The N-terminal region of ABC50 interacts with eukaryotic initiation factor eIF2 and is a target for regulatory phosphorylation by CK2

Sonia PAYTUBI*, Nicholas A. MORRICE†, Jerome BOUDEAU† and Christopher G. PROUD*‡†

*Division of Molecular Physiology, School of Life Sciences, University of Dundee, Dundee, DD1 5EH, U.K., †Medical Research Council Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dundee, DD1 5EH, U.K., and ‡Department of Biochemistry and Molecular Biology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC, V6T 1Z3, Canada

ABC50 is an ABC (ATP-binding cassette) protein which, unlike most ABC proteins, lacks membrane-spanning domains. ABC50 interacts with eIF2 (eukaryotic initiation factor 2), a protein that plays a key role in translation initiation and in its control, and in regulation of ribosomes. Here, we establish that the interaction of ABC50 with eIF2 involves features in the N-terminal domain of ABC50, the region of ABC50 that differs most markedly from other ABC proteins. This region shows no apparent similarity to the eIF2-binding domains of other partners of eIF2. In contrast, the N-terminus of ABC50 cannot bind to ribosomes by itself, but it can in conjunction with one of the nucleotide-binding domains. We demonstrate that ABC50 is a phosphoprotein and is phosphorylated at two sites by CK2. These sites, Ser-109 and Ser-140, lie in the N-terminal part of ABC50 but are not required for the binding of ABC50 to eIF2. Expression of a mutant of ABC50 in which both sites are mutated to alanine markedly decreased the association of eIF2 with 80S ribosomal and polysomal fractions.

Key words: ABC protein, CK2, eIF2, ribosome, translation initiation.

INTRODUCTION

ABC50 (ABCF1), like other members of the ABC (ATP-binding cassette) family of proteins, possesses two such cassettes [1] (Figure 1). It also contains an N-terminal region of unknown function. The two NBDs (nucleotide-binding domains) show (as one would expect) strong similarity to these regions within the other ABC proteins [1,2]. Unlike most other members of the group, however, ABC50 lacks recognizable transmembrane domains.

ABC50 was first identified as a protein whose expression is increased following treatment of synoviocytes with tumour necrosis factor α [1]. ABC50 was subsequently discovered independently as a protein that co-purifies extensively with eIF (eukaryotic initiation factor) 2 [2]. Sequence analysis revealed that ABC50 is a close relative of the yeast protein Gcn20p, which is required for the amino acid control of the yeast eIF2 kinase, Gcn2p. Gcn2p is activated by binding to uncharged tRNA molecules [3]. Gcn20p is thought to co-operate with Gcn1p to bring uncharged tRNAs to Gcn2p during the elongation process: this couples the availability of amino acids for tRNA charging to bring uncharged tRNAs to Gcn2p during the elongation process: this couples the availability of amino acids for tRNA charging to the control of Gcn2p [4]. However, Gcn20p and ABC50 differ in important respects. For example, while Gcn20p associates with ribosomes that are engaged in elongation, ABC50 apparently mainly binds to ribosomes involved in initiation [2]. In addition, while Gcn20p and ABC50 are similar in their ABC domains, they differ markedly in their N-termini: while ABC50 and Gcn20p share 30% identity and 57% similarity across their ABC domains, their N-termini show only 20% identity and 30% similarity. Bearing these points in mind, and considering that it is only the N-terminus of Gcn20p that is required to support the function of Gcn2p in yeast [4], it seemed likely that ABC50 and Gcn20p have distinct functions.

Consistent with this, Tyzack et al. [2] showed that ABC50 stimulates the formation of complexes between eIF2, GTP and the Met-tRNAi, (initiator methionyl-tRNA) in vitro. It did so without affecting the binding of guanine nucleotides to eIF2, indicating the effect is likely to be on the association of Met-tRNA, with eIF2.

eIF2 also interacts with at least two other components of the translational machinery. These are its cognate GAP (GTase-activator protein), eIF5 [5,6] and GEF (guanine-nucleotide-exchange factor), eIF2B (reviewed in [7,8]). The interaction between eIF2 and eIF2B involves ε, the catalytic subunit of eIF2B. The regions of eIF2Bε and eIF5 that bind eIF2 are rich in aromatic and acidic residues [9,10] and contain phosphorylation sites for CK2 (formerly known as casein kinase 2 [11–13]). The CK2 sites in eIF2Bε are required for its stable binding to eIF2 [11] and those in eIF5 are required for the formation of initiation complexes [12].

In this report, we show that ABC50 binds to eIF2 via its N-terminal region and identify in vivo phosphorylation sites within this region that are phosphorylated by CK2. These sites are apparently not required for the binding of ABC50 to eIF2, but do influence the association of eIF2 with ribosomes. This is a further example of the importance of CK2-mediated phosphorylation for the interactions and/or functions of eukaryotic translation factors.

EXPERIMENTAL

Cell culture and transfections

HEK293 (human embryonic kidney) cells were grown in 6- or 10-cm-diameter plates in DMEM (Dulbecco’s modified Eagle’s medium, Gibco BRL) supplemented with 10% (w/v) fetal bovine serum (Gibco BRL). Transient transfections were carried out by calcium phosphate precipitation of 10 µg DNA in BES (N,Nbis(2-hydroxyethyl)-2-aminoethanesulfonic acid)-buffered saline pH 6.69 with cells at a density of approx. 6 × 10^6 to 8 × 10^6 per 6-cm-diameter plate or 1.5 × 10^6 to 2 × 10^6 per 10-cm-diameter plate [14].

Abbreviations used: ABC, ATP-binding cassette; DMEM, Dulbecco’s modified Eagle’s medium; eIF, eukaryotic initiation factor; eIF2Bε, catalytic subunit of eIF2B; GAP, GTase-activator protein; GEF, guanine-nucleotide-exchange factor; HA, haemagglutinin; HEK293 cells, human embryonic kidney cells; Met-tRNAi, initiator methionyl-tRNA; NBD, nucleotide-binding domain.

† To whom correspondence should be addressed (email cgpr@interchange.ubc.ca)
abc50 contains an N-terminal region and two NBDs. Residue numbers are indicated, as are some of features of the ABC domains (e.g., the “Walker boxes”). Also shown is the region involved in binding to eIF2 (residues 1–42) and the two phosphorylation sites identified in this study (Ser-109 and Ser-140). The extreme C-terminus of human eIF2α and eIF5, which bind eIF2α [9], and residues 1–42 of human ABC50, that also bind eIF2, are shown here. For eIF2α and eIF5, the hydrophobic and acidic residues that are involved in binding to eIF2 are shaded light and mid-grey respectively. The alignment is based on that in [9]. The phosphorylation sites for CK2 in eIF2B are shown white on a dark grey background. There are no obvious sequence similarities between the ABC50 sequence and those of eIF2α/eIF5, and a BLAST search showed no significant similarity. Such a search also recorded no similarity between any part of ABC50 and these regions of eIF2α/eIF5.

Cell harvesting and lysis

After treatment, cells were washed once with PBS and harvested in 400 µl of harvesting buffer [20 mM Hepes/KOH (pH 7.4), 50 mM KCl, 50 mM β-glycerophosphate, 0.2 mM EDTA, 10% glycerol, 1% (v/v) Triton X-100, 1 mM diithiothreitol, 1 mM PMSF, 1 mM benzamidine and 1 µg/ml each of leupeptin, antipain and pepstatin]. Cell debris and nuclei were spun down for 1 min at 12,000 g and the supernatant was transferred to a new tube. For the identification of phosphorylation sites in endogenous ABC50, the lysis buffer contained 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM KCl, 50 mM β-glycerophosphate, 0.2 mM EDTA, 10% glycerol, 1% (v/v) Triton X-100, 0.27 M sucrose, 0.1% 2-mercaptoethanol and the protease inhibitors.

Immunoprecipitation

For anti-HA and anti-ABC50 immunoprecipitations, 2 µl of the appropriate antibody was added to cell extracts and incubated for 1 h at 4°C. Subsequently, protein G-Sepharose beads (20 µl of packed beads per immunoprecipitation) in wash buffer (harvesting buffer without Triton X-100) was added and incubated for a further 2 h at 4°C. The beads were then washed three times with 1 ml of wash buffer and finally resuspended in SDS/PAGE loading buffer.

Plasmids and primers

The plasmid containing the different forms of ABC50 used in this work for all the mammalian transient transfections was pCMV5. Primer 5′-GATCTTCCATGATCATATGGTTCACTTGA-3′ and its complementary 3′–5′ partner were self-hybridized and ligated to pCMV5 (5′ end, BgIII; 3′ end, HindIII) to create plasmid pCMV5-HA. The cDNA for the ABC50 full-length protein was amplified from the plasmid pET28c-ABC50 by PCR using primers 5′-CCCAAGCTTCCAGAAGCGCCAAGCAGCAGCCGCCGAGG-3′ (forward) and 5′-TACCCGGGTTCACTTCCGGCCGGCT-3′ (reverse) which contained restriction overhangs (5′ end, HindIII; 3′ end, SmaI respectively). The amplified and digested product was cloned into plasmid pCMV5-HA. The mutations in pCMV5-HA-ABC50 were created using the QuikChange® kit from Stratagene. The N-terminal truncation (from residue 1 to 269) was created by adding a stop codon at position 270 into plasmid pCMV5-HA-ABC50. Plasmid pCMV5-HA containing the truncation N+1 (with the N-terminal region and NBD1; residues 1–569) was created by PCR using primers that contained restriction overhangs (5′ end, HindIII; 3′ end, SmaI respectively). Truncation N+2 (residues 1–269 and 570–end) was created by ligating ABC2 (corresponding to residues 570 to 804, amplified by PCR using primers that contained BamHI at both ends) and introduced into the plasmid encoding the HA (haemagglutinin)-tagged 1–269 fragment. The stop codon after the N-terminal region was then removed using QuikChange®.

Truncations ATP1 and ATP2 were created by PCR using primers that contained restriction overhangs 5′–HindIII/3′–SmaI and 5′–HindIII/3′–BamHI respectively. Also, a truncation containing both NBDs (ATP1 + ATP2) was generated by PCR using primers that contained restriction overhangs 5′, HindIII/3′, SmaI.

Vectors encoding ABC50 residues 1–269, 270–569, 570–804, 92–269, 184–269 and 43–269 were created by PCR. The primers used introduced 5′, EcoRI and 3′, NotI sites respectively. After digestion, these truncated cDNAs were introduced into plasmid pGEX-6P-1. Truncations 1–42, 1–91 and 1–183 were generated in each case to a stop codon. Truncation 92–183 was generated from the vector encoding 1–269 by mutating the next residue at position 184 using the QuikChange® kit.

DNA sequencing was performed by the Sequencing Service [School of Life Sciences, University of Dundee, Scotland, U.K. (http://www.dnaseq.co.uk)] using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers.

Gel electrophoresis and immunoblotting

Proteins were separated on SDS/PAGE (12.5% gel) followed by transfer to Immobilon-P PVDF membranes (Millipore) and detected by Western blot analysis. Blots were visualized by ECL® (enhanced chemiluminescence) (Amersham Pharmacia Biotech).

Antibodies

The antibody for eIF2α was obtained from New England Biolabs. HA and Actin 20-33 antibodies were from Roche and Sigma.
respectively. Antibodies for rpS6 and rpL28 were from Cell Signaling and Santa Cruz Biotechnology respectively.

Rabbit anti-ABC50 antibodies

To prepare antisera for ABC50, rabbits were immunized with 4 doses each of 200 µg recombinant His-ABC50 protein, expressed and purified from Escherichia coli as described below. All immunization procedures were performed at Diagnostics Scotland (Edinburgh, U.K.). Polyclonal antiserum was purified over columns comprising immobilized recombinant His-ABC50. Recombinant His-ABC50 protein (2 mg) was expressed and purified from E. coli, dialysed overnight at 4°C against a dialysis buffer [100 mM NaHCO₃, pH 8.0, 500 mM NaCl, 10% (v/v) glycerol and protease inhibitors] and finally was conjugated to activated CH-Sepharose 4B (GE Healthcare). Antibody purification was performed according to the manufacturer’s instructions.

Production of recombinant proteins in E. coli

E. coli strains BL21 (DE3) or E. coli DH5α were used for protein expression. One litre of culture was grown at 37°C to a D₆₀₀ of 0.6–0.8, and then IPTG (isopropyl β-D-thiogalactoside) was added to 1 mM. Further incubation was carried out for 4 h at 21°C. Cells were collected by centrifugation and resuspended in 20 ml of buffer A [20 mM Hepes (pH 7.9), 10% (v/v) glycerol, 100 mM KCl, 5 mM MgCl₂, 20 mM imidazole] for cultures expressing GST-tagged proteins or lysis buffer [20 mM Hepes (pH 7.4), 200 mM NaCl, 1 mM EDTA, 0.5% (v/v) Nonidet P40, 10% (v/v) glycerol] for those expressing GST-tagged proteins. Cells were lysed by sonication. The extract was centrifuged at 1600 g for 30 min at 4°C. His-tagged proteins were purified by Ni²⁺-NTA technology [15] and eluted in buffer A containing 500 mM imidazole. GST-tagged proteins were further purified by using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and subsequently removed from the resin/GST by cleavage with PreScission protease as described below.

Protein–protein binding experiments

To test the ability of recombinant ABC50 and variants to bind to partner proteins, lysates from bacteria expressing these GST-tagged proteins were incubated with glutathione-Sepharose 4B beads. After incubation for 2 h at 4°C, the beads were washed with PBS and 1 mg of cleared HEK293 cell lysate (obtained as described above) was then added to the same beads and incubated for 90 min at room temperature, then the beads were washed with PBS. The recombinant ABC50 fragment plus any associated proteins were released by cleavage by the addition of 3 units of PreScission protease at 4°C overnight in PP buffer [50 mM Tris/HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol].

Sucrose-cushion centrifugation

HEK293 cells were lysed at 70–80% confluency in ice-cold SDG100 buffer [50 mM Heps/KOH (pH 7.6), 2 mM MgCl₂, 100 mM KCl, 1 µg/ml antipain, 1 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM PMSF, 1 µM microcystin] containing 0.1% (v/v) Triton X-100. Nuclei and membranous material were removed by centrifugation (15 min at 3500 g, 4°C). The cleared lysate (3 ml) was layered gently onto 1.5 ml of 0.8 M sucrose (in SDG100 buffer) and centrifuged (150 min at 49 000 rev./min, 4°C) in a Beckman SW55Ti rotor. After centrifugation, the different layers were removed separately and, after a brief but careful rinse in 200 µl of SDG100, the ribosomal pellet was resuspended in SDG100 buffer or SDG500 (as SDG100 buffer but with 500 mM KCl). The salt-washed ribosomes were then centrifuged as before but using a 0.8 M sucrose cushion in SDG500 buffer.

Sucrose density gradient centrifugation

HEK293 cells were grown in 15-cm-diameter dishes and lysed with SDG100 buffer plus 0.1% (v/v) Triton X-100 as above. The cleared lysate (1.2 ml) was layered on to a 20–50% (w/v) sucrose gradient (10.5 ml, prepared in SDG100 buffer) and centrifuged (3.5 h at 25 000 rev./min, 4°C) in a Beckman SW41Ti rotor. Each gradient was fractionated using a Brandel model 184 fractionator by upward displacement with 60% (w/v) sucrose at a flow rate of 1 ml/min. The A₅₄₆ of the displaced gradient was analysed in a 5-mm path-length ISCO Type 6 Optical Unit and recorded using an ISCO model UA-5 absorbance monitor.

In vitro protein phosphorylation

CK2 (0.2 units; Promega) per reaction were incubated in a total volume of 20 µl in 20 mM Heps/KOH (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 5% glycerol, 200 µM ATP, 1 µCi [γ-³²P]ATP and 10 µl of recombinant His-ABC50 for 30 min at 37°C. The reactions were stopped by adding SDS/PAGE sample buffer and heating for 5 min at 95°C. Samples were analysed by SDS/PAGE and autoradiography.

Mapping of in vivo phosphorylation sites

Growing HEK293 cells were washed three times with phosphate-free DMEM (Gibco BRL) and grown in this medium for 1 h. Then they were washed again three times and [³²P]P, (2 mCi) was added to 5 ml of phosphate-free DMEM and cells were left for 4 h. Cells were lysed and HA-ABC50 was isolated by immunoprecipitation as described above. Samples were analysed by SDS/PAGE and autoradiography. Subsequent treatment was as described below for phosphopeptide mapping.

Phosphopeptide mapping

Phosphorylated ABC50, obtained either by in vivo cell labelling or by in vitro kinase assay, was separated by SDS/PAGE (12.5% gel). Labelled bands detected by autoradiography were excised. The protein was reduced using 10 mM dithiothreitol in 100 mM Tris/HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol.

Phosphoamino acid analysis

Phosphorylated samples obtained as described above were dried. Peptides were hydrolysed by heating at 110°C in 100 µl 6 M HCl for 90 min. The mixture was dried again and dissolved in 2 µl of pyridine acetate pH 3.5 (water/acetic acid/pyridine, 945:50:5, by vol.). Unlabelled phosphotyrosine, phosphothreonine and phosphoserine (markers), each at 5 nmol, were added, and similar mixtures were run alongside as standards. The samples were spotted on to TLC plates (Merck). The samples were run using
RESULTS AND DISCUSSION

ABC50 is a phosphoprotein

Numerous components of the translational machinery are subject to phosphorylation and, in many cases, this is important for their regulation and/or their interactions. To determine whether ABC50 is phosphorylated in living cells, HA-ABC50 was expressed in HEK293 cells and then isolated and subjected to tryptic digestion followed by mass spectrometric analysis. Two phosphopeptides were identified from this analysis: LSVPT-pSDEEDEVPAKPR and GGNVFAALIQDQpSEEEEEEEKHPKPAKPK (Figure 2A). The sequences of these two peptides were confirmed by both nano-LC–MS with precursor ion scanning and by performing a second nano-LC–MS run using an inclusion list for the masses of these peptides in their +2, +3 and +4 charge states. The first peptide was detected in the +2 charge state and the second peptide was detected in +3 and +4 charge states. These phosphopeptides contain phosphorylated forms of Ser-109 and Ser-140, the former being the major phosphorylation site as judged from this LC–MS analysis.

In order to confirm that the phosphorylation of these sites is not an artefact of overexpressing tagged ABC50, we performed a similar analysis for endogenous ABC50 (after immunoprecipitation from HEK293 cells lysates using anti-ABC50). This yielded the two peptides described above plus the peptide KLSVPTpSDEEDEVPAKPR, which is generated by tryptic cleavage and contains Ser-109. This demonstrates that both Ser-109 and Ser-140 are phosphorylated in endogenous ABC50.

Identification of phosphorylation sites in recombinant or endogenous ABC50

HA-ABC50 was transfected to HEK293 cells and immunoprecipitated as described above. For ABC50 analysis, 50 dishes (10-cm-diameter) of HEK293 cells were lysed (see above) at a ratio of 1 ml per dish. Cell debris and nuclei were spun down for 20 min at 20,000 g and the resulting supernatant was mixed with 500 µl of Protein G-Sepharose (pre-equilibrated) for 30 min at 4°C. In parallel, 120 µl of Protein G-Sepharose was mixed with 120 µg anti-ABC50 in 2 ml of PBS at 4°C. After 45 min, the beads were washed once with PBS and the cleared supernatant (188 µg of protein) was then mixed with the antibody-coated beads for 90 min at 4°C. After this time, the beads were washed 4 times with lysis buffer supplemented with 500 mM NaCl and washed 4 more times with buffer B [10 mM Tris/HCl (pH 7.5), 0.1 mM EGTA and 0.1% 2-mercaptoethanol]. Immunoprecipitated protein was eluted in 300 µl of lysis buffer (1:20 dilution) and dried down in a Speed-Vac to a final volume of 40 µl. Dithiothreitol (10 mM) was added to the sample and loaded on to a 4–12% Bis-Tris gel. Following electrophoresis, the gel was stained with colloidal Coomassie Blue and the band corresponding to HA-ABC50 was excised and digested with trypsin. Tryptic digests of overexpressed HA-ABC50 or endogenous ABC50 were separated by nano-LC (nano-liquid chromatography) on a PepMapC18 (0.075 × 150 mm) column, equilibrated with 0.1% (v/v) formic acid in acetonitrile/water (1:49, v/v) at 350 nl/min, connected to an LC-Packings Ultimate HPLC system (Dionex U.K.). Peptides were eluted with a discontinuous acetonitrile gradient over 48 min (0–30 min, 2–30% acetonitrile; 30–35 min, 30–90% acetonitrile; 35–40 min, 90% acetonitrile; 40–45 min, 90–2% acetonitrile; flowrate = 300 nl/min) and the column eluate was connected to an in-line tee (Upchurch) and mixed with 80% (v/v) propan-2-ol in water, delivered from a second pump, at 100 nl/min. The combined flow was analysed by electrospray MS using an Applied Biosystems 4000 Q-TRAP mass spectrometer, as described in [17]. Phosphopeptides were detected by precursor ion scanning in the negative ion mode as described previously [17]. All these phosphopeptides were confirmed by both nano-LC–MS with precursor ion scanning and by performing a second nano-LC–MS run using an inclusion list for the masses of these peptides in their +2, +3 and +4 charge states. The first peptide was detected in the +2 charge state and the second peptide was detected in +3 and +4 charge states. These phosphopeptides correspond to phosphorylated forms of Ser-109 and Ser-140, the former being the major phosphorylation site as judged from this LC–MS analysis.

To test whether ABC50 is indeed a substrate for CK2, recombinant ABC50 made in E. coli was incubated with CK2 in vitro. Use of [γ-32P]ATP confirmed radiolabelling of ABC50 (Figure 2B). To test whether this involved the two potential CK2 sites, we created single and double mutants where these residues were changed to non-phosphorylatable alanines. The S109A and S140A mutants also underwent phosphorylation by CK2, but at reduced levels compared with wild-type ABC50 (Figure 2B). The S109A/S140A double mutant was not labelled at all when incubated with CK2 (Figure 2B). Taken together, these results indicate these sites could be targets for phosphorylation by CK2 in living cells.

To assess whether these sites are also labelled in live cells, wild-type HA-ABC50 and the S109A and S140A mutants were expressed in HEK293 cells, which were then subjected to metabolic labelling using [35S]methionine. The radiolabelled HA-ABC50 proteins were isolated by immunoprecipitation and digested with trypsin and Asp-N as, in pilot experiments (not shown), this ‘double digest’ was found to yield better peptide maps than either protease alone. The resulting peptides were displayed by two-dimensional mapping (Figure 2C). Several phosphopeptides were seen in maps from wild-type ABC50, five major species being indicated by the arrows ‘a–e’ in Figure 2(C). For each of the single mutants S109A and S140A, distinct peptides were missing from the maps generated from the in vivo-labelled ABC50 (Figure 2C). Thus, the map from the S109A mutant lacked phosphopeptides ‘b’, ‘d’ and ‘e’ (there are several overlapping peptides in this region of the map), but still contained peptide ‘a’ (Figure 2C, middle section). The S140A maps lacked peptide ‘a’, but contained peptides ‘b’ and ‘d’ (Figure 2C, bottom section). The reason that mutation of a single site (S109) causes the loss of several peptides may be because there are...
Figure 2  ABC50 is a phosphoprotein

(A) HA-ABC50 was expressed in HEK293 cells and immunoprecipitated using anti-HA antibodies. The purified protein was subjected to tryptic cleavage and tryptic mass fingerprint analysis, leading to the identification of two phosphopeptides. Their sequences and the sites of phosphorylation are shown. The position of the phosphoserine in each peptide is indicated (pS) and the cleavage sites for Asp-N or trypsin are shown (arrows). (B) Bacterially-expressed His-tagged ABC50 wild-type, S109A, S140A or S109A/S140A mutants were purified and then incubated with CK2 in vitro for 0, 5, 15 or 30 min at 37°C in the presence of [γ-32P]ATP. The upper part shows an autoradiograph of the resulting gel. The lower part shows the Coomassie-stained SDS/PAGE from which the autoradiograph was obtained. The relative level of radiolabelling (as % wild-type, normalized for total ABC50) is given below each lane (n.d., not detectable). Similar results were obtained in three replicate experiments. (C, D) Two-dimensional peptide maps, from in vivo phosphorylated HA-tagged (C) and in vitro phosphorylated His-tagged (D) ABC50 wild-type, S109A and S140A mutants were performed after tryptic/Asp-N cleavage as described in the Experimental section. Small-lettered arrows refer to peptides that are discussed in the text. The origin and the position (dotted circles) of the tracking dye (dinitrophenyl-lysine) are indicated. (E) Phospho-amino acid analysis of in vivo labelled HA-ABC50. Immunoprecipitated HA-ABC50 (wild-type) phosphorylated in metabolically labelled HEK293 cells was subjected to SDS/PAGE. The band corresponding to HA-ABC50 was excised, digested with trypsin and Asp-N and the resulting peptides were hydrolysed at 110°C with HCl. Unlabelled phosphotyrosine (P-Tyr), phosphothreonine (P-Thr) and phosphoserine (P-Ser), 5 nmol of each, were loaded in all lanes as markers. On the marker lanes, one drop of diluted [γ-32P]ATP was added on the spots corresponding to phosphoserine, phosphothreonine and phosphotyrosine to facilitate alignment of the markers with the autoradiograph. The panel on the left shows the ninhydrin staining to visualize the phosphoamino acids and the right panel shows the autoradiograph. The polarity (+/−) and the direction of electrophoresis (long vertical arrow) are shown.
multiple possible cleavage sites for trypsin and Asp-N adjacent to S109 (see Figure 2A), so that partial cleavage events give rise to multiple phosphopeptides.

To assess whether S109 and S140 are actually phosphorylated by CK2, we expressed wild-type ABC50 and the S109A and S140A mutants in E. coli, incubated them with CK2 and [γ-32P]ATP, and again prepared peptide maps (Figure 2D). For wild-type ABC50, four major phosphopeptides were seen, corresponding to four of the main phosphopeptides observed in the ‘in vivo’ maps (peptides ‘a’–‘d’; compare top sections of Figure 2C and 2D). As already observed in the ‘in vivo’ labelling experiments, peptides ‘b’–‘d’ were absent from the map derived for CK2-labelled ABC50[S109A], consistent with the interpretation that this residue is a phosphorylated by CK2 in vivo. Similarly, the map for the S140A mutant lacked peptide ‘a’, which was also absent from the ‘in vivo’ map for this mutant. This map also lacked peptide ‘d’, which is one of multiple peptides derived from S109 (presumably by partial cleavages, as mentioned above): this probably reflects differences in the cleavage patterns between digests and/or between ABC50 in mammalian cells compared with bacteria. Thus, one can conclude that Ser-109 and Ser-140: (i) are labelled in vivo; (ii) correspond to peptides ‘a’ and ‘b’–‘d’ respectively; and (iii) are indeed substrates for CK2, at least in vitro.

Peptide ‘e’ and some minor species (Figure 2C) were still seen in the S109A mutant (and probably the S140A mutant) labelled in HEK293 cells, but is absent from the maps derived from ABC50 phosphorylated by CK2 in vitro. It must therefore contain a site that is phosphorylated by a kinase other than CK2. Indeed, phosphoamino acid analysis of ABC50 labelled in cells revealed the presence of phosphothreonine and phosphoserine residues, showing that additional phosphorylation site(s) must exist in ABC50 (Figure 2E) since both CK2 sites are serine residues.

Given that the CK2 phosphorylation sites in the C-termini of elf2βε and elf5 are important for their interaction with elf2 [11,12], we tested whether mutation of the two CK2 sites in ABC50 affected its ability to bind elf2. The results in Figure 3(A) show that immunoprecipitation of lysates from HEK293 cells with anti-ABC50 results in the co-immunoprecipitation of endogenous ABC50 with elf2, as detected with an antibody to its α-subunit. No elf2α was detected in negative controls lacking HEK293 cell lysate or from which anti-ABC50 was omitted (Figure 3A). This demonstrates that endogenous ABC50 and elf2 are indeed components of a protein complex in cell lysates, consistent with our earlier conclusions [2]. The fact that a small amount of elf2 was also retained on beads in the absence of anti-ABC50 immunoprecipitation reflects the fact that elf2 is a ‘sticky’ protein prone to non-specific binding, for example to the walls of microfuge tubes.

CK2 phosphorylation sites in elf5 and elf2βε are required for their interactions with elf2 and/or other initiation factors [11,12]. To test the role of the CK2 sites in this interaction, we expressed either wild-type (HA-tagged) ABC50 or the S109A/S140A mutant in HEK293 cells, immunoprecipitated these proteins with anti-HA and performed Western blots to detect associated elf2 (Figure 3B). Similar amounts of wild-type and mutant ABC50 were expressed, and similar amounts of elf2 were co-immunoprecipitated with them (Figure 3B). Comparison of the ratios of the anti-HA and anti-elf2α signals for the lysate and the immunoprecipitate indicated that about 40% of the total elf2 co-immunoprecipitated with HA-ABC50. Thus, in contrast with the situation for elf2B [11] and elf5 [12], the CK2 sites in ABC50 are not required for its interaction with elf2. In those proteins, the CK2 sites lie within the region that binds elf2. In ABC50, the elf2-binding region has not yet been defined.
Association of ABC50 with ribosomes requires at least one NBD

ABC50 has previously been shown to associate with ribosomes, including polyribosomes [2], although the features of ABC50 that are required for ribosome binding have not been identified. To address this, ABC50, or domains of this protein (as indicated in Figure 1), were expressed as HA-tagged polypeptides in HEK293 cells. Two days later, cells were lysed and the lysates were layered on to a sucrose cushion and subjected to differential centrifugation to generate ribosomal pellets and post-ribosomal supernatant fractions. The upper phases were collected as several distinct fractions. These samples were then analysed by SDS/PAGE and Western blotting to detect the HA-tagged fragments, ribosomal protein S6 (as a marker for ribosomes) and, in some cases, the \( \alpha \)-subunit of eIF2 (Figure 5). Ribosomal protein S6 was almost entirely found in the pellet fraction indicating that the ribosomes had been sedimented to the bottom of the tube as expected. In some cases, a trace of S6 was seen in the cushion fraction ‘C’, perhaps because some of the slower-sedimenting 40S subunits remained in this fraction. As shown in Figure 5(A), full-length ABC50 was found in the ribosomal pellet. Fractionation in the presence of 0.5 M KCl eliminated the association of ABC50 with ribosomes (Figure 5B) showing that ABC50 is not an intrinsic component of the ribosome, but rather binds through interactions that are disrupted at high ionic strength, as is also the case for translation factors. As expected, almost all of the S6, an intrinsic ribosomal protein, remained in the pellet (Figure 5B).

Like eIF2, HA–ABC50 is also found in the non-ribosomal fractions, perhaps because it transiently associates with ribosomes. This may also in part be a consequence of the expression of the protein in excess. The N-terminal fragment of ABC50 expressed well in HEK293 cells, but was not found in the ribosomal pellet indicating that it cannot associate with ribosomes on its own (Figure 5A). We were unable to express the first and second ABC domains of ABC50 individually in HEK293 cells (results not shown). Each could, however, readily be expressed as fusions

\[ \text{Figure 4B still bound eIF2 (Figure 4A), showing that a region} \]
\[ \text{outside this extreme N-terminal part of ABC50 can also support} \]
\[ \text{the interaction with eIF2 (Figure 4A). Based on the other} \]
\[ \text{results shown here, this region must presumably be the one} \]
\[ \text{between residues 92 and 183. We created a vector encoding} \]
\[ \text{this region} \]
\[ \text{but, unfortunately, it did not express in} \ E. \ coli. \]

It is not clear why ABC50(1–42) binds eIF2 better than longer fragments: one possibility is that other parts of the N-terminal region actually contain features that impair this interaction. It is perhaps more likely that this observation reflects some misfolding problems of the longer sub-fragments when they are expressed in \( E. \ coli \). Thus ABC50 can interact with eIF2 through a region (residues 1–42) that is distinct from that which contains the CK2 phosphorylation sites. In this regard, it differs from other partners of eIF2, such as eIF5 and eIF2B. Indeed, while the regions of eIF5 and eIF2B by which eIF2 interacts with ABC50 have a strong sequence similarity [9], there is no such similarity between them and the N-terminal part of ABC50 (Figure 1B). In particular, residues 1–42, which are sufficient to bind eIF2, do not contain the aromatic/acidic motif that is found in eIF5 and eIF2B by which eIF2 interacts with ABC50 in a way that is distinct from the way it binds to eIF5 or eIF2B. Thus mutating the CK2 sites in ABC50 to non-phosphorylatable residues does not affect their ability to bind eIF2, because ABC50 can bind eIF2 through a region that is distinct from the one containing the CK2 sites. Nevertheless, it remained possible that the CK2 sites in ABC50 were functionally significant, for example in the binding of ABC50 to ribosomes.
with the N-terminus. Each of these bound to ribosomes (as shown in Figure 5A), and the N-terminus + NBD1 fragment appeared to bind better to the ribosomes than full-length ABC50, as almost all this material was found in the ribosomal fraction (Figure 5A).

We conclude that each ABC domain can bind to ribosomes independently of the other one, but cannot tell from our results whether the N-terminus is actually needed for this. These results also do not inform us whether ABC50/ribosome binding is direct or is mediated via another protein.

**The CK2 sites in ABC50 affect the association of eIF2 with polyribosomes**

Wild-type HA-ABC50 or the S109A/S140A mutant were expressed in HEK293 cells. Samples of cell lysate were subjected to sucrose density gradient analysis to resolve ribosomal subunits (40S and 60S), 80S ribosomes and polysomes, as depicted in Figure 6(A). Fractions were collected and their positions in the gradient were confirmed by immunoblotting for protein components of the 40S (rpS6) and 60S (rpL28) subunits. Expression of ABC50(S109A/S140A) had very little effect on the overall ribosome profile (results not shown). The distributions of endogenous and ectopically expressed ABC50 and actin were detected by Western blotting of cell lysates (Figure 6B). Wild-type ABC50 was found across the gradient, including fractions that correspond to polyribosomes engaged in elongation. This may indicate that ABC50 can actually bind to elongating ribosomes, but this could also include association with 48S initiation complexes attached to the 5′-ends of polysomes rather than with the ribosomes that are actually engaged in elongation.

The S109A/S140A mutant, which lacks the CK2 phosphorylation sites, still bound to polyribosomes and there was little difference in its overall distribution across the gradient (Figure 6B). However, in cells expressing this variant, there was a very marked decrease in the association of eIF2 with the fractions in the gradient that contain 60S/80S subunits and small polysomes. The decrease in eIF2 binding to ribosomes is especially striking when normalized to the levels of ribosome protein S6 in each fraction (Figure 6B). This effect, which was seen in three independent experiments, suggests that the CK2 phosphorylation sites in
ABC50 do play an important role in its function. Further work is clearly required to address the overall function of ABC50 in translation, including the role of the CK2 sites.

Expression of the S109A/S140A mutant of ABC50 did not affect overall protein synthesis (Figure 6C) indicating that it does not exert a dominant interfering effect, even when expressed at levels that are substantially higher than those of the endogenous ABC50 (right hand panels of Figure 6C).

CONCLUDING COMMENTS

In this study we show that the interaction between ABC50 and eIF2 involves the N-terminal region of ABC50. This region is well conserved between ABC50 proteins from different species, but not between ABC50 and other members of the ABC group of proteins. This is consistent with the fact that ABC50 is the only one of these proteins known to bind eIF2. The minimal region of ABC50 that can bind to eIF2 (residues 1–42) show no similarity at all to the regions of two other eIF2-partners (eIF2βε, eIF5) that are needed for their binding to eIF2. As the GEF and the GAP respectively, for eIF2, one would expect their binding to eIF2 to be mutually exclusive; eIF5 preferentially binds eIF2-GTP [6], while (physiologically) eIF2B is expected to bind eIF2-GDP. It is therefore not surprising that their binding to eIF2 involves similar motifs which presumably dock with the same feature(s) in eIF2 (probably lysine boxes in eIF2βε; see [18] and references cited therein). The fact that the eIF2-binding motif of ABC50 is distinct suggests that it binds differently to eIF2 and also it may not compete with (obstruct) the binding of eIF5/eIF2βε. In preliminary experiments, however, we have so far been unable to observe, for example, ternary eIF2–ABC50–eIF2βε complexes.

We also show that the N-terminus of ABC50 cannot bind to ribosomes on its own and that at least one of the NBDs of ABC50 is required for this. This accords with the ability of ATP to promote the association of ABC50 with ribosomes [2], but further work is required to substantiate how ATP promotes this interaction.

The other major findings of this study are that (i) ABC50 is a phosphoprotein, (ii) it is phosphorylated at two sites by CK2 in vitro, and probably in vivo, and (iii) mutation of the CK2 phosphorylation sites modulates the association of eIF2 (but not ABC50) with ribosomes. Mutation of these sites did not appear to affect the ability of ABC50 to bind eIF2, even though they are within the N-terminal region of ABC50 that is involved in eIF2 binding. This observation reflects the fact that they are outside the minimal eIF2-binding fragment so far identified, i.e. residues 1–42. The CK2 sites are completely conserved in all mammalian species for which information is available and in the presumed Xenopus laevis orthologue (GI:148226825). They do lie within the second eIF2-binding region we identified here (92–183) but this part is not essential for binding to eIF2 and also lacks similarity to the eIF2-binding regions of eIF2βε and eIF5 (see also comments in the legend of Figure 1B).

These findings further underline the difference between the modes of eIF2-binding that are employed by eIF2βε/eIF5 on one hand, and ABC50 on the other. In the case of eIF2βε, for example, the CK2 sites within the eIF2-binding region are required for the interaction [11]. Further work is needed to study the importance of ABC50 in mRNA translation and/or its control.

This work was supported by a project grant (to C. G. P.) from the U.K. Biotechnology and Biological Sciences Research Council. We thank other members of the Proud laboratory, especially Dr Maria Buxadé, Dr Josep Lluis Liarrà, Dr Andrew Tee and Dr Xuemin Wang, for their technical help and advice. We are grateful to Professor D. R. Alessi (University of Dundee, Dundee, U.K.) for allowing us to use facilities in his laboratory.

REFERENCES