identified were accounted for by some 20 proteins. These included a few proteins already known to bind to AREs,

such as TIA-1 (Gueydan *et al.*, 1999), which is reported to be a negative regulator of TNF- α translation (Piecnyk *et al.*, 2000), hnRNP A0 (Myer and Steitz, 1995), hnRNP A1 (Hamilton *et al.*, 1993) and hnRNP D (Loflin *et al.*, 1999).

The specificity of interaction of these proteins with the ARE was demonstrated by their failure to bind to a control RNA oligonucleotide of the same length that did not contain an ARE (data not shown). The pattern of proteins binding to the TNF- α ARE in LPS-stimulated and untreated RAW cells was similar (data not shown).

Phosphorylation of ARE-binding proteins by MAPKAP-K2

The proteins binding to the TNF- α ARE were incubated with Mg-[γ - 32 P]ATP in the absence or presence of MAPKAP-K2, and subjected to SDS-PAGE and autoradiography. These experiments revealed that two major bands were phosphorylated by MAPKAP-K2 with apparent M_r s of 38 and 36 kDa, together with two minor 32 P-labelled bands of apparent M_r s 80 and 45 kDa (Figure 2). The 38 kDa 32 P-labelled band was excised from the one-dimensional gel and identified as hnRNP A0 by tryptic mass fingerprinting. It also co-migrated with hnRNP A0 on two-dimensional gels (data not shown). Preliminary results suggest that the 36 kDa substrate is RNA-binding motif 7 protein (RBM7), but further experiments are needed to confirm this. The 90 kDa protein phosphorylated weakly by MAPKAP-K2 (Figure 2) has not yet been identified.

The human form of hnRNP A0 was cloned and expressed as a GST fusion protein. This protein could be phosphorylated to 0.7 mol/mol by incubation for 60 min with 150 U/ml MAPKAP-K2. The initial rate of phosphorylation was 3–5 times slower than that of heat shock protein 27 (HSP27), an established physiological substrate of MAPKAP-K2 (Eyers *et al.*, 1999; Kotlyarov *et al.*, 1999). Digestion with trypsin, followed by chromatography on a C₁₈ column showed one major 32 P-labelled peptide (Figure 3A). The mass of the peptide

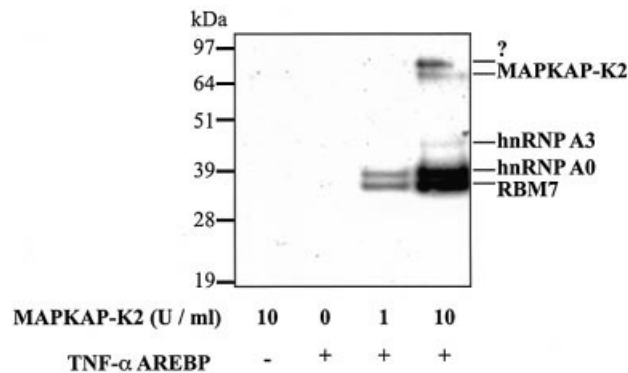


Fig. 2. MAPKAP-K2 phosphorylates two major proteins binding to the TNF- α ARE. Untreated RAW cell extracts were incubated with a biotinylated RNA oligonucleotide corresponding to the TNF- α ARE. RNA-binding proteins were then phosphorylated in the absence or presence of 1 or 10 U/ml of MAPKAP-K2 and 20 nM [γ - 32 P]ATP (lanes 1–4). Proteins were then separated by SDS-PAGE, transferred to nitrocellulose membranes and autoradiographed.

The mRNA encoding macrophage inflammatory protein 2 is bound by hnRNP A0

The finding that hnRNP A0 was a physiological substrate for MAPKAP-K2 raised the question of whether its phosphorylation played an important role in LPS-stimulated cytokine production. In order to investigate whether this was the case, we immunoprecipitated hnRNP A0 from RAW cell extracts and examined which mRNAs were bound to this protein (see Materials and methods). The method used was verified by the demonstration that TNF- α mRNA could be identified after immunoprecipitation of TIA-1 (data not shown), a protein known to bind to the ARE of the TNF- α mRNA (Piecyk *et al.*, 2000). Using the same conditions, RNA bound to hnRNP A0 immunoprecipitates was amplified using random primer set 1 (see Materials and methods). This led to the amplification of one band that was present in the hnRNP A0 immunoprecipitated from extracts prepared from LPS-stimulated cells, but not from unstimulated cells or a control immunoprecipitation performed in the absence of the hnRNP A0 antibody (Figure 5A). This band was cut out, re-amplified, cloned and sequenced to determine its identity. The region retrieved was the 3'-UTR of MIP-2, which contains an ARE not dissimilar to that of TNF- α mRNA (Figure 5B). MIP-2 is a member of the CXC chemokine family and is involved in the recruitment of neutrophils to the site of inflammation (Nick *et al.*, 2000).

hnRNP A0 interaction with specific cytokine mRNAs is dependent on the SAPK2a/p38 pathway

The identification of MIP-2 mRNA in hnRNP A0 immunoprecipitates was confirmed in a separate experiment using primers specific for the coding sequence of MIP-2 (Figure 6, lane 3). These experiments confirmed that this interaction only occurred in LPS-stimulated RAW cells and that it was prevented by SB 203580, but not by PD 184352 (Figure 6).

Since MAPKAP-K2 has been implicated in the LPS-induced post-transcriptional regulation of COX-2, TNF- α and IL-6 (see Introduction), we used specific primers to examine whether any of these mRNAs could be amplified from the hnRNP A0 immunoprecipitates. These experi-

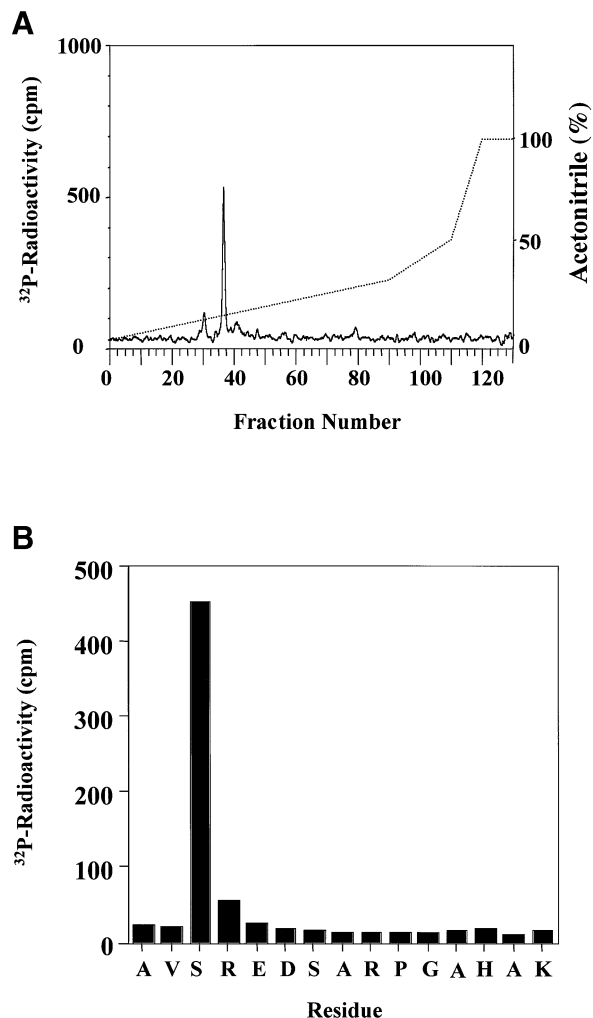


Fig. 3. Mapping of the site in hnRNP A0 phosphorylated by MAPKAP-K2. (A) Purified GST-hnRNP A0 was phosphorylated for 60 min *in vitro* with 150 U/ml of MAPKAP-K2 and 0.1 mM [γ - 32 P]ATP, and subjected to SDS-PAGE. The band corresponding to GST-hnRNP A0 was excised, digested with trypsin and the resulting peptides separated by reverse phase hydrophobic interaction chromatography on a C₁₈ column equilibrated in 0.1% trifluoroacetic acid. 32 P-radioactivity is shown by the full line, and the acetonitrile gradient by the broken line. (B) The mass of the major tryptic phosphopeptide from A ($m/z = 1631.8$) corresponds to that of a mono-phosphorylated form of a tryptic peptide comprising residues 82–96. The sequence of the peptide was confirmed by Edman sequencing and the site of phosphorylation was identified as the first serine in the peptide in a solid phase sequencing experiment. The methodology is detailed elsewhere (Stokoe *et al.*, 1992).

ments revealed that COX-2 mRNA and TNF- α mRNA were also present in the hnRNP A0 immunoprecipitates, and that binding was markedly stimulated by LPS and inhibited by SB 203580. PD 184352 had no effect on the binding of TNF- α mRNA, but moderately decreased the binding of COX-2 mRNA. No IL-6 mRNA could be detected in hnRNP A0 immunoprecipitates even though it could be amplified from the extracts of LPS-stimulated cells in control experiments (Figure 6; data not shown).

The induction of MIP-2 protein and stability of the MIP-2 mRNA are prevented by inhibition of SAPK2a/p38 and the classical MAP kinase cascade

The MIP-2 protein is up-regulated following treatment with LPS (50 ng/ml). Induction can be observed after 1 h

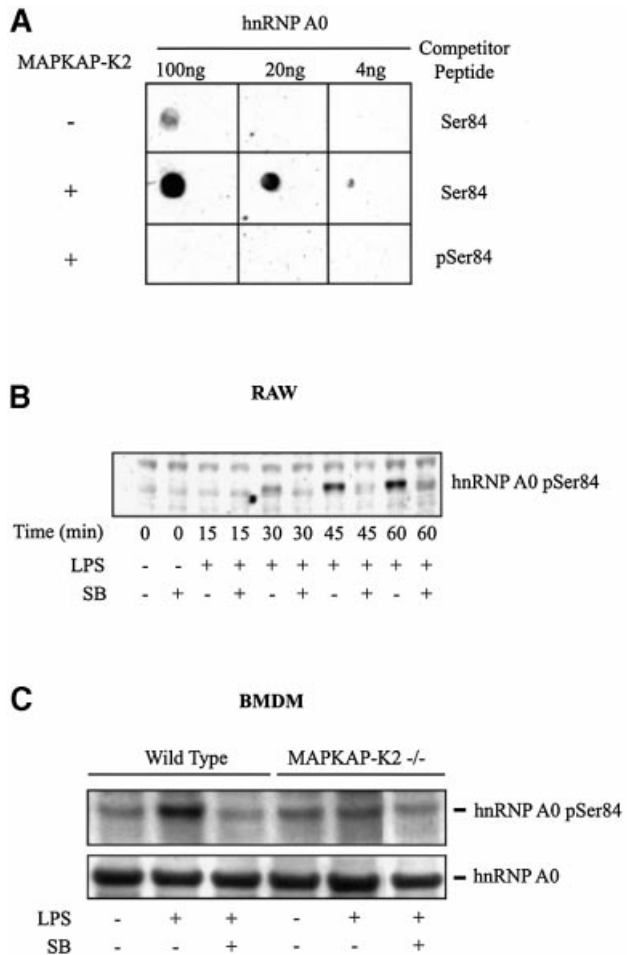


Fig. 4. hnRNP A0 is phosphorylated at Ser84 in LPS-stimulated macrophages. (A) GST-hnRNP A0 was phosphorylated for 60 min *in vitro* with 10 U/ml of MAPKAP K2 (+) and 0.1 mM ATP or left unphosphorylated (-). The proteins were spotted onto a nitrocellulose membrane in the amounts indicated. Blots were probed with the phospho-specific antibody that recognizes hnRNP A0 phosphorylated at Ser84 in the presence of the phosphorylated peptide antigen (pSer84) or the unphosphorylated Ser84 peptide (both at 10 μ g/ml). (B) RAW cells were pre-treated or not for 15 min with 10 μ M SB 203580 (SB) and either left untreated or stimulated with 50 ng/ml LPS for the times indicated. Lysates were separated by SDS-PAGE and, after transfer to nitrocellulose, the membranes were probed with the phospho-specific Ser84 antibody. (C) BMDMs from wild-type and MAPKAP-K2-deficient mice were pre-treated or not for 15 min with 10 μ M SB 203580 (SB) and either left untreated or stimulated with 50 ng/ml LPS for 1 h. The cells were lysed and hnRNP A0 immunoprecipitated and immunoblotted as described in (B). The upper panel was probed with the Ser84 phospho-specific antibody and the lower panel with an antibody that recognizes the phosphorylated and dephosphorylated forms of hnRNP A0 equally well.

and is maximal after 2–4 h (data not shown). This up-regulation is partially inhibited by pre-treatment with SB 203580 or PD 184352, but blocked completely when both inhibitors are used at a concentration of 1.0 μ M. At 1.0 μ M SB 203580, half-maximal inhibition of MIP-2 induction by PD 184352 occurred at 0.3–0.5 μ M, while at 1.0 μ M PD 184352, half-maximal inhibition by SB 203580 occurred at 0.3 μ M (Figure 7A). These concentrations are similar to those that block the LPS-induced activation of MAPKAP-

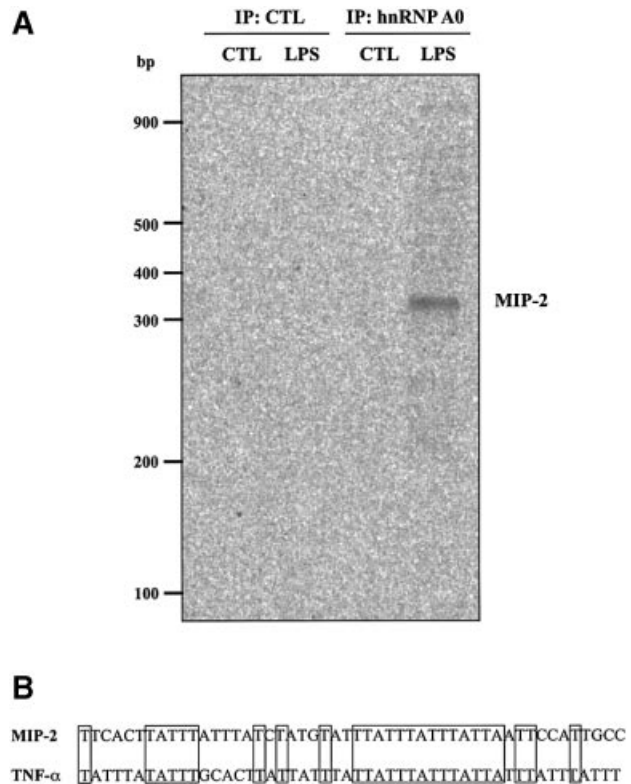


Fig. 5. MIP-2 mRNA is bound by hnRNP A0 in extracts from LPS-stimulated cells. (A) RAW cells were left untreated or treated for 1 h with 50 ng/ml LPS and lysed. Then immunoprecipitation with (lanes 3–4) or without (lanes 1–2) the hnRNP A0 antibody was carried out from clarified extracts and the RNA bound identified using a differential display-based assay with random primer set 1 (see Materials and Methods). CTL, control experiments. (B) Sequences of part of the 3'-UTR of murine MIP-2 mRNA (accession No. X53798) and TNF- α mRNA (accession No. X02611).

K2 and the classical MAP kinase cascade, respectively (data not shown).

In order to investigate the mechanism by which LPS stimulates the production of MIP-2, RAW cells were treated for 15 min with or without SB 203580 and/or PD 184352 and then for 1 h with LPS. This caused an enormous increase in the level of MIP-2 mRNA, which was only reduced by 25% in the presence of SB 203580 or PD 184352, but by 65% in the presence of both inhibitors (see time zero in Figure 7B). This indicates that at least one other signal transduction pathway (resistant to SB 203580 plus PD 184352) is required for maximal induction of MIP-2 mRNA, probably at the level of gene transcription.

One hour after stimulation with LPS, transcription was inhibited by the addition of actinomycin D, and the level of MIP-2 RNA was quantified at various times using an RNase protection assay (Figure 7). Pre-treatment with either SB 203580 or PD 184352 had little effect on mRNA stability in the 4 h subsequent to the addition of actinomycin D. In contrast, pre-treatment with SB 203580 plus PD 184352 drastically decreased MIP-2 mRNA stability, the half-life decreasing to <2 h (Figure 7).

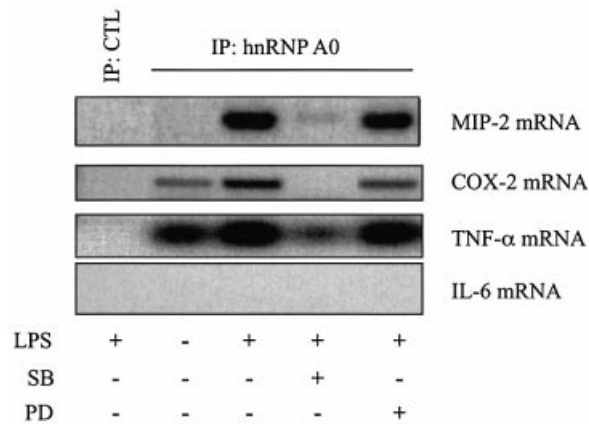


Fig. 6. SB 203580 prevents the LPS-induced interaction of hnRNP A0 with the mRNAs of MIP-2, COX-2 and TNF- α . RAW cells were pre-treated or not for 15 min with 10 μ M SB 203580 (SB) or 1 μ M PD 184352 (PD) and either left untreated or stimulated for 1 h with 50 ng/ml LPS. hnRNP A0 was immunoprecipitated as described in Figure 5 and mRNAs for MIP-2, TNF- α , COX-2 and IL-6 were amplified by RT-PCR with specific primers. CTL, control experiment.

Discussion

Elucidating the mechanisms by which LPS stimulates the production of inflammatory mediators involves understanding how the mRNAs encoding these proteins are regulated at the post-transcriptional level. Numerous studies have shown how the ARE found in the 3'-UTR of many of these mRNAs is critical in controlling their stability and translation (Kontoyiannis *et al.*, 1999; Dumitru *et al.*, 2000; Lasa *et al.*, 2000; Neinger *et al.*, 2002). However, the mechanism by which this is accomplished in macrophages remains to be elucidated. In order to understand this phenomenon better, we initially characterized the proteins that bind to the TNF- α ARE in macrophages using a proteomic-based approach. Interestingly, some of the proteins known to bind to the ARE (hnRNP A0, hnRNP A1, hnRNP D and TIA-1) and to be involved in TNF- α production (TIA-1) were identified in our analysis, although others (such as TTP) were not. Most of the proteins also bound to the c-fos ARE as well as the AREs in the mRNAs encoding COX-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Most of the proteins we characterized contain RNA recognition motifs (RRMs) present in 1–3 copies per protein, but the KH-type RNA-binding domain was found in the FUSEBP family. A subset of the RNA-binding proteins, which included members of the hnRNP A/B family, also possessed an arginine/glycine-rich domain (RGG) that is thought to be involved in protein-protein interaction as well as RNA binding (Krecic and Swanson, 1999). The largest group of proteins (~50%) belonged to the hnRNP family, known to shuttle between the nucleus and the cytoplasm and to be involved in many aspects of RNA processing. The members of this group are also known to exist in multiple forms, resulting from both alternative splicing and post-translational modifications (Krecic and Swanson, 1999), which is in accordance with our observations (Figure 1). This gives the cell an

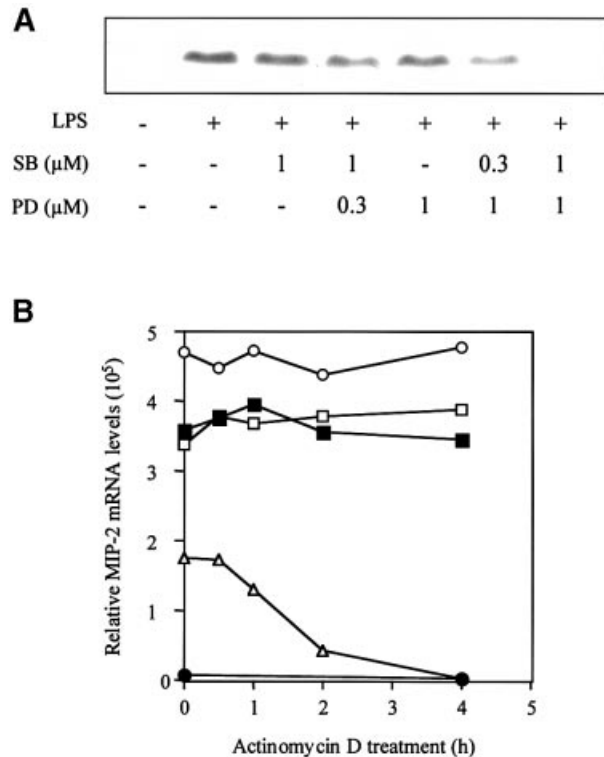


Fig. 7. The SAPK2a/p38 pathway and the classical MAP kinase cascade are both required for MIP-2 production at the mRNA and protein level. (A) RAW cells were pre-treated or not for 15 min with the indicated concentrations of SB 203580 (SB) and/or the indicated concentrations of PD 184352 (PD), and either left untreated or stimulated with 50 ng/ml LPS for 2 h. Lysates were separated by SDS-PAGE and, after transfer to nitrocellulose, the membranes were probed with a murine MIP-2-specific antibody. (B) RAW cells were treated for 15 min without (open and closed circles), with SB 203580 (closed squares), with PD 184352 (open squares) or SB 203580 plus PD 184352 (open triangles) and then for 1 h without (closed circles) or with (other symbols) LPS (time = 0 h). Transcription was then inhibited by the addition of actinomycin D and the level of MIP-2 RNA quantified at various times up to 4 h using an RNase protection assay.

impressive pool of regulatory proteins with which to control the different steps of RNA processing. Other proteins found are better known for their involvement in splicing (FUSEBP, nuclear matrix protein 55), but might also be part of RNA-protein complexes shuttling between the nucleus and the cytoplasm. Our identification of most of the proteins binding specifically to the ARE should facilitate further experiments aimed at understanding the nature of the protein complexes that bind to the ARE.

The post-translational modifications present in ARE-binding proteins (AREBPs) may be involved in the LPS-mediated post-transcriptional control of inflammatory mediators. Since signalling through MAPKAP-K2 appears to be crucial for the production of TNF- α , IL-6, interferon- γ and COX-2 (see Introduction), we investigated whether MAPKAP-K2 was able to phosphorylate any of the AREBPs. Two proteins were phosphorylated strongly by MAPKAP-K2 *in vitro*, one of which was identified as hnRNP A0 (Figure 2). The phosphorylation site was mapped to Ser84 within a consensus phosphorylation sequence for MAPKAP-K2 found only in hnRNP A0 and not in other hnRNP A/B. For example, although the equivalent serine (Ser113) is present in hnRNP A3, it is not

phosphorylated by MAPKAP-K2 *in vitro* (data not shown), probably because of the lack of a large hydrophobic residue five amino acids N-terminal to the site of phosphorylation (Stokoe *et al.*, 1993).

A phospho-specific antibody was developed against Ser84 of hnRNP A0. With this antibody, the LPS-stimulated phosphorylation of Ser84 was observed in both RAW cells and BMDMs. This phosphorylation was sensitive to the SAPK2a/p38 inhibitor SB 203580, unaffected by PD 184352, which blocks the classical MAPK pathway, or wortmannin, which inhibits class I phosphatidylinositol 3-kinases (data not shown), and greatly decreased in BMDMs from MAPKAP-K2-deficient mice (Figure 4C). Although these results suggest that MAPKAP-K2 is the major protein kinase phosphorylating hnRNP A0 at Ser84 *in vivo*, it has been reported that the level of SAPK2a/p38 protein is diminished in primary macrophages from MAPKAP-K2-deficient mice for reasons that are not yet understood (Kotlyarov *et al.*, 2002). It could therefore be argued that the decrease in SAPK2a/p38 activity in BMDMs reduces the activity of other protein kinases that are switched on by SAPK2a/p38 and that it is these enzymes, rather than MAPKAP-K2, that phosphorylate Ser84 *in vivo*. Indeed, we have found that hnRNP A0 can also be phosphorylated at Ser84 by two such enzymes, MAPKAP-K3 and p38-regulated and activated kinase (PRAK, also called MAPKAP-K5) (McLaughlin *et al.*, 1996; New *et al.*, 1998) *in vitro* (data not shown).

In order to address this problem, we studied the LPS-induced phosphorylation of the transcription factor CREB, which is known to be catalysed by mitogen and stress-activated protein kinases 1 and 2 (MSK1 and MSK2; Caivano and Cohen, 2000; Wiggin *et al.*, 2002), two closely related protein kinases that are activated by SAPK2a/p38 (Deak *et al.*, 1998). In the presence of PD 184352 to prevent the activation of MSK1/MSK2 by the MAP kinases of the classical MAP kinase cascade (Deak *et al.*, 1998), we found that the LPS-induced phosphorylation of CREB was not decreased in BMDMs from MAPKAP-K2-deficient mice (data not shown). This indicates that, although the level of SAPK2a/p38 protein is reduced in MAPKAP-K2-deficient BMDMs, its activity towards at least one of its substrates (MSK1/MSK2) is not impaired.

The residual phosphorylation of hnRNP A0 at Ser84 in BMDMs from MAPKAP-K2-deficient mice was also sensitive to SB 203580 (Figure 4C). This suggests that the weak phosphorylation of Ser84 observed under these conditions may be mediated MAPKAP-K3 and/or PRAK.

In order to investigate which mRNAs bind specifically to hnRNP A0, we immunoprecipitated this protein from macrophage extracts. These studies revealed that the mRNAs encoding TNF- α , COX-2 and MIP-2 could all be detected in the immunoprecipitates. Strikingly, the mRNA encoding MIP-2 could only be detected after immunoprecipitation of hnRNP A0 from extracts of LPS-stimulated cells, but not if the cells were pre-incubated with SB 203580 prior to stimulation with LPS (Figure 6), even though the level of MIP-2 mRNA in the macrophage extracts was only decreased slightly by SB 203580 (Figure 7B). The amount of mRNA encoding TNF- α and COX-2 was also stimulated by LPS and suppressed by

SB 203580. The simplest explanation for these findings is that the phosphorylation of hnRNP A0 at Ser84 by MAPKAP-K2 enhances binding to the AREs of these mRNAs or allows hnRNP A0 to displace another protein(s) from the AREs.

The LPS-induced stabilization of the MIP-2 mRNA was unaffected by the inhibition of SAPK2a/p38 (Figure 7), even though the interaction of hnRNP A0 with the MIP-2 mRNA was blocked (Figure 6). Our working hypothesis is that at least one other phosphorylation event is sufficient for MIP-2 stabilization, because the LPS-induced stabilization of MIP-2 mRNA is only blocked by SB 203580 in combination with PD 184352 (Figure 7), an inhibitor of a distinct signal transduction pathway. This situation is likely to apply to the post-transcriptional regulation of other inflammatory mediators, since inhibition of both the classical MAP kinase cascade and SAPK2a/p38 is also required to prevent the LPS-induced production of the mRNA and protein encoding COX-2 and IL-1 in RAW cells (Caivano and Cohen, 2000).

In contrast to MIP-2, COX-2 and IL-6, where post-transcriptional regulation occurs mostly at the level of mRNA stabilization, the LPS-induced production of TNF- α is thought to be due mainly to the release of inhibition of mRNA translation, resulting from the interaction of proteins with the ARE (Kontoyiannis *et al.*, 1999; Neining *et al.*, 2002). Moreover, this is largely suppressed by inhibition of SAPK2a/p38 alone (Lee *et al.*, 1994) and in the MAPKAP-K2-deficient mice (Kotlyarov *et al.*, 1999). This raises the possibility that the MAPKAP-K2-catalysed phosphorylation of hnRNP A0 and/or the other major substrate for this protein kinase that we have detected in the ARE-binding fraction (Figure 2) may play a key role in stimulating translation of the TNF- α message. Interfering with endogenous hnRNP A0 either with small interfering RNA (siRNA) or by replacement of wild-type hnRNP A0 with the Ser84Ala mutant will be important to establish whether the phosphorylation of this site is critical for cytokine production.

Gene regulation through post-transcriptional mechanisms is likely to play an important role in controlling many steps of inflammation and other physiological processes. In order to understand this mechanism better, it will be important to comprehend the dynamics of protein complex formation on specific mRNAs and how they are regulated by signal transduction pathways. In this study, we have addressed this question by, on the one hand, purifying many AREBPs bound to a prototypical ARE and, on the other hand, by identifying specific mRNAs bound by one AREBP. It will be interesting to find out if different members of the hnRNP A/B family bind the same or distinct mRNAs. Moreover, as we have observed for MIP-2 and COX-2, a network of signal transduction pathways clearly operates to control gene regulation at different levels.

Materials and methods

Materials

[γ -³²P]ATP, [α -³³P]dATP, PreScission protease and materials for two-dimensional gel analysis were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK), except for the pre-cast SDS-polyacrylamide gels, running buffer, transfer buffer and colloidal

blue staining kit which were obtained from Invitrogen (Groningen, The Netherlands). Unlabelled ATP, Pefabloc, dithiothreitol (DTT) and 'complete protease inhibitor set EDTA-free' were from Roche Molecular Biochemicals (Lewes, UK), cell culture media from BioWhittaker (Wokingham, UK) and SB 203580 from Calbiochem (Nottingham, UK). Maxiscript T7 kit, Retroscript kit, RPA III kit, RNase-free buffers and solutions were purchased from Ambion (Abingdon, UK). Streptavidin-coated magnetic beads and MPC-1 magnet were from Dynal (Bromborough, UK). The access RT-PCR kit and RNasin were from Promega (Southampton, UK). The RNAimage kit for differential display was purchased from GenHunter Corporation (Kimbolton, UK). The RNAeasy Mini and Midi kit were purchased from Qiagen (Crawley, UK). Sypro-Orange and Sypro-Ruby gel and blot stains were from Molecular Probes (Leiden, The Netherlands). Other chemicals were purchased from Merck (Poole, UK) or Sigma-Aldrich (Poole, UK).

Antibodies

Phospho-specific antibodies were affinity purified from antisera on phosphopeptide antigen-Sepharose columns, followed by passage through an unphosphorylated peptide-Sepharose column. The flow-through fractions were collected and used for experimentation. The antibodies were used at 0.1–1.0 µg/ml in the presence of 10 µg/ml of the unphosphorylated peptide antigen. The antibody that recognizes hnRNP A0 phosphorylated at Ser84 was raised against the peptide clkravs*red-sarpga (synthesized by Dr Graham Blumberg, University of Bristol, UK), where the phosphorylated serine is represented by S*. The peptide was conjugated separately to both bovine serum albumin and keyhole limpet haemocyanin via the N-terminal cysteine before injection into sheep at Diagnostics Scotland (Edinburgh, UK). The antibody that recognizes hnRNP A0 was raised by injecting GST-hnRNP A0 and purifying the antisera through an affinity column followed by passage down a GST column. These antibodies were provided by Drs Jane Leitch and Chris Armstrong (Division of Signal Transduction Therapy, University of Dundee). Antibodies against mouse TNF-α and mouse MIP-2 were purchased from R&D systems (Abingdon, UK). The antibody against mouse TIA-1 was purchased from BD Biosciences (Oxford, UK). Rabbit anti-sheep IgG antibodies and goat anti-rabbit IgG, both conjugated to peroxidase, were obtained from Perbio Science Ltd. (Tattenhall, UK).

Plasmids

Image clone 755060 was amplified using primers 5' HA_Bam (ggatccgccaccatgtaccatacagatgtgcagataaccgagaaattctcagttgtgtaagctgttc) and 3' Bam (ggatccttagaaggagctgctccatagccacc) with the GC-Rich PCR System (Roche) following the manufacturer's instructions, which produced a single product of 963 bp. These primers introduced *Bam*HI sites at both ends along with a Kozak sequence and 5' haemagglutinin (HA) tag. The PCR product was cloned into pCR2.1 TOPO (Invitrogen) and fully sequenced. pCR2.1A0 was digested with *Bam*HI and cloned into the same site in pGEX6P-1 (Amersham Pharmacia Biotech) to produce bacterially expressed GST/HA tag hnRNP A0.

Proteins

All recombinant proteins used were expressed as GST fusion proteins in *Escherichia coli* strain BL21. Where indicated, the GST was cleaved using the PreScission protease. MAPKAP-K2 was activated by incubation with GST-ERK2 (extracellular signal-regulated kinase-2) and the activator removed by chromatography on S-Sepharose; alternatively, MAPKAP-K2 was activated with GST-SAPK2a/p38 and used in the presence of 10 µM SB 203580. These enzymes were prepared in the Division of Signal Transduction Therapy, Dundee. HSP27 was a generous gift from Professor Roy Quinlan, University of Durham, UK. One unit of MAPKAP-K2 activity was that amount that catalysed the incorporation of 1 nmol of phosphate into the peptide substrate KKLNRTLSVA in 1 min under standard assay conditions (Stokoe *et al.*, 1993).

Cell culture

RAW264 macrophages were maintained in a 95% air, 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. The day before stimulation, the macrophages were plated at a density of 2×10^6 cells/6 cm plate. BMDMs were isolated by flushing mice femurs with RPMI-1640, passed through a cell strainer (70 µm; Falcon), centrifuged and re-suspended in RPMI-1640 containing 10% (v/v) heat-inactivated FCS, 30% L929 conditioned media, 100 U/ml penicillin and 100 µg/ml streptomycin.

They were plated on 9 cm bacteriological plastic and left to grow for a week. One day prior to plating, the cells were washed with fresh media.

Cell lysis

The cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in buffer A [50 mM Tris-HCl pH 7.4, 150 mM KCl, 0.1 mM EDTA, 1% (w/v) NP-40, 4 mM DTT, 20 mM NaF, 40 mM sodium β-glycerophosphate, 2 mM sodium orthovanadate, 1 mM Pefabloc, complete EDTA-free protease inhibitors]. The lysates were centrifuged for 10 min at 13 000 *g* and the supernatants removed, and used immediately or frozen in liquid nitrogen and stored at -80°C.

RNA affinity chromatography

Streptavidin-coated magnetic beads were made RNase free and bound to different biotinylated RNA oligonucleotides (Xeragon) according to the manufacturer's instructions (Dynal). Lysates from RAW cells were incubated for 30 min at 4°C with a biotinylated RNA oligonucleotide corresponding to the AU-rich region of TNF-α (UUAUUUUUUUUUUAUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU), *c-fos* (GUUUUUUUUUUUUUUUUUUUUUUUUUAUUUUUAAGAUGGAUUUcU) or an irrelevant RNA (AAGCUUGGGCUGCAGGUCGACUCUAGAGGA) in binding solution. This consisted of equal volumes of buffer A and buffer B [50 mM KCl, 10 mM MgCl₂, 10% (v/v) glycerol, 2 mg/ml heparin, 2 mg/ml tRNA, 50 U/ml of RNasin]. The beads were washed extensively in buffer C [25 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 5% (v/v) glycerol], and the AREBPs were eluted in one of two ways. For two-dimensional gel analysis, the AREBPs were eluted directly in the re-hydration solution (8 M urea, 2 M thiourea, 2% CHAPS, 3 mM TCEP), while for analysis of phosphorylation the AREBPs were eluted with 1.5 M NaCl in buffer C.

Two-dimensional gel analysis

AREBP were resolved on IPG 6–11 strips for the first dimension using the IPGphor isoelectric focusing system (Amersham). This pH range was selected because most of the proteins that were retained by the RNA affinity column had pI values within that region due to their affinity for RNA (data not shown). The second dimension was performed on 4–12% ZOOM gels from Invitrogen using standard electrophoresis technique. The gels were stained with Sypro-Ruby and the image captured using a Fuji Imager with LAS1000IR software.

Phosphorylation of AREBPs

The mixture of AREBPs from the RNA affinity column was diluted 10-fold in buffer D [50 mM Tris-HCl pH 7.5, 0.1% (v/v) β-mercaptoethanol, 0.1 mM sodium orthovanadate, 5 mM sodium β-glycerophosphate] and activated MAPKAP-K2 added to a final concentration of 1 or 10 U/ml. The reactions (0.03 ml) were initiated by the addition of 15 mM MgCl₂ and γ-³²P-labelled ATP (2.5×10^6 c.p.m.) at the concentrations specified in the figure legends, and incubated for 7.5 min at 30°C. Purified hnRNP A0 and HSP27 were phosphorylated at a final concentration of 1 µM.

Identification of RNA bound to immunoprecipitated hnRNP A0

Cells were lysed in 10 mM Tris-HCl pH 7.6, 1 mM potassium acetate, 1.5 mM magnesium acetate, 0.5 % (w/v) NP-40, 2 mM DTT, 1000 U/ml of RNasin, 1 mM Pefabloc, complete EDTA-free protease inhibitors. The lysates were centrifuged for 10 min at 13 000 *g* and the supernatants removed and used immediately for immunoprecipitation. This was carried out using the Seize-X immunoprecipitation kit from Pierce according to the manufacturer's instructions. Briefly, 25 µg of hnRNP A0 antibody was cross-linked with disuccinimidyl suberate (DSS) to protein G-Sepharose. Lysates were diluted with an equal volume of 8 mM Na₂PO₄, 2 mM KH₂PO₄, 140 mM NaCl, 10 mM KCl pH 7.4 and incubated for 1 h at 4°C in the presence of the bound antibody. They were then washed four times with 25 mM Tris-HCl pH 7.2 containing 150 mM NaCl, eluted with Immunopure Elution buffer (Pierce, pH 2.8) and neutralized by the addition of 10 µl of 1 M Tris base to 190 µl of eluate. RNA in the eluate was extracted using the RNAeasy Kit (Qiagen).

For the identification of unknown RNA species present in hnRNP A0 immunoprecipitates, differential display was carried out using the GenHunter RNAimage kit number 4. The 3' primer used for reverse transcription was a mix of the three primers H-T₁₁G, H-T₁₁A and H-T₁₁C. The PCR was then carried out with these primers in the presence of [α-³³P]dATP (3000 Ci/mmol) and a mix of the following four 5' primers, H-AP25, H-AP26, H-AP27 and H-AP28 (termed random primer set 1). The mixture was resolved on 6% denaturing polyacrylamide gels and specific bands were cut out and re-amplified before being cloned into pCR2.1 TOPO (Invitrogen) and sequenced. The identification was

confirmed by RT-PCR using specific primers for identified genes (see below).

RNAse protection assay and RT-PCR

Relative levels of MIP-2 RNA were quantified by RNase protection with the RPA III kit (Ambion). The MIP-2 probe was synthesized with the following primers (5', ATGGCCCTCCACCTGCC; 3', TCAGTTAGCCTTGCCTTTGTTCAGTATCTTTTG; 303 bp product) and the cyclophilin probe using the pTRI template (Ambion). RNA levels were determined with a phosphoimager and normalized to cyclophilin control. In some cases, RNA was amplified using the Access RT-PCR kit from Promega with specific primers for TNF- α , COX-2 and IL-6 from Ambion and those for MIP-2 as described above for synthesis of the RPA probe.

Mass spectrometry

Tryptic peptides were analysed on a Perseptive Biosystems (Framingham, MA) Elite STR matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometer with saturated α -cyanocinnamic acid as the matrix. The mass spectrum was acquired in the reflector mode and was mass calibrated internally. The tryptic peptide ions obtained were scanned against the Swiss-Prot and Genpep databases using the MS-FIT program of Protein Prospector. Where there was an ambiguity in the identity of a protein sample, LC-MS/MS was performed. The tryptic digest was injected onto a 0.075 \times 100 mm PepMap C18 capillary column, equilibrated with 0.1% formic acid in water, attached to a LC-Packings Ultimate HPLC system (Dionex UK; Camberley, UK). The column was developed with a discontinuous acetonitrile gradient at 0.2 μ l/min and the column was interfaced to a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The peptide ions generated by the electrospray interface were fragmented automatically using machine-defined collision voltages. The resultant peak lists were searched using the Sonar search engine (Genomic Solutions, Ann Arbor, MI) against the NCBI nr database.

Acknowledgements

We thank the Protein Production Team of the Division of Signal Transduction Therapy at Dundee for providing activated MAPKAP-K2, and the Dundee Transgenic Mouse Facility for maintaining the colonies of MAPKAP-K2-deficient mice. S.R. is supported by a postdoctoral fellowship from the Canadian Institute of Health Research. The work was supported by the UK Medical Research Council, the Royal Society, AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, NovoNordisk and Pfizer.

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*Received July 8, 2002; revised September 24, 2002;
accepted October 10, 2002*