

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

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A protein in RAW 264.7 macrophages, which became phosphorylated in response to LPS (lipopolysaccharide), was identified as the RNA-binding protein called DAZAP1 [DAZ (deleted in azoospermia)-associated protein 1]. The phosphorylation of this protein was prevented by specific inhibition of MKK1 [MAPK (mitogen-activated protein kinase) kinase 1], indicating that it was phosphorylated via the classical MAPK cascade. Further experiments showed that DAZAP1 was phosphorylated stoichiometrically *in vitro* by ERK2 (extracellular-signal-regulated protein kinase 2) at two Thr-Pro sequences (Thr<sup>269</sup> and Thr<sup>315</sup>), and that both sites became phosphorylated in HEK-293 (human embryonic kidney 293) cells in response to PMA or EGF (epidermal growth factor), or RAW 264.7 macrophages in response to LPS. Phosphorylation induced by each stimulus was prevented by two structurally distinct inhibitors of MKK1 (PD184352 and U0126), demonstrating that DAZAP1 is a physiological substrate for

ERK1/ERK2. The mutation of Thr<sup>269</sup> and Thr<sup>315</sup> to aspartate or the phosphorylation of these residues caused DAZAP1 to dissociate from its binding partner DAZ. DAZ interacts with PABP [poly(A)-binding protein] and thereby stimulates the translation of mRNAs containing short poly(A) tails [Collier, Gorgoni, Loveridge, Cooke and Gray (2005) *EMBO J.* **24**, 2656–2666]. In the present study we have shown 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.

**Key words:** AU-rich element (ARE), deleted in azoospermia (DAZ), deleted-in-azoospermia-associated protein 1 (DAZAP1), extracellular-signal-regulated protein kinase (ERK), mRNA stability, mRNA translation.

## INTRODUCTION

Inflammation is an important process in the defence of invasion by pathogens, and is mediated in part by the production of pro-inflammatory cytokines, such as TNF $\alpha$  (tumour necrosis factor- $\alpha$ ), IL-6 (interleukin-6) and IL-8. Normal expression of cytokines is vital for the inflammatory response, but their overexpression can lead to a number of inflammatory diseases, such as rheumatoid arthritis [1], inflammatory bowel disease [2] and certain 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 signalling pathways are switched on in response to pathogen-derived molecules, such as bacterial LPS (lipopolysaccharide), through the activation of Toll-like receptors [4]. Engagement of Toll-like receptor 4 by LPS triggers the activation of numerous protein kinases, including transforming-growth-factor- $\beta$ -activated kinase 1 [5,6] and COT/Tpl2 (tumour progression locus 2) [7,8]. In turn, transforming-growth-factor- $\beta$ -activated kinase 1 induces the activation of NF- $\kappa$ B (nuclear factor  $\kappa$ B), as well as c-Jun N-terminal kinase and p38 MAPK (mitogen-activated protein kinase) [9], whereas COT/Tpl2 activates the classical 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 $\alpha$  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 ARE (AU-rich element) found in the 3'-untranslated regions of a number of pro-inflammatory cytokines, such as TNF $\alpha$  [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 AREs are suppressed when macrophages are stimulated by LPS. To investigate how LPS exerts this effect, much effort has been directed towards identifying relevant AREBPs (ARE-binding proteins) [14–17]. In some instances, AREBPs, such as KSRP (K-homology splicing regulatory protein), 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, PABP [poly(A)-binding protein] not only stabilizes mRNAs by protecting them from deadenylation, but also initiates protein translation via interaction with eIF4G (eukaryotic protein

Abbreviations used: ARE, AU-rich element; AREBP, ARE-binding protein; DAZ, deleted in azoospermia; DAZAP1, DAZ-associated protein 1; DAZL, DAZ-like protein; EGF, epidermal growth factor; ERK, extracellular-signal-regulated protein kinase; GST, glutathione S-transferase; HA, haemagglutinin; HEK-293, human embryonic kidney 293; hnRNP, heterogeneous nuclear ribonucleoprotein; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; PABP, poly(A)-binding protein; RRM, RNA-recognition motif; TNF $\alpha$ , tumour necrosis factor  $\alpha$ ; Tpl2, tumour progression locus 2.

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synthesis initiation factor 4G), 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 stabilize 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 $\alpha$  and other cytokines [24], and that mice which are deficient in COT/Tpl2, and therefore cannot activate the MAPKs ERK1 (extracellular-signal-regulated protein kinase 1) and ERK2, do not produce TNF $\alpha$  in response to LPS [7]. Although several AREBPs that are substrates for the p38 MAPK–MAPKAP-K2 (MAPK-activated protein kinase 2) pathway have been identified, such as TTP (tri-tetraproline) [25] and hnRNP A0 (heterogeneous nuclear ribonucleoprotein 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. In the present study, we identify an AREBP, DAZAP1 [DAZ (deleted in azoospermia)-associated protein 1], 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

### Materials

PD184352 [26a], a specific inhibitor of MKK1 (MAPK kinase 1) and MKK2, was synthesized by an improved method [27]. U0126 was purchased from Calbiochem (Nottingham, U.K.), *Escherichia coli* LPS, PMA and DMSO were from Sigma (Poole, Dorset, U.K.), Complete™ protease inhibitor tablets were from Roche (Lewes, Sussex, U.K.), EGF (epidermal growth factor) and cell culture media were from Gibco (Paisley, Renfrewshire, Scotland, U.K.), precast Bis-Tris gradient SDS polyacrylamide gels, running buffer and transfer buffer were from Invitrogen (Paisley, Renfrewshire, Scotland, U.K.), and Protein G–Sepharose, glutathione–Sepharose and ECL (enhanced chemiluminescence) reagent were from Amersham (Little Chalfont, Bucks., U.K.). All the peptides utilized in this study were synthesized by Dr Graham Bloomberg (Department of Biochemistry, University of Bristol, Bristol, U.K.). Activated ERK2 was produced and assayed as described previously [28].

### Plasmids

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 with 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 Quikchange® site-directed mutagenesis kit (Stratagene), whereas the T315D mutation was

introduced using oligonucleotides NM122 and NM123. The T269A and T315A mutations were made using oligonucleotides MP1436/MP1437 and MP1438/MP1439 respectively. PABP-CI (PABP cytoplasmic I) was amplified in a similar manner from IMAGE clone 5597273, then cloned with the oligonucleotides NM163 and NM164. It was subcloned into pEBG6P to form pEBG6P-1-PABP-CI. DAZ was amplified from IMAGE clone 5297459 with oligonucleotides MP1845 and MP1846, as described above, and subcloned into pCMV-FLAG-1 to form pCMV-FLAG-DAZ.

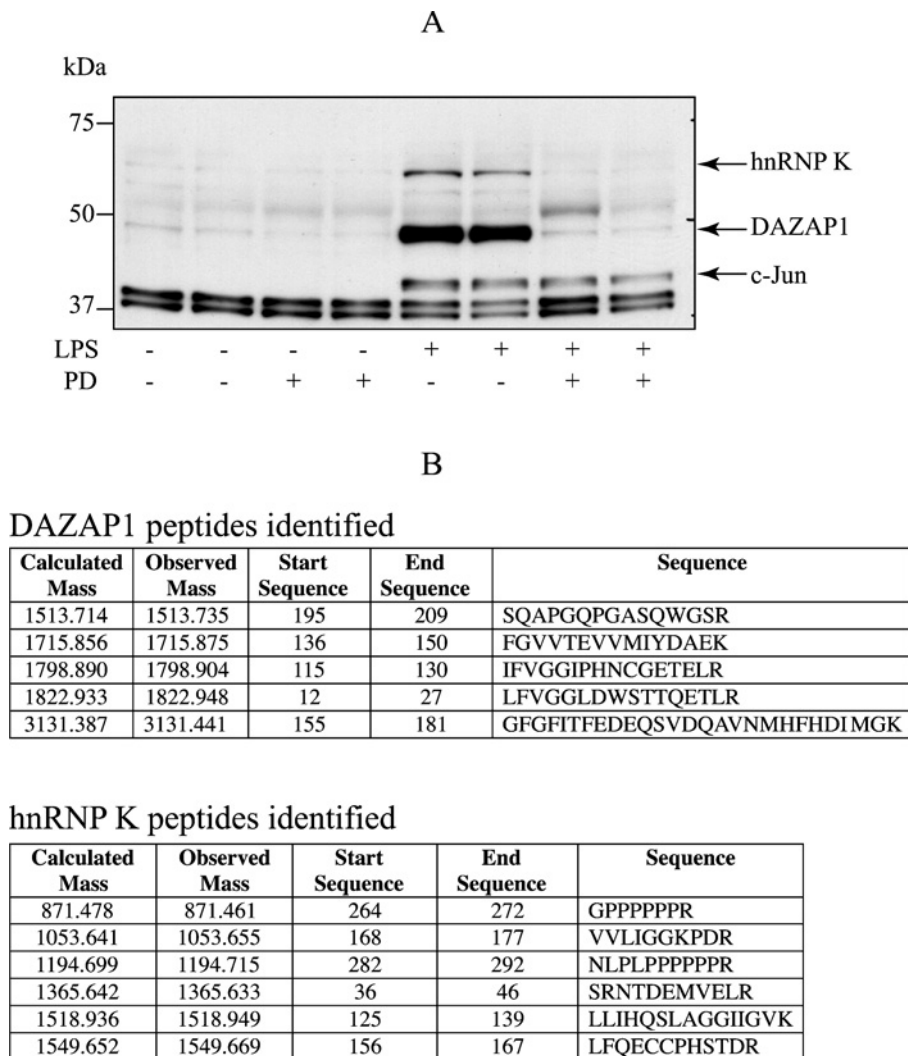
The oligonucleotides used were: MP1233, GCG GAT CCA ACA ACT CGG GCG CCG ACG AG; MP1235, GCG GAT CCC TAG CGT CGG TAG GGG TGG AAC; MP1436, TCC TAC ATC GTG TCC GCC CCT CCT GGA GGC TTT; MP1437, AAA GCC TCCA GGA GGG GCG GAC ACG ATG TAG GA; MP1438, CCT CCT CCA CCA GCC GCT CCC GGG GCA GCA CCT; MP1439, AGG TGC 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 *E. coli*

The plasmid pGEX6P-1-DAZAP1, encoding a GST (glutathione S-transferase) fusion protein of full-length human DAZAP1, was expressed in BL21 cells and induced with 50  $\mu$ M IPTG (isopropyl  $\beta$ -D-thiogalactoside) 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 BSA and keyhole-limpet haemocyanin, then injected into sheep at Diagnostics Scotland (Edinburgh, Scotland, U.K.). The antisera were affinity purified on CH–Sepharose to which the relevant phosphorylated peptide had been coupled covalently. Antibodies capable of recognizing 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 recognize HA (haemagglutinin) 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, and antibodies that recognized the active phosphorylated forms of ERK1 and ERK2 were from Cell Signalling Technologies (Hitchin, Herts., U.K.). Rabbit-, mouse- and sheep-specific secondary antibodies conjugated to horseradish peroxidase were purchased from Pierce (Rockford, IL, U.S.A.).



**Figure 1** Identification of two novel proteins phosphorylated in response to LPS in RAW 264.7 macrophages

(A) Macrophages were incubated for 1 h with or without 2  $\mu$ M PD184352 and then stimulated for 30 min with LPS (100 ng/ml) and lysed. c-Jun, immunoprecipitated from the lysates, was denatured in SDS, subjected to SDS/PAGE and transferred on to nitrocellulose. The membranes were immunoblotted with an antibody that recognizes c-Jun phosphorylated at Thr<sup>91</sup>. This detected not only c-Jun, but also two other proteins indicated by arrows that were identified in (B) as DAZAP1 and hnRNP K. (B) RAW 264.7 cell extract (2 mg of protein) was incubated for 1 h at 4 °C with Protein G–Sepharose (60  $\mu$ l) and the immunoprecipitation carried out as described in the Experimental section. The stained protein bands corresponding to the proteins recognized by the anti-phospho-c-Jun(Thr<sup>91</sup>) antibody in (A), were excised, alkylated with iodoacetamide and digested with trypsin. The resultant peptides were analysed by MALDI-TOF/TOF (matrix-assisted laser-desorption ionization–tandem time-of-flight) MS or LCMS (liquid chromatography MS) as described previously [44].

### Cell culture, transfection and cell lysis

HEK-293 (human embryonic kidney 293) cells and murine RAW 264.7 macrophages (purchased from the European Tissue Culture Collection) were maintained in a 95 % air/5 % CO<sub>2</sub> atmosphere at 37 °C. HEK-293 cells and RAW 264.7 macrophages were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10 % foetal calf serum (HEK-293 cells) or heat-inactivated foetal calf serum (RAW 264.7 cells) plus 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-glutamine. The HEK-293 cells were transfected with the indicated amounts of the different DNA constructs using poly(ethyleneimine) [29]. The cells were lysed in buffer composed of 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium  $\beta$ -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<sup>TM</sup> protease inhibitor cocktail (1 tablet/50 ml of cell lysis buffer). The lysates were

centrifuged at 13 000 g for 15 min at 4 °C and the supernatants were collected. Protein concentrations were determined using the Bradford method. Lysates (1 mg of extract) were incubated for 10 min at 37 °C with 10 units of benzonuclease to digest RNA and DNA (Novagen).

### Immunoprecipitation and immunoblotting

Cell extract (1 mg) was incubated for 2 h at 4 °C with 10  $\mu$ l of anti-HA antibody–agarose or 10  $\mu$ l of glutathione–Sepharose 4B. The beads were washed twice with 1 ml of 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 on to nitrocellulose, the membrane was immunoblotted with anti-HA, anti-FLAG or anti-GST antibodies, each at 1  $\mu$ g/ml, using the ECL<sup>®</sup> system.

### Phosphorylation of DAZAP1 by ERK2

Bacterially expressed GST-DAZAP1 was incubated for 30 min at 30°C alone or with 2 units/ml ERK2 in presence of 0.1 mM ATP and 10 mM MgCl<sub>2</sub>. The reaction was stopped by the addition of SDS to denature the samples, and the samples were subjected to SDS/PAGE as described in the Results section.

## RESULTS

### Identification of two proteins that become phosphorylated via the classical MAPK cascade in LPS-stimulated macrophages

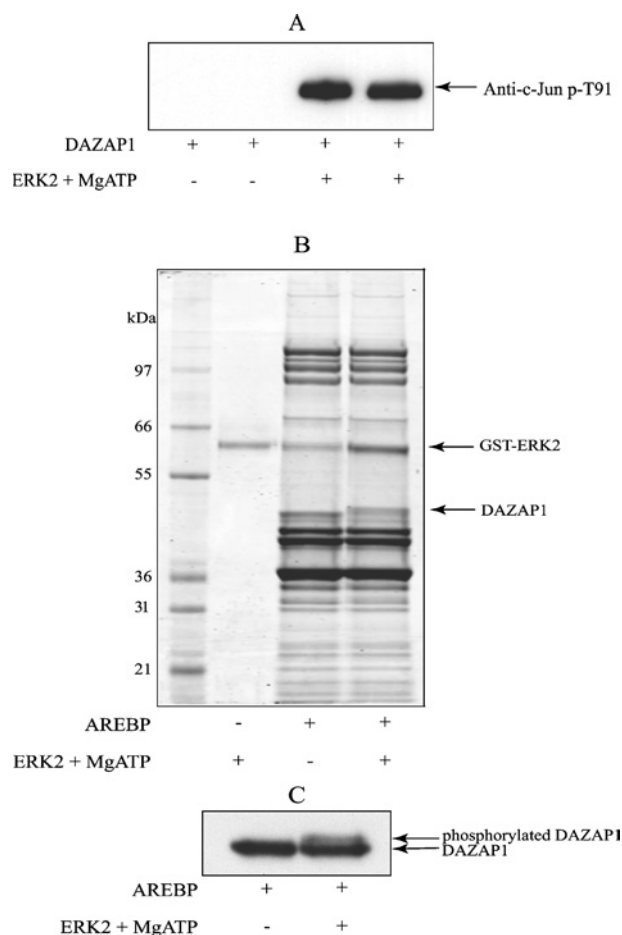
While re-investigating the multisite phosphorylation of the transcription factor c-Jun [30], we identified two proteins, distinct from c-Jun, that were present in c-Jun immunoprecipitates and were recognized 'non-specifically' by another antibody that detects c-Jun only when it is phosphorylated at Thr<sup>91</sup> (Figure 1A). However, in contrast with the LPS-induced phosphorylation of c-Jun at Thr<sup>91</sup>, which is catalysed by the MAPK family member c-Jun N-terminal kinase and therefore unaffected by PD184352, a specific inhibitor of MKK1, the LPS-induced phosphorylation of the other two proteins was prevented by this inhibitor (Figure 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 (results not shown).

The two proteins were purified from the RAW 264.7 cell extracts by affinity chromatography with Protein G-Sepharose followed by SDS/PAGE, and were identified as DAZAP1 and hnRNP K by tryptic mass fingerprinting (Figure 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 PD184352. In contrast, DAZAP1 was a novel downstream target for this pathway and therefore subjected to a more detailed analysis.

### DAZAP1 is phosphorylated at Thr<sup>269</sup> and Thr<sup>315</sup> by ERK2 *in vitro* and binds to the ARE of the mRNA encoding TNF $\alpha$

MAPKs nearly always phosphorylate serine or threonine residues that are followed by a proline, suggesting that the c-Jun Thr<sup>91</sup> phospho-specific antibody might be recognizing a phospho-Thr-Pro 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 Figure 2(A), this proved to be the case, as incubation with ERK2 and MgATP led to the recognition of this protein by the anti-Thr<sup>91</sup> antibody (Figure 2A).

We have previously identified DAZAP1 as a protein that binds specifically to the ARE of the mRNA encoding TNF $\alpha$  [17]. In order to examine whether DAZAP1 could still be phosphorylated by ERK2 when bound to this ARE, we incubated the immobilized AREBPs from RAW 264.7 macrophages with MgATP and ERK2. As shown in Figure 2(B), this led to a decrease in the mobility of DAZAP1 during SDS/PAGE, indicative of stoichiometric phosphorylation at one or more sites. MS analysis established that the band whose mobility was decreased by phosphorylation with ERK2 was indeed DAZAP1 (Figure 2B, and results not shown). Immunoblotting also showed that DAZAP1 underwent a decrease in electrophoretic mobility after phosphorylation by ERK2 (Figure 2C). Strikingly, DAZAP1 was the only major AREBP that underwent this band shift. After phosphorylation by ERK2, DAZAP1 was still retained by the ARE-conjugated



**Figure 2** Phosphorylation of DAZAP1 *in vitro* by the MAPK ERK2

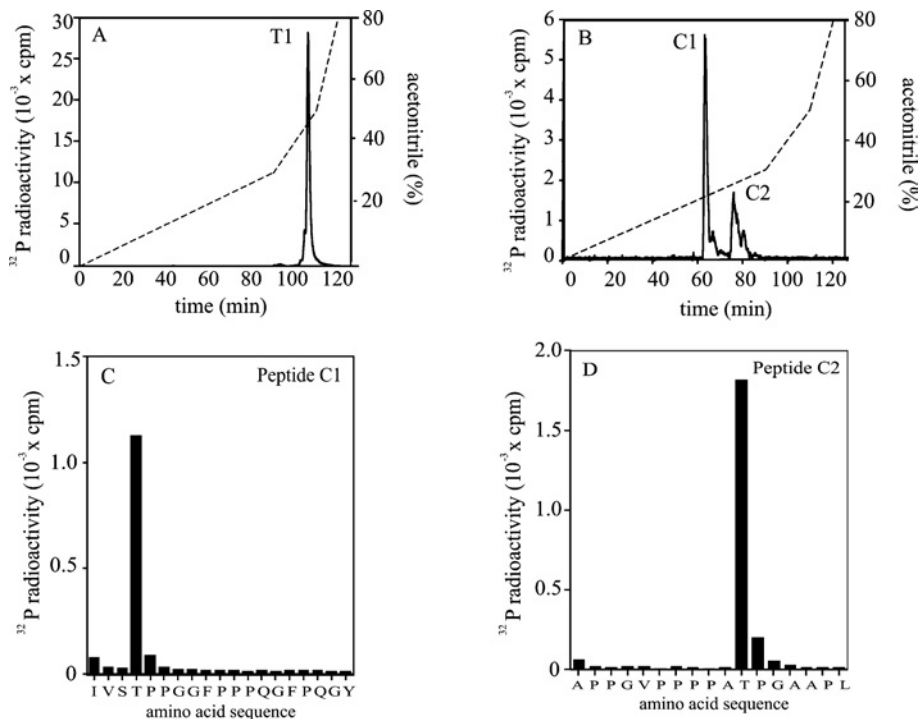
(A) Bacterially expressed GST-DAZAP1 was phosphorylated with ERK2, subjected to SDS/PAGE and immunoblotted with the anti-phospho-Thr<sup>91</sup> antibody as in Figure 1(A). (B, C) AREBPs were purified from 5 mg of RAW 264.7 macrophage extract by affinity chromatography [17], then phosphorylated as described in (A), subjected to SDS/PAGE, and the proteins were revealed 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).

beads, indicating that phosphorylation did not disrupt its ability to interact with this element (results not shown).

DAZAP1 that had been phosphorylated maximally by ERK2 was digested with trypsin and the resulting peptides were separated on a C<sub>18</sub> column. One <sup>32</sup>P-labelled peptide was detected, which was eluted at nearly 50% acetonitrile (Figure 3A), indicating that it was very large. As the size precluded identification of the site(s) of phosphorylation by MS, it was re-digested with chymotrypsin. Chromatography was carried out again on the C<sub>18</sub> column and now resolved two <sup>32</sup>P-labelled peptides, denoted C1 and C2 (Figure 3B). Analysis by MS showed that C1 and C2 were the peptides comprising residues 266–284 and 305–321 of DAZAP1, each containing one covalently bound phosphate (results not shown). Solid-phase sequencing revealed that the sites of phosphorylation in peptides C1 and C2 were located at Thr<sup>269</sup> and Thr<sup>315</sup> respectively (Figures 3C and 3D).

### DAZAP1 is phosphorylated in cells by ERK1/ERK2 at Thr<sup>269</sup> and Thr<sup>315</sup>

In order to investigate whether DAZAP1 was phosphorylated at Thr<sup>269</sup> and Thr<sup>315</sup> in cells, we generated phospho-specific



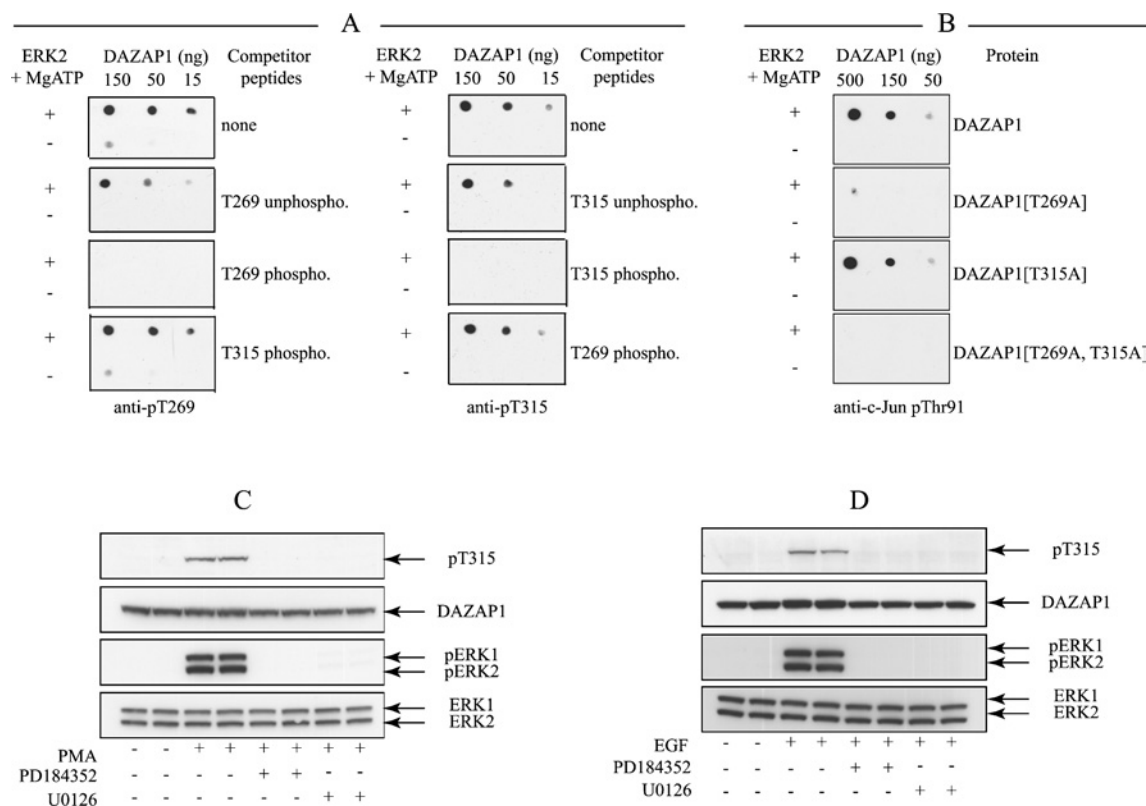
**Figure 3** Identification of the residues on DAZAP1 phosphorylated by ERK2

GST-DAZAP1 was maximally phosphorylated with GST-ERK2, as in Figure 2, and subjected to SDS/PAGE. The band corresponding to  $^{32}\text{P}$ -labelled DAZAP1 was revealed by staining with Coomassie Blue, excised and digested with trypsin. (A) The tryptic phosphopeptides were separated by HPLC on a Vydac  $\text{C}_{18}$  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 continuous line. (B) The major  $^{32}\text{P}$ -labelled peptide T1 from (A) was re-digested with chymotrypsin and re-chromatographed on the  $\text{C}_{18}$  column as in (A). 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 MS analysis.

antibodies that recognize these phosphorylated epitopes. The anti-phospho-Thr<sup>269</sup> 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 Thr<sup>315</sup> phosphorylated site (Figure 4A). Similarly, the anti-phospho-Thr<sup>315</sup> 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 Thr<sup>269</sup> phosphorylated site (Figure 4A). The anti-phospho-Thr<sup>269</sup> 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 (Figure 4A, top left-hand panel). Interestingly, the antibody that recognized c-Jun phosphorylated at Thr<sup>91</sup> only recognized DAZAP1 phosphorylated at Thr<sup>269</sup>, as shown by experiments using variants of DAZAP1 in which either Thr<sup>269</sup> or Thr<sup>315</sup> had been replaced by an alanine residue. (Figure 4B). The recognition of DAZAP1 by this antibody in LPS-stimulated macrophages (Figure 1A) therefore establishes that DAZAP1 is phosphorylated by ERK1/ERK2 at Thr<sup>269</sup> in these cells. Similarly, the anti-phospho-Thr<sup>315</sup> antibody also recognized DAZAP1 in PMA- (Figure 4C) or EGF-stimulated (Figure 4D) HEK-293 cells. In each case phosphorylation was prevented by PD184352 or U0126, the latter being another MKK1 inhibitor that is structurally unrelated to PD184352. Thus DAZAP1 becomes phosphorylated at both Thr<sup>269</sup> and Thr<sup>315</sup> by ERK1/ERK2 in response to agonists that activate the classical MAPK cascade.

### Phosphorylation of DAZAP1 induces its dissociation from DAZ

DAZAP1 was originally identified as a binding partner for 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. Similar to DAZAP1, DAZ is an RNA-binding protein containing RRM motifs (RNA-recognition 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 along with forms of HA-DAZAP1 in which Thr<sup>269</sup> and/or Thr<sup>315</sup> 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 (Figure 5A). Further experiments using mutants of DAZAP1 in which either Thr<sup>269</sup> or Thr<sup>315</sup> was changed to Asp showed that each single mutation greatly reduced the interaction with DAZ, although the



**Figure 4** DAZAP1 is phosphorylated in cells at Thr<sup>269</sup> and Thr<sup>315</sup> 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 recognized DAZAP1 phosphorylated at either Thr<sup>269</sup> (anti-pT269) or Thr<sup>315</sup> (anti-pT315) in the absence (none) or presence of 10  $\mu$ g/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 Thr<sup>91</sup> (anti-c-Jun pThr91) in the presence of 10  $\mu$ g/ml of the unphosphorylated form of the phosphopeptide immunogen [30]. (C, D) HEK-293 cells were pre-incubated for 1 h without or with 2  $\mu$ M PD184352 or 10  $\mu$ M 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 on to nitrocellulose membranes and immunoblotted with an antibody that recognized DAZAP1 phosphorylated at Thr<sup>315</sup> (pT315) and with an antibody that recognises the phosphorylated and unphosphorylated forms of DAZAP1 equally well (DAZAP1). Further samples of the cell lysates were immunoblotted using antibodies that recognized phosphorylated ERK1 and ERK2 (pERK1, pERK2) or the phosphorylated and unphosphorylated forms of ERK1/ERK2 equally well (ERK1, ERK2). Similar results were obtained three different experiments.

Thr<sup>269</sup>→Asp mutation had the strongest effect (Figure 5A). The combined mutation of Thr<sup>269</sup> and Thr<sup>315</sup> to Asp also decreased significantly the interaction between full-length DAZAP1 and DAZ (Figure 5B). 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 HEK-293 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 with control incubations performed in the absence of MgATP and/or ERK2 (Figure 6A). However, this dissociation did not occur when the non-phosphorylatable form of DAZAP1(T269A/T315A) was co-transfected with DAZ (Figure 6B).

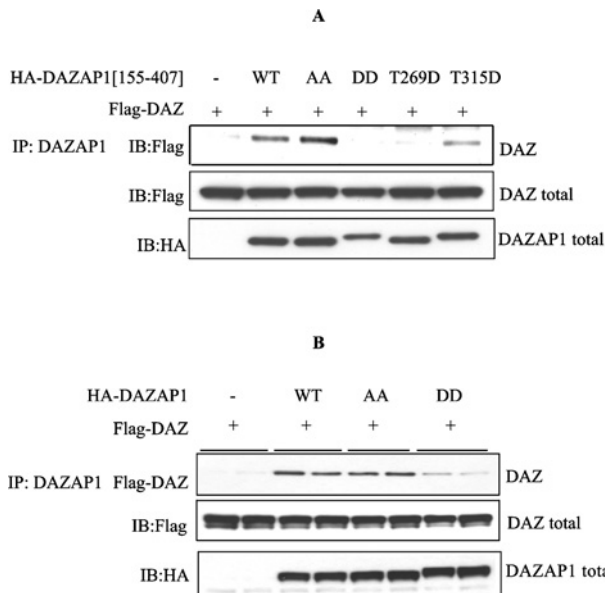
#### Evidence that DAZAP1 cannot bind simultaneously to both DAZ and PABP1

It has recently been reported that DAZ also interacts with PABP1, thereby stimulating the translation of mRNAs containing short poly(A) 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 was immunoprecipitated from the extracts. Interestingly, DAZAP1 was absent from PABP1 immunoprecipitates (Figure 7A) and PABP1 was absent from DAZAP1 immunoprecipitates (Figure 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 the present study, we identified DAZAP1 as a new physiological substrate for ERK1/ERK2 because of its cross-reactivity with an antibody that recognizes the Thr<sup>91</sup> phosphorylation site on c-Jun and by its ‘non-specific’ binding to Protein G–Sepharose. We have found that approx. 2% of the proteins in cell extracts are pulled down by Protein G–Sepharose, many of which we have identified by MS as RNA-binding proteins (results not shown). Indeed the ‘sticky’ nature of these proteins has been well documented, and procedures for minimizing their interaction with chromatographic matrices have been published [36]. Interestingly,



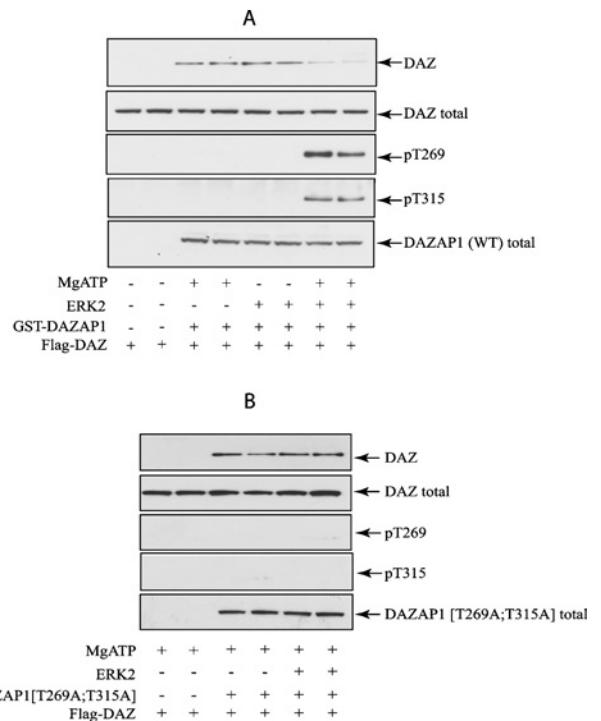
**Figure 5** A phospho-mimetic form of DAZAP1 does not interact with DAZ

(A) HEK-293 cells were transfected without (–) or with 2.5 μg of FLAG-DAZ plasmid DNA (+), 2.5 μg of DNA expressing unmodified HA-DAZAP1(155–407) (WT), HA-DAZAP1(155–407) in which Thr<sup>269</sup> and Thr<sup>315</sup> had both been mutated to Ala (AA), HA-DAZAP1(155–407) in which Thr<sup>269</sup> and Thr<sup>315</sup> had both been mutated to Asp (DD) or singly mutated forms of DAZAP1 in which either Thr<sup>269</sup> or Thr<sup>315</sup> had been mutated to Asp (T269D and T315D). After 36 h, DAZAP1 was immunoprecipitated (IP) from 1 mg of cell extract protein with an anti-HA antibody and immunoblotted (IB) with anti-HA and anti-FLAG antibodies. (B) As described for (A), except that the cells were transfected with full-length forms of DAZAP1.

we have previously identified DAZAP1 as one of the more abundant proteins that interacts specifically with the ARE of the TNFα mRNA [17] and the c-Fos mRNA (S. Rousseau, unpublished work), and other investigators have shown that its RRM binds preferentially to poly(U) RNA homopolymers compared with poly(G) or poly(A) [32]. These findings raise the possibility that DAZAP1 might play a role in the regulation of mRNA stability/translation by the classical MAPK cascade.

DAZAP1 was first identified as a protein that interacts with DAZ and the structurally related DAZL (DAZ-like 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 polyribosomes 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 poly(A) tails [26].

The key finding of our present study is that the phosphorylation of DAZAP1 at Thr<sup>269</sup> and Thr<sup>315</sup> 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 RRMs. Deletion of the RRMs disrupts RNA binding [32], but not the interaction with DAZ (Figure 5), indicating that the sites of phosphorylation may be in the vicinity of the DAZ interaction site [32,35].

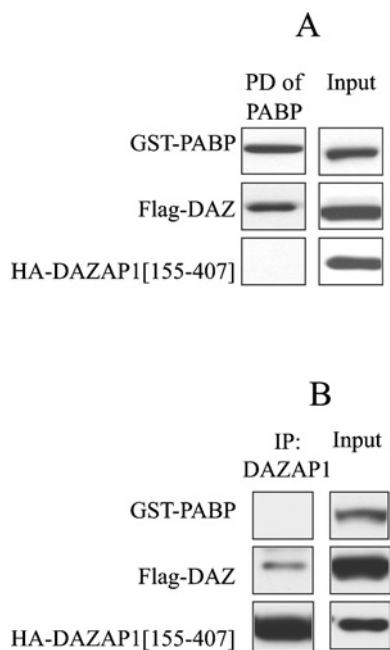


**Figure 6** Phosphorylation of full-length DAZAP1 by ERK2 impairs its binding to DAZ

HEK-293 cells were transfected with 1 μg of DNA encoding FLAG-DAZ and with 0.5 μg of 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 of protein) was incubated for 2 h at 4 °C with glutathione-Sepharose (20 μl). 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 on to nitrocellulose membranes and immunoblotted. The GST-DAZAP1 in the extract (DAZAP1 total) was revealed with an anti-GST-antibody (DAZAP1) and FLAG-DAZ with an anti-FLAG antibody (DAZ). The membranes were also immunoblotted with the phospho-specific antibody that recognizes DAZAP1 phosphorylated at Thr<sup>315</sup> (pT315) and with the phospho-specific antibody that not only recognizes c-Jun phosphorylated at Thr<sup>91</sup>, but also DAZAP1 phosphorylated at Thr<sup>269</sup> (pT269).

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 circularization (see the Introduction). Consistent with this hypothesis, we found that DAZ could not bind simultaneously to both DAZAP1 and PABP (Figure 7), and others have reported that, in contrast with 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 expressed not only 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 264.7 macrophages [17] (Figure 1A). As no other protein with DAZ motifs has been found by searching databases (results not shown), this raises the interesting question as to the identity of the binding partner(s) for



**Figure 7** DAZAP1 and PABP exist as distinct complexes with DAZ

(A) HEK-293 cells were transfected with 0.2  $\mu$ g of DNA encoding FLAG-DAZ, 1  $\mu$ g of DNA encoding GST-PABP or 1  $\mu$ g of 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  $\mu$ l of glutathione-Sepharose beads to pull down (PD) GST-PABP. After extensive washing (see the Experimental section), the proteins were released from the beads by denaturation in SDS and immunoblotted with anti-HA antibody to detect DAZAP1, anti-GST antibody to detect PABP or anti-FLAG antibody to detect DAZ. (B) As described for (A), except that DAZAP1 was immunoprecipitated (IP) with an anti-HA antibody.

DAZAP1 in macrophages and other cells which do not express DAZL. 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 the Introduction), it is clearly now critical to find its binding partners in macrophages.

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