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Phosphorylation of the ARE-binding protein DAZAP1 by ERK2 induces its dissociation from DAZ.

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Abbreviations: ARE, AU-rich element; AREBP, ARE-binding protein; DAZ, deleted in azoospermia; DAZL, DAZ-like protein; DAZAP1, DAZ-associated protein 1; EGF, epidermal growth factor; eIF4G, eukaryotic protein synthesis initiation factor 4G; ERK, extracellular signal-regulated protein kinase; HEK293, human embryonic kidney 293 cells; IL, interleukin; JNK, c-Jun N-terminal kinase; KSRP, K-Homology Splicing Regulatory Protein; MAPK, mitogen-activated protein kinase; MAPKAP-K2, MAPK-activated protein kinase 2; MKK, mitogen-activated protein kinase kinase; LPS, lipopolysaccharide; PABP, Poly(A)-binding protein; PMA, phorbol 12-myristate 13-acetate; RRM, RNA-recognition motif; TAK1, TGF- β Activated Kinase 1; TLR, Toll-like receptor; TNF, tumour necrosis factor.

Synopsis

A protein in RAW macrophages, which became phosphorylated in response to LPS, was identified as the RNA-binding protein termed Deleted in AZoospermia (DAZ) Associated Protein 1 (DAZAP1). The phosphorylation of this protein was prevented by specific inhibition of MKK1, indicating that it was phosphorylated via the classical MAP kinase cascade. Further experiments showed that DAZAP1 was phosphorylated stoichiometrically *in vitro* by ERK2 at two Thr-Pro sequences (Thr269 and Thr315) and that both sites became phosphorylated in HEK293 cells in response to PMA or EGF, or RAW macrophages in response to LPS. Phosphorylation induced by each stimulus was prevented by two structurally distinct inhibitors of MKK1 (PD 184352 and U0126), demonstrating that DAZAP1 is a physiological substrate for ERK1/ERK2. The mutation of Thr269 and Thr315 to Asp or the phosphorylation of these residues caused DAZAP1 to dissociate from its binding partner DAZ. DAZ interacts with Poly(A) binding protein (PABP) and thereby stimulate the translation of mRNAs containing short Poly(A) tails (Collier et al, 2005, EMBO J. 24, 2656-2666). Here we showed that DAZ cannot bind simultaneously to DAZAP1 and PABP and suggest that the phosphorylation-induced dissociation of DAZ and DAZAP1 may allow the former to stimulate translation by interacting with PABP.

Introduction

Inflammation is an important process in the defense to invasion by pathogens, and is mediated in part by the production of pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- α , interleukin-6 (IL-6) and IL-8. Normal expression of cytokines is vital for the inflammatory response, but their over-expression can lead to a number of inflammatory diseases, like rheumatoid arthritis [1], inflammatory bowel disease [2] and neurodegenerative disorders [3]. It is therefore crucial that the production of cytokines is tightly regulated.

The expression of cytokines is controlled at multiple levels, including transcriptional and post-transcriptional mechanisms, protein translation and secretion. Several intracellular signaling pathways are switched on in response to pathogen-derived molecules, such as bacterial lipopolysaccharide (LPS), through the activation of Toll-like receptors (TLRs) [4]. Engagement of TLR4 by LPS triggers the activation of numerous protein kinases including TGF- β Activated Kinase 1 (TAK1) [5, 6] and COT/Tpl2 [7, 8]. In turn, TAK1 induces the activation of NF- κ B, as well as c-Jun N-terminal kinase (JNK) and p38 MAPK [9], while COT/Tpl2 activates the classical Mitogen Activated Protein Kinase (MAPK) cascade [7, 8]. These pathways all contribute to pro-inflammatory cytokine production at one or more of the different levels mentioned above. For example, it has been suggested that the classical MAPK cascade is involved in regulating TNF- α production at the post-transcriptional level [7, 8].

Two post-transcriptional mechanisms involve regulation of the rate of turnover of mRNA and regulation of the translation of these messages via *cis*-elements found along the mRNA, both of which are affected by *trans* acting factors. One such regulatory element is the AU-rich element (ARE) found in the 3'-untranslated regions (3'UTR) of a number of pro-inflammatory cytokines, such as TNF- α [10], IL-6 [11] and IL-8 [12] and immediate early genes which has been shown to confer mRNA instability [13]. The destabilizing effects of the ARE are suppressed when macrophages are stimulated by LPS. To investigate how LPS exerts this effect, much effort has been directed towards identifying relevant ARE-binding proteins (AREBPs) [14-17]. In some instances, AREBPs, such as K-Homology Splicing Regulatory Protein (KSRP), have been reported to recruit the exosome, a complex of 3'-5' exonucleases involved in the degradation of ARE-containing mRNAs [18]. However,

it is also believed that other AREBPs might stabilize mRNA by competing with other ARE-binding factors that destabilize mRNAs [19-21]. Taken together, these findings suggest that AREs can act as RNA instability determinants and/or translational modulators.

The control of protein translation is also crucial to post-transcriptional regulation. For example, by binding to the poly(A) tails Poly(A) binding protein (PABP) not only stabilises mRNAs by protecting them from deadenylation, but also initiates protein translation via interaction with eukaryotic protein synthesis initiation factor 4G (eIF4G), a protein which interacts with the eIF4E that binds to the 5' cap structure of mRNA. Through these interactions, a model has been proposed in which PABP protects against degradation and also contributes to the circularization of mRNAs, which is thought to enhance their stability and promote their translation [22, 23].

The ability of LPS to stabilise mRNAs and stimulate their translation is likely to be mediated by the phosphorylation of proteins that regulate these processes. This is supported by the observation that relatively specific inhibitors of p38 MAPK decrease the production of TNF α and other cytokines [24], and that mice which are deficient in COT/Tpl2, and therefore cannot activate the MAPKs termed Extracellular signal-Regulated protein Kinases 1 and 2 (ERK1 and ERK2), do not produce TNF α in response to LPS [7]. Although several AREBP that are substrates for the p38 MAP kinase-MAPKAP-K2 pathway have been identified, such as tri-tetraproline (TTP) [25] and hnRNP A0 [17], which may regulate mRNA stability/translation, the identity of the key substrates for ERK1/ERK2 that contribute to such regulation is still lacking. Here, we identify an AREBP, termed Deleted in Azoospermia (DAZ)-Associated Protein 1 (DAZAP1) as a novel physiological substrate for ERK1/ERK2 and demonstrate that its phosphorylation induces dissociation from DAZ, a protein which has been shown to initiate protein translation by interacting with PABP [26].

Experimental Procedures

Materials

PD 184352 [Sebolt-Leopold et al, 1999], a specific inhibitor of MAP kinase kinases 1 and 2 (MKK1/2), was synthesised by an improved method [27]. U0126, was purchased from Calbiochem (Nottingham, U.K.), *Escherichia coli* LPS, phorbol 12-myristate 13-acetate (PMA) and dimethylsulphoxide (DMSO) from Sigma (Poole, U.K.), 'Complete' Protease Inhibitor tablets from Roche (Lewes, Sussex), EGF and cell culture media from Gibco (Paisley, UK), precast Bis-Tris gradient SDS polyacrylamide gels, running buffer and transfer buffer from Invitrogen (Paisley, U.K.) and Protein G-Sepharose, glutathione-Sepharose and enhanced chemiluminescence (ECL) reagent from Amersham (Bucks, UK). All the peptides utilised in this study were synthesised by Dr. Graham Bloomberg (University of Bristol, UK). Active ERK2 was produced and assayed as described previously [28].

Plasmids

Deleted in azoospermia (DAZ) associated protein 1b (DAZAP1b) was amplified from IMAGE clone 4549444 with the GC Rich PCR System (Roche) using the oligonucleotides MP1233 and MP1235 shown below. DAZAP1b differs slightly from DAZAP1; they share the same amino acid sequence from residues 1 to 349, but DAZAP1b has a distinct and longer C-terminal region (terminating at residue 408) compared to DAZAP1a (terminating at residue 379), these two species presumably arising from alternative splicing. The resulting fragment was cloned into pCR2.1 (Invitrogen) sequenced and then ligated into pCMV HA-1 to form pCMV HA DAZAP1b or into pGEX6P-1 to make pGEX6P-1 DAZAP1b. pCMV HA DAZAP1b[155-407] was made in a similar way using oligonucleotides MP1820 and MP1235. The T269D mutation was introduced using oligonucleotides NM120 and NM121 with the Quickchange Site Directed Mutagenesis kit (Stratagene), while the T315D mutation was introduced using NM122 and NM123. The T269A and T315A mutations were made using MP1436/MP1437 and MP1438/MP1439, respectively. Poly(A) binding protein cytoplasmic I (PABP-CI) was amplified in a similar manner from IMAGE clone 5597273, then cloned with the oligonucleotides NM163 and NM165. It was sub-cloned into pEBG6P to form pEBG6P-1 PABP-CI. DAZ was amplified from IMAGE clone 5297459 with oligos MP1845 and MP1846 as described above and subcloned into pCMV FLAG-1 to form pCMV FLAG DAZ.

The oligonucleotides used were:- MP1233 (GCG GAT CCA ACA ACT CGG GCG

CCG ACG AG); **MP1235** (GCG GAT CCC TAG CGT CGG TAG GGG TGG AAC); **MP1436** (TCC TAC ATC GTG TCC GCC CCT CCT GGA GGC TTT); **MP1437** (AAA GCC TCCA GGA GGG GCG GAC ACG ATG TAG GA); **MP1438** (CCT CCT CCA CCA GCC GCT CCC GGG GCA GCA CCT); **MP1439** (AGG TGC TGC CCC GGG AGC GGC TGG TGG AGG AGG); **MP1820** (GCG GAT CCG GTT TTG GAT TTA TTA CTT TCG AGG ACG AAC AAT); **MP1845** (GCG GAT CCA TGT CTG CTG CAA ATC CTG AGA CTC C); **MP1846** (GCG CGG CCG CTC AGT CTC TTC TCT GGA TTA AAC AGA CAA GAT AC); **NM120** (TCC TAC ATC GTG TCC GAC CCT CCT GGA GGC TT); **NM121** (AAG CCT CCA GGA GGG TCG GAC ACG ATG TAG GA); **NM122** (CCT CCT CCA CCA GCC GAT CCC GGG GCA GCA CC), **NM123** (GGT GCT GCC CCG GGA TCG GCT GGT GGA GGA GG); **NM163** (GCG GAT CCA ACC CCA GTG CCC CCA GCT ACC CCA T); **NM164** (GCG CGG CCG CTT AAA CAG TTG GAA CAC CGG TGG CAC TG).

Expression of DAZAP1 in Escherichia coli

The plasmid pGEX6P-1-DAZAP1, encoding a glutathione S-transferase (GST) fusion proteins of full-length human DAZAP1, was expressed in BL21 cells and induced with 50 μ M isopropyl β -D-thiogalactoside (IPTG) and grown overnight at 26 °C. The bacterial extracts were purified at 4 °C by affinity chromatography on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). The bound fusion protein was eluted with 20 mM glutathione in 150 mM Tris-HCl pH 7.5, dialysed overnight in 150 mM Tris-HCl pH 7.5, 0.1 % (v/v) 2-mercaptoethanol and 50 % sucrose and stored at -80°C.

Antibodies

The peptides SYIVST*PPGG and VPPPPAT*PGAA (where T* is phosphothreonine), corresponding to residues 264-273 and 309-319 of human DAZAP1, were coupled to both bovine serum albumin and keyhole limpet haemocyanin, then injected into sheep at Diagnostics Scotland (Edinburgh, UK). The antisera were affinity purified on CH-Sepharose to which the relevant phosphorylated peptide had been coupled covalently. Antibodies capable of recognising the unphosphorylated and phosphorylated forms of DAZAP1 equally well were raised against GST-DAZAP1 and the antisera affinity purified on CH-Sepharose to which GST-DAZAP1 had been coupled covalently followed by passage through GST-Sepharose to remove

anti-GST antibodies. The antibodies that recognise HA and GST were also raised in sheep and used for immunoblotting. Anti-HA antibodies coupled covalently to agarose and anti-Flag antibodies were purchased from Sigma (Poole, UK) and antibodies that recognize the active phosphorylated forms of ERK1 and ERK2 were from Cell Signalling Technologies (Hitchin, U.K). Rabbit, mouse and sheep-specific secondary antibodies conjugated to horseradish peroxidase were purchased from Pierce (Rockford, IL).

Cell culture, transfection and cell lysis

Human embryonic kidney (HEK) 293 cells and murine RAW 264.7 macrophages (purchased from the European Tissue Culture Collection) were maintained in a 95% air/5% CO₂ 37°C atmosphere. HEK293 cells and RAW macrophages were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (293 cells) or heat-inactivated foetal calf serum (RAW cells) plus 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. The HEK293 cells were transfected with the indicated amounts of the different DNA constructs using Polyethylenimine [29]. The cells were lysed in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol and 'Complete' Protease Inhibitor cocktail (one tablet/50 ml cell lysis buffer). The lysates were centrifuged for 15 min at 13000 x g at 4°C and supernatants collected. Protein concentrations were determined using the Bradford method. Lysates (1 mg extract) were incubated for 10 min at 37°C with 10 U benzonuclease to digest RNA and DNA (Novagen).

Immunoprecipitation and immunoblotting

1 mg of cell extract was incubated for 2 h at 4 °C with 10 µl of anti-HA-agarose or 10 µl of glutathione Sepharose 4B. The beads were washed twice with 1 ml 50 mM Tris-HCl pH 7.5, 150 mM NaCl and twice with 1 ml of 50 mM Tris-HCl pH 7.5, 0.5 M NaCl. The beads were heated in SDS for 3 min at 90°C and after pelleting the beads, the supernatant was subjected to SDS-PAGE on 4-12% gradient gels. After transfer to nitrocellulose, the membrane was immunoblotted with anti-HA, anti-Flag or anti-GST antibodies, each at 1 µg/ml, using the ECL chemiluminescence system.

Phosphorylation of DAZAP1 by ERK2

Bacterially expressed GST-DAZAP1 was incubated for 30 min at 30°C alone or with 2U/ml ERK2 in presence of 0.1 mM ATP and 10 mM MgCl₂. The reaction was stopped by adding SDS to denature the samples, and the aliquots subjected to SDS-PAGE as described under results.

Results

Identification of two proteins that become phosphorylated via the classical MAP kinase cascade in LPS-stimulated macrophages.

While reinvestigating the multisite phosphorylation of the transcription factor c-Jun [30] we identified two proteins, distinct from c-Jun that were present in c-Jun immunoprecipitates and were recognized “non-specifically” by another antibody that detects c-Jun only when it is phosphorylated at Thr91 (Fig 1A). However, in contrast to the LPS-induced phosphorylation of c-Jun at Thr91, which is catalysed by the MAPK family member c-Jun N-terminal kinase (JNK) and therefore unaffected by PD 184352, a specific inhibitor of MKK1, the LPS-induced phosphorylation of the other two proteins was prevented by this inhibitor (Fig 1A). Control experiments in which the antibody used to immunoprecipitate c-Jun was omitted then revealed that, unlike c-Jun, the other two proteins still adhered to Protein G-Sepharose, demonstrating that they were not c-Jun-interacting proteins (data not shown).

The two proteins were purified from the RAW extracts by “affinity chromatography” on Protein G-Sepharose followed by SDS-PAGE and identified as DAZ-associated protein 1 (DAZAP1) and hnRNP K by tryptic mass fingerprinting (Fig 1B). It has previously been established that hnRNP K is a physiological substrate for ERK1/ERK2 [31], the two MAPKs that are activated by MKK1, the target of PD 184352. In contrast, DAZAP1 was a novel downstream target for this pathway and therefore subjected to a more detailed analysis.

DAZAP1 is phosphorylated at Thr269 and Thr315 by ERK2 in vitro and binds to the ARE of the mRNA encoding TNF.

MAPKs nearly always phosphorylate serine or threonine residues that are followed by proline, suggesting that the c-Jun Thr91 phospho-specific antibody might be recognizing a phospho-threonyl proline sequence present in DAZAP1 and that the phosphorylation of this site might be catalysed by ERK1/ERK2. We therefore expressed and purified DAZAP1 and examined whether it could be phosphorylated by ERK2 in vitro. As shown in Fig 2A, this proved to be the case, incubation with ERK2 and MgATP leading to the recognition of this protein by the anti-Thr91 antibody (Fig 2A).

We have previously identified DAZAP1 as a protein that binds specifically to the ARE of the mRNA encoding TNF α [17]. In order to examine whether DAZAP1 could still be phosphorylated by ERK2 when bound to this ARE, we incubated the

immobilized ARE-binding proteins from RAW macrophages with MgATP and ERK2. As shown in Fig 2B, this led to a decrease in the mobility of DAZAP1 during SDS-PAGE, indicative of stoichiometric phosphorylation at one or more sites. Mass spectrometric analysis established that the band whose mobility was decreased by phosphorylation with ERK2 was indeed DAZAP1 (Fig 2B and data not shown). Immunoblotting also showed that DAZAP1 underwent a decrease in electrophoretic mobility after phosphorylation by ERK2 (Fig 2C). Strikingly, DAZAP1 was the only major ARE-binding protein that underwent this band-shift. After phosphorylation by ERK2, DAZAP1 was still retained by the ARE-beads, indicating that phosphorylation did not disrupt its ability to interact with this element (data not shown).

DAZAP1 that had been phosphorylated maximally by ERK2 was digested with trypsin and the resulting peptides separated on a C₁₈ column. One ³²P-labelled peptide was detected, which eluted at nearly 50% acetonitrile (Fig 3A), indicating that it was very large. As the size precluded identification of the site(s) of phosphorylation by mass spectrometry, it was sub-digested with chymotrypsin. Re-chromatography on the C₁₈ column now resolved two ³²P-labelled peptides, termed C1 and C2 (Fig 3B). Analysis by mass spectrometry showed that C1 and C2 were the peptides comprising residues 266-284 and 305-321 of DAZAP1, each containing one covalently bound phosphate (data not shown). Solid phase sequencing revealed that the sites of phosphorylation in peptides C1 and C2 were located at Thr269 and Thr315, respectively (Figs 3C and 3D).

DAZAP1 is phosphorylated in cells by ERK1/ERK2 at Thr269 and Thr315.

In order to investigate whether DAZAP1 was phosphorylated at Thr269 and Thr315 in cells, we generated phospho-specific antibodies that recognize these phosphorylated epitopes. The anti-phosphoThr269 antibody recognized DAZAP1 only after phosphorylation by ERK2 and was neutralized by the phosphopeptide immunogen, but not by the unphosphorylated form of the peptide or a distinct peptide containing the Thr315 phosphorylated site (Fig 4A). Similarly, the anti-phosphoThr315 antibody recognized DAZAP1 only after phosphorylation by ERK2 and was neutralized by the phosphopeptide immunogen, but not by the unphosphorylated form of the peptide or a distinct peptide containing the Thr269 phosphorylated site (Fig 4A). The anti-phosphoThr269 antibody was used in the presence of the unphosphorylated form of the peptide immunogen, because, in its absence, it showed slight cross reactivity with unphosphorylated DAZAP1 (see top

left hand panel in Fig 4A). Interestingly, the antibody that recognizes c-Jun phosphorylated at Thr91 only recognized DAZAP1 phosphorylated at Thr269, as shown by experiments using variants of DAZAP1 in which either Thr269 or Thr315 had been replaced by Ala. (Fig 4B). The recognition of DAZAP1 by this antibody in LPS-stimulated macrophages (Fig 1A) therefore establishes that DAZAP1 is phosphorylated by ERK1/ERK2 at Thr269 in these cells. Similarly, the phosphoThr315 antibody also recognized DAZAP1 in PMA-stimulated (Fig 4C) or epidermal growth factor (EGF) stimulated (Fig 4D) HEK 293 cells. In each case phosphorylation was prevented by either PD 184352 or U0126, the latter being another MKK1 inhibitor that is structurally unrelated to PD 184352. Thus DAZAP1 becomes phosphorylated at both Thr269 and Thr315 by ERK1/ERK2 in response to agonists that activate the classical MAP kinase cascade.

Phosphorylation of DAZAP1 induces its dissociation from DAZ.

DAZAP1 was originally identified as a binding partner for Deleted in Azoospermia (DAZ), a protein whose gene is frequently deleted in cases of male sterility [32]. Since the phosphorylation of DAZAP1 did not disrupt its ability to interact with the ARE, as described above, we next examined whether phosphorylation affected its ability to bind to DAZ. Like DAZAP1, DAZ is an RNA-binding protein containing RRM motifs and such RNA-binding motifs can sometimes act as protein-protein interaction motifs [33, 34]. Therefore initial experiments were carried out using an N-terminally truncated form of DAZAP1 (DAZAP1[155-407]), which lacks its RNA binding motifs, but retains the region thought to interact with DAZ [32, 35]. In these experiments, we co-transfected Flag- DAZ with forms of HA-DAZAP1 in which Thr269 and/or Thr315 had been mutated to Ala to prevent phosphorylation or to Asp to mimic phosphorylation by introducing a negative charge at the same position. After immunoprecipitation of unmutated DAZAP1[155-407] from the cell extracts, DAZ was present in the anti-HA immunoprecipitates, confirming that DAZ interacts with DAZAP1 outwith the RNA-binding regions [32]. DAZ was also present in the anti-HA immunoprecipitates of DAZAP1[155-407] in which the two phosphorylation sites of DAZAP1 were mutated to Ala, but was absent when these residues had been mutated to Asp (Fig 5A). Further experiments using mutants of DAZAP1 in which either Thr269 or Thr315 were changed to Asp showed that each single mutation greatly reduced the interaction with DAZ, although the Thr269Asp mutation had the strongest effect (Fig 5B). The combined mutation of

Thr269 and Thr315 to Asp also decreased significantly the interaction between full length DAZAP1 and DAZ (Fig 5C). Taken together, these experiments suggested that the phosphorylation of DAZAP1 might induce its dissociation from DAZ.

In order to investigate whether phosphorylation did indeed trigger the dissociation of these two proteins, vectors expressing GST-DAZAP1 or GST-DAZAP1[T269A;T315A] and Flag-DAZ were co-transfected into HEK293 cells. After purification on glutathione-Sepharose, the immobilized DAZAP1 and its associated DAZ were incubated with MgATP and ERK2. This caused a substantial release of DAZ from DAZAP1 in comparison to control incubations performed in the absence of MgATP and/or ERK2 (Fig 6A). However this dissociation did not occur when the non-phosphorylatable form of DAZAP1[T269A;T315A] was co-transfected with DAZ (Fig. 6B).

Evidence that DAZAP1 cannot bind simultaneously to both DAZ and PABP1.

It has recently been reported that DAZ also interacts with polyA-binding protein 1 (PABP1), thereby stimulating the translation of mRNAs containing short polyA tails in *Xenopus* oocytes [26]. It was therefore of interest to investigate whether DAZ could interact with DAZAP1 and PABP1 simultaneously. DNA expressing epitope-tagged versions of each protein were therefore co-transfected into HEK 293 cells and PABP1 or DAZAP1 immunoprecipitated from the extracts. Interestingly, DAZAP1 was absent from PABP1 immunoprecipitates (Fig 7A) and PABP1 was absent from DAZAP1 immunoprecipitates (Fig 7B), although DAZ was present in both. These results, which imply that the binding of DAZAP1 and PABP to DAZ is mutually exclusive, are considered further in the Discussion.

Discussion

In this paper we fortuitously identified DAZAP1 as a new physiological substrate for ERK1/ERK2 because of its cross-reactivity with an antibody that recognizes the Thr91 phosphorylation site on c-Jun and by its “non-specific” binding to Protein G-Sepharose. We have found that about 2% of the proteins in cell extracts are “pulled down” on Protein G-Sepharose, many of which we have identified by mass spectrometry as RNA binding proteins (data not shown). Indeed the “sticky” nature of these proteins has been well documented and procedures for minimising their interaction with chromatographic matrices have been published [36]. Interestingly, we had previously identified DAZAP1 as one of the more abundant proteins that interacts specifically with the ARE of the TNF α mRNA [17] and the c-Fos mRNA (S. Rousseau unpublished work), and other investigators have shown that its RRM motifs bind preferentially to polyU RNA homopolymers, compared to polyG or polyA [32]. These findings raise the possibility that DAZAP1 might play a role in the regulation of mRNA stability/translation by the classical MAP kinase cascade.

DAZAP1 was first identified as a protein that interacts with DAZ and the structurally related DAZ-like (DAZL) protein [32]. DAZ and DAZL, as well as another family member called BOULE, are all highly expressed in germ cells. Indeed, the DAZ gene is located on the Y chromosomes of humans, great apes, and Old World monkeys [37-39] and is frequently deleted in male sterility [40]. There is evidence that members of this family play a role in stimulating protein translation. For example, in *Drosophila*, BOULE is essential for the translation of the mRNA encoding Twine, a Cdc25-like phosphatase involved in the regulation of meiosis [41]. Moreover, DAZ and DAZL have been found associated with poly-ribosomes that are actively engaged in protein synthesis [42]. Furthermore, DAZ, DAZL and BOULE have all been shown to interact with PABP in *Xenopus* oocytes and thereby to stimulate the translation of mRNAs with short polyA tails [26].

The key finding of our paper is that the phosphorylation of DAZAP1 at Thr269 and Thr315 does not disrupt its interaction with the ARE, but induces its dissociation from DAZ. This is consistent with the location of the phosphorylation sites, which are in a proline-rich region located C-terminal to the RRM motifs. Deletion of the RRM motifs disrupts RNA binding [32] but not the interaction with DAZ (Fig 5), indicating that the sites of phosphorylation may be in the vicinity of the DAZ interaction site [32, 35].

The observation that phosphorylation of DAZAP1 induces its dissociation from DAZ suggested that this might regulate the function of either or both proteins. For example, it could be envisaged that the DAZ-DAZAP1 complex confers mRNA instability by binding to the ARE and/or prevents DAZ from interacting with other binding partners. Following the phosphorylation of DAZAP1, the ensuing release of DAZ may reduce mRNA instability and now allow DAZ to interact with other proteins involved in promoting translation, such as PABP. Additionally, the interaction of DAZ with PABP may further confer mRNA stability by promoting mRNA circularisation (see Introduction). Consistent with this hypothesis, we found that DAZ could not bind simultaneously to both DAZAP1 and PABP (Fig 7), and others have reported that, in contrast to PABP, DAZ and DAZL, DAZAP1 is absent from polyribosomes [35].

DAZ and DAZL have so far only been found in germ cells, whereas BOULE has also been found in the nervous system of *Drosophila* [43]. In contrast DAZAP1 is not only expressed in the tissues where members of the DAZ family are present, but also in many other tissues [35], as demonstrated by our original identification of DAZAP1 in RAW macrophages [17] (Fig 1A). As no other protein with DAZ motifs has been found by mining databases (data not shown), this raises the interesting question as to the identity of the binding partner(s) for DAZAP1 in macrophages and other cells which do not express a DAZ-like protein. To understand if and how the ERK1/ERK2-catalysed phosphorylation of DAZAP1 is involved in regulating the stability and/or translation of ARE-containing mRNAs encoding pro-inflammatory cytokines, which is the major interest of our laboratory (see Introduction), it is clearly now critical to find its binding partners in macrophages.

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Figure Legends

Figure 1. Identification of two novel proteins phosphorylated in response to LPS in RAW macrophages.

A. Macrophages were incubated for 1h with or without 2 μ M PD 184352 and then stimulated for 30 min with LPS (100 ng/ml) and lysed. c-Jun was immunoprecipitated from the lysates denatured in SDS, subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were immunoblotted with an antibody that recognizes c-Jun phosphorylated at Thr91. This not only detected c-Jun but also two other proteins indicated by arrows that were identified in B as DAZAP1 and hnRNP K. **B.** RAW extract (2 mg protein) was incubated for 1h at 4°C with Protein G-Sepharose (60 μ l) and the immunoprecipitation carried out as described under Methods. The protein staining bands corresponding to the proteins recognized by the anti-phospho-c-Jun[Thr91] antibody in A, were excised, alkylated with iodoacetamide and digested with trypsin. The resultant peptides were analysed by MALDI-TOF-TOF or LCMS as described previously [44].

Figure 2. Phosphorylation of DAZAP1 *in vitro* by the MAP kinase ERK2.

A. Bacterially expressed GST-DAZAP1 was phosphorylated with ERK2, subjected to SDS-PAGE and immunoblotted with the anti-phospho(p)Thr91 as in Fig 1A. **B, C.** The AU-rich element binding proteins were purified from 5 mg of RAW extract by affinity chromatography [17] then phosphorylated as in A, subjected to SDS-PAGE and proteins visualized by staining with Colloidal Coomassie Blue (B) or immunoblotted with an anti-DAZAP-1 antibody that recognizes DAZAP-1 phosphorylated and unphosphorylated forms equally well (C).

Fig. 3. Identification of the residues on DAZAP1 phosphorylated by ERK2.

GST-DAZAP1 was maximally phosphorylated with GST-ERK2 as in Fig 2 and subjected to SDS-PAGE. The band corresponding to ³²P-labelled DAZAP1 was visualised by staining with Coomassie Blue, excised and digested with trypsin. **(A)** The tryptic phosphopeptides were separated by HPLC on a Vydac C₁₈ column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (broken line). Radioactivity is indicated by the full line. **(B)** The major ³²P-labelled peptide T1 from A was sub-digested with chymotrypsin and rechromatographed on the C₁₈ column as in A. **C, D** Peptide C1

(C) and peptide C2 (D) were subjected to solid phase sequencing to determine the sites of phosphorylation [45]. Amino acid sequences shown are those inferred from mass spectrometry analysis.

Figure 4. DAZAP1 is phosphorylated in cells at Thr269 and Thr315 in response to PMA or EGF. **A.** GST-DAZAP1 (0.3 mg/ml) was maximally phosphorylated with ERK2 in the presence (+) or absence (-) of MgATP. The indicated amounts of each preparation were spotted on to a nitrocellulose membrane and immunoblotted with the antibodies that recognise DAZAP1 phosphorylated at either Thr269 (anti-pThr269) or Thr315 (anti-pThr315) in the absence (none) or presence of 10 µg/ml of the unphosphorylated (unphospho) or phosphorylated (phospho) peptide antigen or the phosphorylated peptide antigen raised against the other phosphorylation site on DAZAP1. **B.** The wild type or mutant forms of DAZAP1 shown were phosphorylated as in A, spotted on to nitrocellulose membranes and immunoblotted with the phospho-specific antibody that recognises c-Jun phosphorylated at Thr91 (anti-pThr91) in the presence of 10µg/ml of the unphosphorylated form of the phosphopeptide immunogen [30]. **C, D.** HEK 293 cells were pre-incubated for 1h without or with 2 µM PD 184352 or 10 µM U0126, then stimulated for 30 min with 100 ng/ml PMA (C) or for 10 min with 100 ng/ml EGF (D) and lysed. Cell lysates were denatured in SDS, subjected to SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an antibody that recognises DAZAP1 phosphorylated at Thr315 (pT315) and with an antibody that recognises the phosphorylated and unphosphorylated forms of DAZAP1 equally well (DAZAP1). Further aliquots of the cell lysates were immunoblotted using antibodies that recognise phosphorylated ERK1 and ERK2 (pERK1, pERK2) or the phosphorylated and unphosphorylated forms of ERK1/2 equally well (ERK1, ERK2). Similar results were obtained three different experiments.

Fig. 5. A phospho-mimetic form of DAZAP1 does not interact with DAZ.

A. HEK 293 cells were transfected for 36 h without (-) or with 2.5 µg Flag-DAZ DNA (+) and 2.5 µg DNA expressing unmodified HA-DAZAP1[155-407] (WT), HA-DAZAP1[155-407] in which Thr269 and Thr315 had both been mutated to Ala (AA), or HA-DAZAP1 [155-407] in which Thr269 and Thr315 had both been mutated to

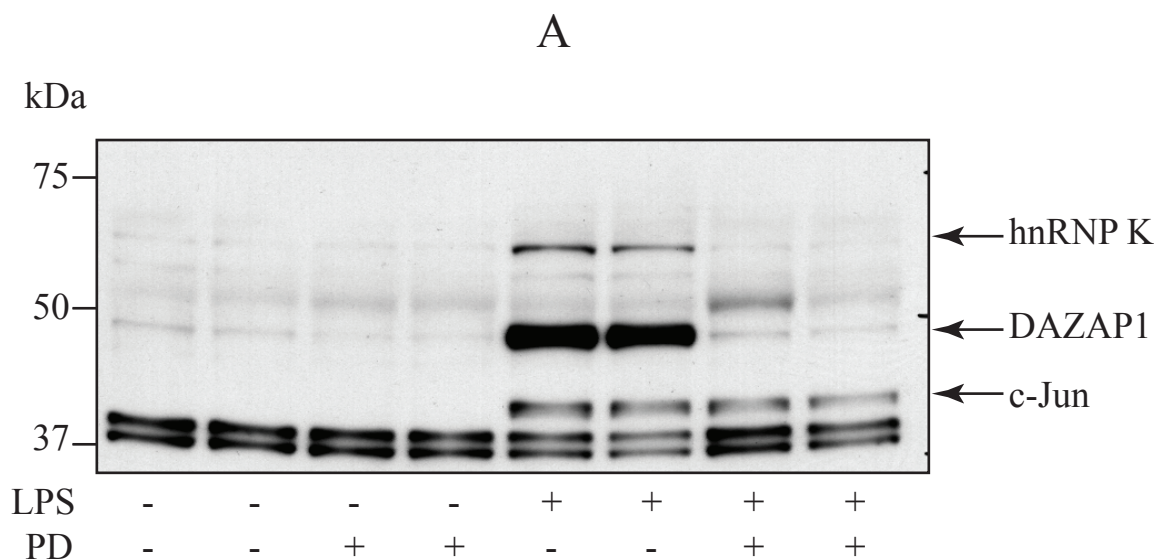
Asp (DD) or singly mutated forms of DAZAP1 in which either Thr269 or Thr315 had been mutated to Asp (T269D, T315D). DAZAP1 was immunoprecipitated (IP) from 1 mg cell extract protein with an anti-HA antibody and immunoblotted (IB) with anti-HA and anti-Flag antibodies. **B.** Same as A, except that the cells were transfected with full-length forms of DAZAP1.

Fig. 6. Phosphorylation of full-length DAZAP1 by ERK2 impairs its binding to DAZ. **A, B.** HEK 293 cells were transfected with 1 μ g DNA encoding Flag-DAZ and with 0.5 μ g DNA encoding GST-DAZAP1[WT] (A) or a non-phosphorylatable form GST-DAZAP1[T269A;T315A] (B). After 36 h, the cells were lysed and the extract (1 mg protein) was incubated for 2 h at 4°C with glutathione-Sepharose (20 μ l). The beads were washed, and phosphorylated with ERK2 in the presence (+) or absence (-) of MgATP, before being washed again and denatured in SDS. Protein released from the beads was subjected to SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted. The GST-DAZAP1 in the extract (DAZAP1 total) was visualised with an anti-GST-antibody (DAZAP-1) and Flag-DAZ with an anti-Flag antibody (DAZ). The membranes were also immunoblotted with the phospho-specific antibody that recognises DAZAP1 phosphorylated at Thr315 (pT315) and with the phospho-specific antibody that not only recognises c-Jun phosphorylated at Thr91 but also DAZAP1 phosphorylated Thr269 (pT269).

Fig. 7. DAZAP1 and PABP exist as distinct complexes with DAZ.

A. HEK 293 cells were transfected with 0.2 μ g DNA encoding Flag-DAZ, 1 μ g DNA encoding GST-PABP and 1 μ g DNA encoding HA-DAZAP1[155-407]. After 48 h, the cells were lysed, and 1 mg of extract protein was incubated for 1 h with 10 μ l glutathione-Sepharose beads to pull down GST-PABP. After extensive washing (see Methods), the proteins were released from the beads by denaturation in SDS and immunoblotted with anti-HA antibodies to detect DAZAP1, anti-GST antibodies to detect PABP or anti-Flag antibody to detect DAZ. **B.** Same as A, except that DAZAP1 was immunoprecipitated with an anti-HA antibody.

Figure 1



B

DAZAP1 peptides identified

| Calculated Mass | Observed Mass | Start Sequence | End Sequence | Sequence |
|-----------------|---------------|----------------|--------------|------------------------------|
| 1513.714 | 1513.735 | 195 | 209 | SQAPGQPGASQWGSR |
| 1715.856 | 1715.875 | 136 | 150 | FGVVTEVVMYDAEK |
| 1798.890 | 1798.904 | 115 | 130 | IFVGGIPHNCGETELR |
| 1822.933 | 1822.948 | 12 | 27 | LFVGGLDWSTTQETLR |
| 3131.387 | 3131.441 | 155 | 181 | GFGFITFEDEQSVDQAVNMHFHDI MGK |

hnRNP K peptides identified

| Calculated Mass | Observed Mass | Start Sequence | End Sequence | Sequence |
|-----------------|---------------|----------------|--------------|-----------------|
| 871.478 | 871.461 | 264 | 272 | GPPPPPPR |
| 1053.641 | 1053.655 | 168 | 177 | VVLIGGKPDR |
| 1194.699 | 1194.715 | 282 | 292 | NLPLPPPPPPR |
| 1365.642 | 1365.633 | 36 | 46 | SRNTDEMVELR |
| 1518.936 | 1518.949 | 125 | 139 | LLIHQSLAGGIIGVK |
| 1549.652 | 1549.669 | 156 | 167 | LFQECCPHSTDR |

Figure 2

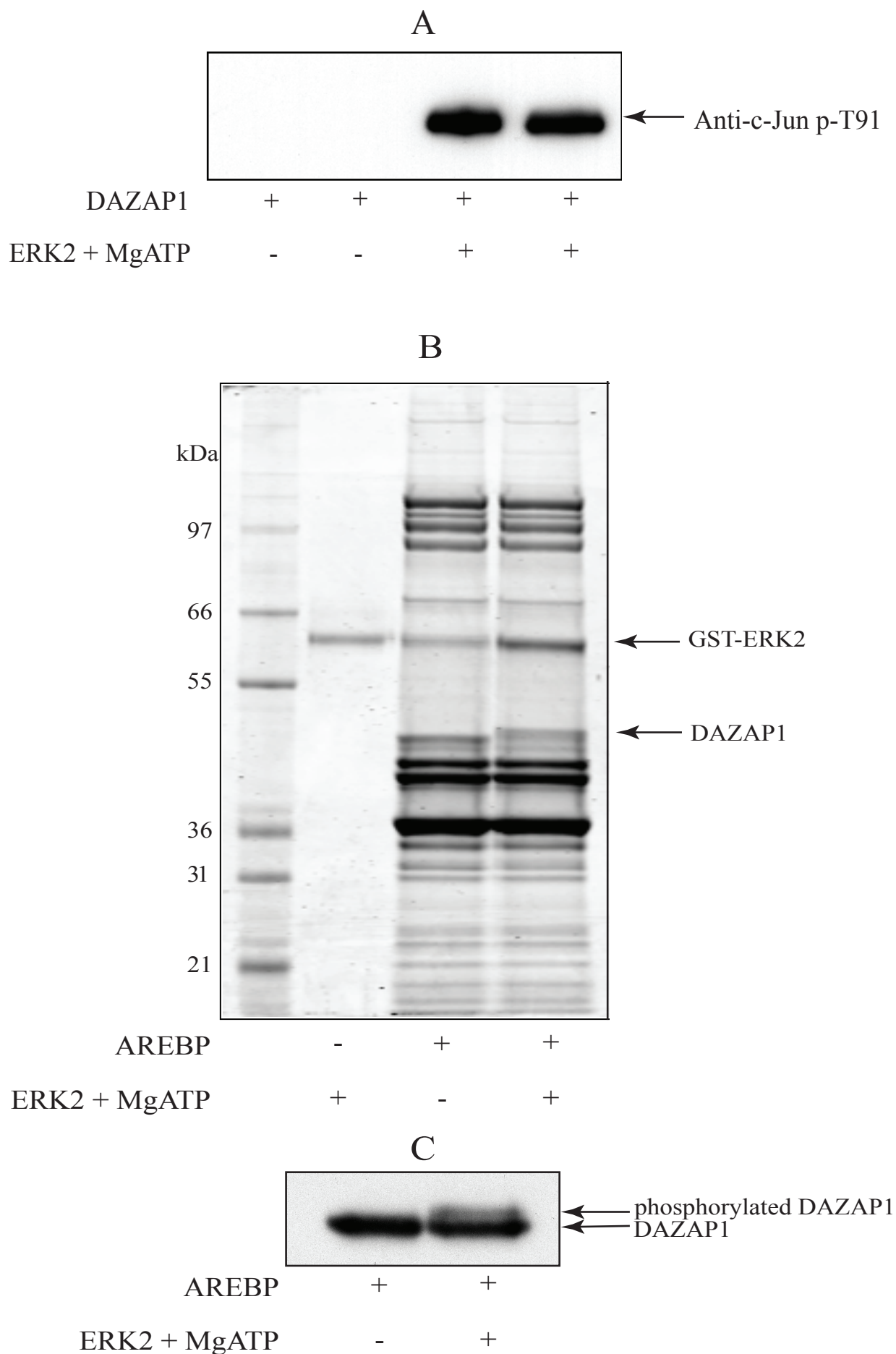


Figure 3

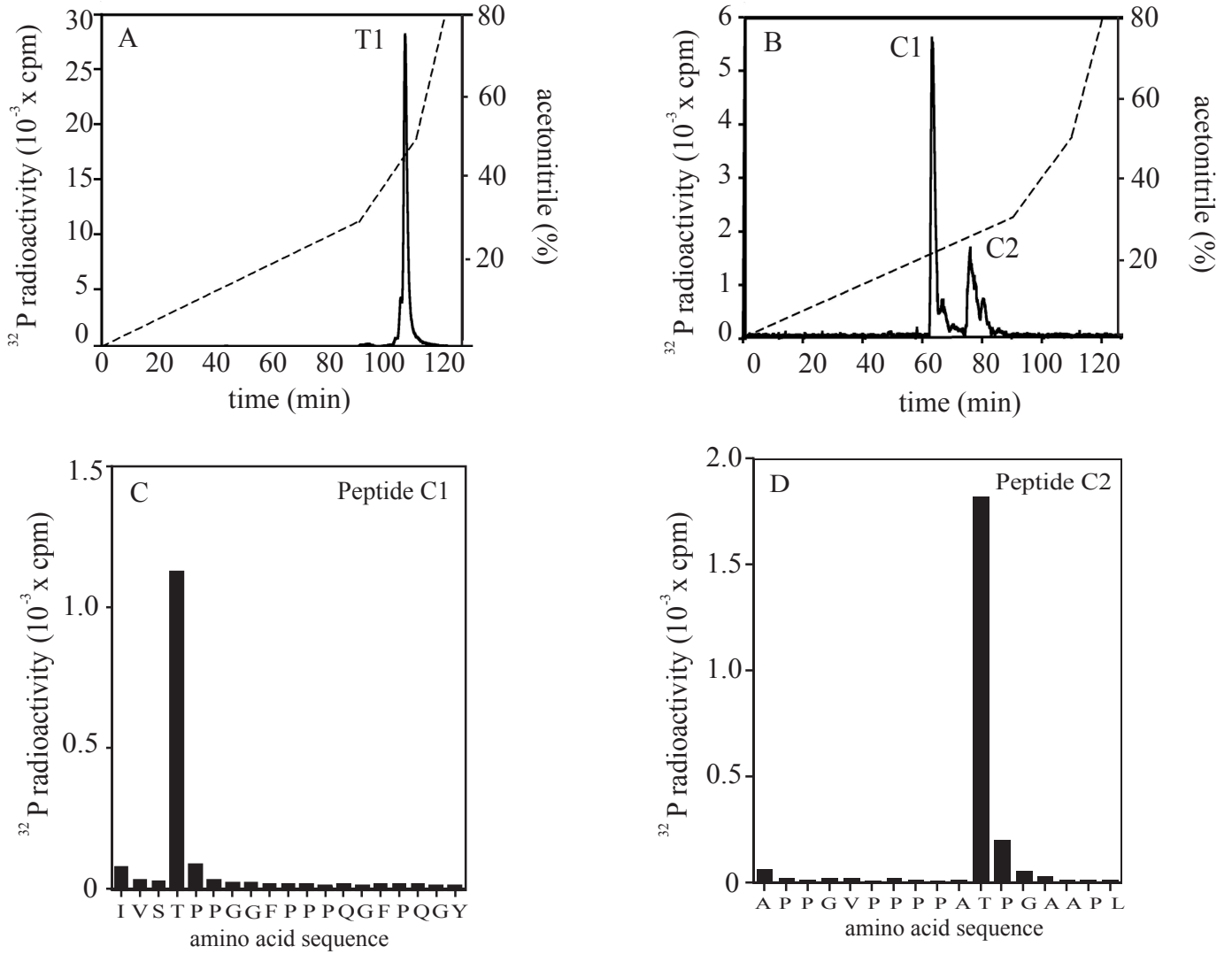


Figure 6

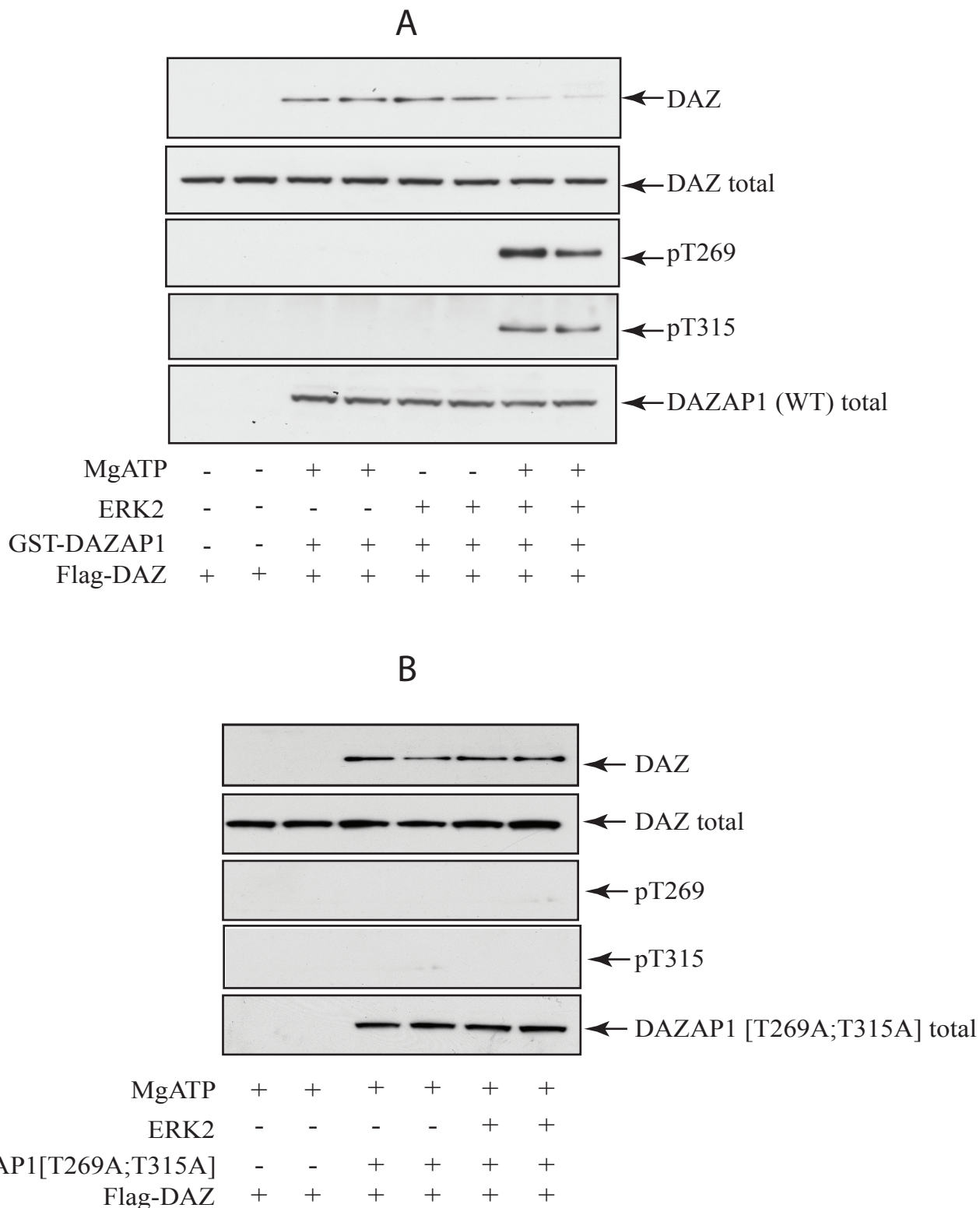


Figure 7

