ABC50 Interacts with Eukaryotic Initiation Factor 2 and Associates with the Ribosome in an ATP-dependent Manner*

Received for publication, April 5, 2000, and in revised form, August 7, 2000
Published, JBC Papers in Press, August 7, 2000, DOI 10.1074/jbc.M002868200

Jonathan K. Tyzack‡§, Xuemin Wang‡, Graham J. Belsham¶, and Christopher G. Proud‡‖

From the §MSI/WTB Complex, University of Dundee, Dundee, DD1 5EH and the ¶Institute for Animal Health, Pirbright, Woking, Surrey, GU24 0NF, United Kingdom

Eukaryotic initiation factor 2 (eIF2) plays a key role in the process of translation initiation and in its control. Here we demonstrate that highly purified mammalian eIF2 contains an additional polypeptide of apparent molecular mass of 110 kDa. This polypeptide co-purified with eIF2 through five different chromatography procedures. A cDNA clone encoding the polypeptide was isolated, and its sequence closely matched that of a protein previously termed ABC50, a member of the ATP-binding cassette (ABC) family of proteins. Antibodies to ABC50 co-immunoprecipitated eIF2 and vice versa, indicating that these two proteins interact. The presence of ABC50 had no effect upon the ability of eIF2 to bind GDP but markedly enhanced the association of methionyl-tRNA with the factor. Unlike the majority of ABC proteins, which are membrane-associated transporters, ABC50 associates with the ribosome and co-sediments in sucrose gradients with the 40 and 60 S ribosomal subunits. The association of ABC50 with ribosomal subunits was increased by ATP and decreased by ADP. ABC50 is related to GCN20 and eIF3, two yeast ABC proteins that are not membrane-associated transporters and are instead implicated in mRNA translation and/or its control. Thus, these data identify ABC50 as a third ABC protein with a likely function in mRNA translation, which associates with eIF2 and with ribosomes.

The initiation of translation in eukaryotes is a complex process involving approximately 15 different initiation factor proteins, the ribosome, methionyl-tRNA, and mRNA. One of the key initiation factors is eukaryotic initiation factor 2 (eIF2), a heterotrimeric protein (subunits α, β and γ) that binds guanine nucleotides and mediates the transfer of the initiator Met-tRNA to the 40 S ribosomal subunit (1). This step is required for all translation initiation events, and eIF2 is known to play an important role in the regulation of translation initiation under a variety of conditions (1, 2).

At a late stage in the initiation process the GTP bound to eIF2 is hydrolyzed to GDP plus P, and an inactive eIF2-GDP complex is released from the ribosome (reviewed in Refs. 1 and 3). GTP hydrolysis may be activated by the interaction of eIF2 with another translation factor, eIF5 (1). Because the rate of dissociation of GDP from eIF2 is very low, regeneration of active eIF2-GTP requires an additional protein termed eIF2B (a guanine nucleotide exchange factor; reviewed in Ref. 3). eIF2B is a heteropentameric protein (subunits α–e) and also plays an important role in the regulation of translation initiation (see below).

The γ-subunit of eIF2 binds GDP or GTP (4), whereas eIF2α plays a regulatory role; phosphorylation of eIF2α at Ser51 results in inhibition of the eIF2B-catalyzed recycling of eIF2 (reviewed in Refs. 2 and 3). Phosphorylation of eIF2α is important in the overall regulation of translation in response to numerous different types of cell stress as discussed below. The role of eIF2β is less clear. There is data suggesting that it is required for Met-tRNA binding (5) and is involved in the selection of the start site for initiation of translation (6). Other studies have indicated that it interacts with eIF5 (7) and with eIF2B (8).

Phosphorylation of Ser51 in eIF2α is involved in the regulation of the activity of eIF2 and the overall rate of protein synthesis under a variety of conditions in eukaryotic organisms (9). When phosphorylated on its α-subunit, eIF2 is a potent competitive inhibitor of eIF2B and thus causes inhibition of overall peptide chain initiation (9). A number of kinases capable of phosphorylating Ser51 have been identified, these include the double-stranded RNA-activated eIF2α kinase RNA-dependent protein kinase (10), the heme regulated kinase haem-regulated inhibitor (11), and most recently, the endoplasmic reticulum-associated eIF2α kinase (RNA-dependent protein kinase-like endoplasmic reticulum kinase/pancreatic eIF2α kinase (12, 13)). In yeast, there is a further well characterized regulatory mechanism involving the phosphorylation of eIF2 by the protein kinase GNC2, which is believed to be activated by uncharged tRNA in response to amino acid starvation (14). GNC2 contains both a kinase domain and a region similar to histidinyl-tRNA synthetase that may bind tRNA. In yeast, inhibition of eIF2B through the phosphorylation of eIF2α leads to up-regulation of the translation of the mRNA encoding the transcriptional regulator GNC4, thus promoting transcription of genes encoding enzymes involved in amino acid biosynthesis (14). The genetic tractability of yeast has allowed the identification of several other genes involved in this regulatory mechanism. These include GNC1 and GNC20, which are both required for the activation of GNC4 synthesis, and, in particular, the activation of the kinase GNC2, in response to amino acid deprivation (15–17). GNC20 is a member of the ATP-binding cassette (ABC) family of proteins and forms a complex with
ABC50 Interacts with eIF2 and Associates with the Ribosome

GCN1 that appears to be present upon elongating ribosomes, but the precise roles played by these proteins in the regulation of GCN2 remains to be established. Recent data indicate that higher eukaryotes also possess GCN2-like enzymes (18–20) because cDNAs for such enzymes have been cloned from fruit flies and mammals. Indeed, it has been known for some years that manipulations that lead to accumulation of uncharged tRNA (e.g. incubation of rat liver cells without essential amino acids (21), treatment of rat liver cells with an inhibitor of histidinyl-tRNA synthetase (histidinol) (22), or the use of mammalian cell lines harboring a temperature-sensitive mutant of the leucyl-tRNA synthetase (23, 24)) lead to increased phosphorylation of eIF2α, indicative of the operation of a GCN2-like system.

Here we identify a protein that co-purifies and interacts with mammalian eIF2 and show that it is the rabbit homologue of human ABC50 which had previously been identified as a protein (with no known function) whose expression is elevated in tumor necrosis factor α-stimulated synovocytes (25). It contains the motifs of the ABC family of proteins and exhibits sequence similarity to the members of the eEF3 subfamily (which includes the Saccharomyces cerevisiae proteins eEF3 and GCN20) (25). We show that mammalian ABC50, like yeast eEF3 and GCN20, is associated with the translational machinery.

**MATERIALS AND METHODS**

**Chemicals and Biochemicals—**Unless otherwise indicated all chemicals and biochemicals were obtained from Sigma or Merck.

**Protein Purification—**eIF2 and eIF2B was purified from rabbit reticulocyte lysates or HeLa cell lysate essentially as described previously (26).

**Gel Electrophoresis and Immunoblotting—**SDS-polyacrylamide gel electrophoresis and immunoblotting (using Immobilon-P polyvinylidene difluoride membranes, Millipore) were carried out as detailed earlier (27). Blots were developed using ECL reagents (Amersham Pharmacia Biotech).

**Peptide Sequence Analysis—**To obtain internal peptide sequence data for p110, preparations of eIF2 rich in this polypeptide (as assessed by Coomassie Brilliant Blue staining) were subjected to SDS-PAGE, and the region of the gel containing p110 was excised. Cleavage of the protein using CNBr treatment, separation of the fragments by Tricine-SDS-PAGE, and transfer to Immobilon-P membrane were performed as described earlier (28, 29). Automated Edman degradations were performed by K. Howland (Department of Biosciences, University of Kent at Canterbury) on a Procise 492 Protein Sequenator (PerkinElmer Biosystems) and phenylthiohydantoin-derivatives were identified by on-line reverse-phase high pressure liquid chromatography utilizing a phenylthiohydantoin C18 column (2.1 x 220 mm, 5-mm particle size; PerkinElmer Biosystems). PerkinElmer Biosystems reagents and solvents were used wherever available.

**Data Base Searching—**DNA or protein sequences were used to search the data bases available at the National Center for Biotechnology Information web site using the Advanced Gapped Basic Local Alignment Search Tool (BLAST) program (30). DNA and protein sequences identified by these searches were recovered from the GenBank™ and GenProt data bases using the National Center for Biotechnology Information Entrez program.

**Alignment of DNA and Protein Sequences—**DNA and protein sequences were aligned using the ClustalW Multiple Sequence Alignment program at the European Bioinformatics Institute web site or the BLAST 2 sequences program at the National Center for Biotechnology Information web site. Shading and editing of these alignments was performed using the GeneDoc Multiple Sequence Alignment Editor and Shading Utility obtained from the Pittsburgh Supercomputing Center web site.

**Generation of Rat Testis cDNA—**Total RNA (about 2 mg) was isolated from 1 g of rat testis tissue using TRIZOL® reagent (Life Technologies, Inc.) and the mRNA (about 20 μg) was isolated from this total RNA using the Oligotex mRNA mini-prep kit (Qiagen). First strand cDNA synthesis, using oligo(dT)15 as the primer was performed using Expand™ Reverse Transcriptase (Roche Molecular Biochemicals).

**DNA Screening—**The production of an [α-32P]dCTP (Amersham Pharmacia Biotech) radiolabeled DNA probe for p110/ABC50 was achieved using the Random Primed Labeling Kit (Roche Molecular Biochemicals) with the primers p110 FOR 3 (5′-GTA CCA GAA GCA GAA GAA GAA-3′) and p110 REV 2 (5′-CAG TCA GCA GGA GTA GCA-3′) and the rat testis cDNA generated above. DNA screening of a Stratagene Uni-ZAP® rat skeletal muscle cDNA library was performed essentially as described by the supplier using this probe. Phage found to contain the p110 cDNA had the pBlueScript II vector sequence identified and isolated using the ExAssist/SOLR™ protocol (Stratagene). The isolated phagemids were sequenced using T7 and T3 primers and the following primers: p110 FOR 1 (5′-AGTCATGGTCGAACGACCCTC-3′), p110 FOR 2 (5′-GCGTCTTTTAAACCGAGTTA-3′), p110 FOR 3 (5′-GACCTGCTCCTGCAGAC-3′), p110 REV 3 (5′-CTGGAATCCCCTACCTGAG-3′), p110 REV 4 (5′-TTGTTTCTAGCCGGCAGTA-3′), and p110 REV 5 (5′-GGCTTGAGACAGAGAGTTC-3′).

**Polymerase Chain Reaction Amplification and Cloning of the 5′ End of the p110 cDNA—**The region of the rat p110 cDNA corresponding to nucleotides 116–1216 of GenBank™ sequence AF027302 was amplified using the polymerase chain reaction from the rat testis cDNA using the p110 FOR 5 (5′-CAAAGTAGCAGAGAAGGCG-3′) and p110 REV 2 primers and Taq DNA polymerase. The products were ligated into the pGEM-T Easy vector (Promega) and insert DNA sequenced as above. Rapid amplification of the 5′ end of the rat p110 cDNA was performed essentially as described previously (31) using the p110 REV 4, p110 REV 3, or p110 REV 1 primer. The products were cloned into the pGEM-T Easy vector as above. Plasmid DNA was isolated, and insert DNA was sequenced.

**Antibody Production—**A peptide with sequence DEESQAPELLKRPKEC was prepared by Kevin Howland (University of Kent at Canterbury) and used to generate a rabbit anti-p110 antisera. The anti-p110 immunoglobulins were purified from this serum using the peptide cross-linked to Affi-gel (Bio-Rad) as described (32).

**Immunoprecipitation—**Purified rabbit anti-eIF2p110 obtained from the purification procedure above (5–10 μg) was added to a mixture (final volume, 75 μl) consisting of IP buffer (phosphate-buffered saline (PBS), pH 7.6, 0.07% (w/v) SDS, 0.7% (w/v) sodium deoxycholate, 0.7% (v/v) Triton X-100) and a 1/5 volume of either PBS, pH 7.6, or the following antigens as indicated in the results: preimmune antiserum from rabbits prior to injection with antigen, mouse monoclonal anti-eIF2α antisemur, or purified rabbit anti-p110 peptide antisemur. The reactions were mixed briefly and incubated at room temperature for 1 h. PANSORBIN® cell suspension (20 μl) in RIPA buffer (50 mM HEPES/KOH, pH 7.6, 150 mM KCl, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100) was then added to each reaction, mixed briefly, and incubated at room temperature for 20 min. The suspensions were then centrifuged in a benchtop microcentrifuge to pellet the antigen/antibody/PANSORBIN® cell complexes. The supernatants were removed, and the pellets were resuspended and washed in 500 μl of PBS buffer. This wash procedure was repeated a further two times and, finally, in one 500 μl of PBS buffer. SDS-PAGE (2 ×) sample buffer (25 μl) was added to each of the final pellets which were then frozen at -80 °C and thawed to aid resuspension of the pellet. The samples were boiled for 5 min and centrifuged at 13,000 rpm prior to analysis by SDS-PAGE/immunoblotting.

**Sucrose Cushion Centrifugation—**HEK 293 cells were maintained and passaged in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10% (v/v) and antibiotic/antimycotic (1% (v/v); Life Technologies, Inc.). Lysis of cells at 70–80% confluency was achieved in ice-cold SDG100 buffer (50 mM HEPES/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 phenylmethylsulphonyl fluoride, 1 μM microcystin) containing 0.1% (v/v) Triton X-100. Nuclei and membranous material was removed by centrifugation (15 min at 3,500 × g, 4 °C). The cleared lysate was layered gently onto 0.8 ml sucrose (in SDG100 buffer) and centrifuged (2 h at 290,000 × g, 4 °C) in a Beckman SW28.1 rotor. After centrifugation, the cleared lysate at the top of the cushion and the sucrose layer were removed separately, and after a brief rinse in 100 μl of PBS, pH 7.6, the ribosomal pellet was resuspended in SDG500 buffer (as SDG100 buffer except 500 mM KCl). The salt washed ribosomes were then centrifuged as before except using a 0.8 ml sucrose cushion in SDG500 buffer.

**Sucrose Density Gradient Centrifugation—**HEK 293 cells were maintained and lysed into SDG100 buffer (except at 7 mM MgCl2) + 0.1% (v/v) Triton X-100 as above. After the lysate had been cleared, it was layered onto 20–50% (w/v) sucrose gradients (15 ml, prepared in SDG100 buffer) and centrifuged (3.5 h at 110,000 × g, 4 °C) in a Beckman SW28.1 rotor. Where indicated in the results, ATP or ADP was added to the cleared lysate to give a final concentration of 5 mM, and this was then layered onto a gradient that contained 5 mM ATP or...
ADP as appropriate. Each gradient was fractionated using a Bandel model 184 Fractionator by displacement with 60% (w/v) sucrose at a flow rate of 1 ml/min. The A$_{260}$ of the displaced gradient was analyzed in a 5-mm-path length ISCO Type 6 Optical Unit and recorded using an ISCO model UA-5 Absorbance/Fluorescence meter. Fractions (0.35 ml) were collected using a Gilson Microfraction model 203 fraction collector. Trichloroacetic acid precipitation of each of the fractions obtained was performed, and the precipitated samples were resolved by SDS-PAGE for immunoblotting analysis.

Gel Filtration—Gel filtration was performed using an Amersham Pharmacia Biotech Superose 6 HR10/30 column on a Bio-Rad Biologic FPLC System. Protein samples were dialyzed into the column running buffer (50 mM HEPES/KOH, pH 7.6, 150 mM KCl, 1 mM DTT, 5% (v/v) glycerol, unless otherwise indicated), and the dialysate (250 µl) was loaded onto the column and eluted in the running buffer at a flow rate of 0.3 ml/min. Fractions (300 μl) were collected, and peak fractions were analyzed by SDS-PAGE and immunoblotting using the antisera indicated in the results. The column was calibrated using the standards from the molecular mass marker kit for gel filtration chromatography (molecular mass range, 12,000–200,000 Da; Sigma). The kit was supplied in the results. The column was calibrated using the standards from the molecular mass marker kit for gel filtration chromatography (molecular mass range, 12,000–200,000 Da; Sigma). The kit was supplied in the results. The column was calibrated using the standards from the molecular mass marker kit for gel filtration chromatography (molecular mass range, 12,000–200,000 Da; Sigma). The kit was supplied in the results. The column was calibrated using the standards from the molecular mass marker kit for gel filtration chromatography (molecular mass range, 12,000–200,000 Da; Sigma). The kit was supplied in the results. The column was calibrated using the standards from the molecular mass marker kit for gel filtration chromatography (molecular mass range, 12,000–200,000 Da; Sigma). The kit was supplied in the results.

GDP Binding and Ternary Complex Formation Assays—These assays were performed essentially as described previously (5).

RESULTS

Identification of p110—SDS-PAGE analysis of several preparations of eIF2 that had been isolated from rabbit reticulocytes as described previously (26) indicated the consistent presence of a protein with an apparent molecular mass of approximately 110 kDa in addition to the three subunits of eIF2 (Fig. 1A). This polypeptide (henceforth termed p110) appeared to co-elute with eIF2 from the later ion exchange column steps of our standard purification procedure (Fig. 1B). This co-purification of p110 with eIF2 was analyzed further as described below.

To obtain information on the identity of this protein, the p110 protein band was excised from a SDS-polyacrylamide gel and cleaved with CNBr. After transfer to Immobilon-P membrane and Amido Black staining, five distinct bands were observed. These were excised, and the derived peptides were sequenced as described under “Materials and Methods” (Table I).

The data for bands 2 and 5, which gave the longest and clearest sequences, were used to search the data bases available at the National Center for Biotechnology Information web site. Note, band 3 appears to be a more complete digest of the same peptide fragment as band 2 because they both contain the same sequence in their first five residues, and band 4 appears to be a mixture of multiple fragments. At the time of the initial analysis, no sequences were identified in the nonredundant data base of sequences, suggesting that the p110 protein was novel. However, tBLASTn analysis of the data base of expressed sequence tags (dbEST) led to the identification of several mouse and human ESTs encoding for peptide sequences showing 100% identity to the band 5 sequence (when a Y residue was used at the location of the indicated ambiguity in Table I). Alignment of all the ESTs identified in this manner suggested that they all encoded the same protein (data not shown). The largest sequences were a 2,207-nt-long human EST (GenBank™ accession number U66677) and a 491-nt-long murine EST (GenBank™ accession number AA014138). No EST sequences containing the band 2 sequence were identified at this time.

Cloning of the cDNA Encoding for p110—The polypeptide sequence predicted from the EST alignment appeared to be the C-terminal end of p110 because many of the ESTs identified contained an in-frame stop codon and a polyadenylation signal (AATAAA) downstream. To clone and sequence the cDNA encoding for p110, primers were designed for the production of an oligonucleotide probe by the polymerase chain reaction amplification of the cDNA corresponding to nucleotides 18–418 of the AA014138 sequence. This probe was used to screen a rat skeletal muscle cDNA library for phage containing the p110 cDNA. One clone was identified and the cDNA insert of its phagemid sequenced (GenBank™ accession number AF293383). Analysis of the insert sequence demonstrated that it was a chimeric molecule because approximately 700 nt encoded for rat parvalbumin and the remaining 2400 nt were found to show ∼95% identity to the alignment of the previously identified EST sequences and also 89% identity to a newly submitted human cDNA sequence for a protein of unknown function that had been termed ABC50 (with GenBank™ accession number AF027302 and GenProt accession number AAC70891). The mRNA for this protein had been identified by virtue of its increased expression in synovioctyes stimulated with tumor necrosis factor α (25). The bands 2/strand 3 and band 5 sequences in Table I were all present in the ABC50 protein sequence. Thus, the rabbit and rat p110 proteins were identified as homologues of the human ABC50 protein. Alignment of the rat p110 cDNA sequence from the phagemid insert with the ABC50 cDNA indicated that the rat sequence lacked approximately 800 nt from its 5’ end. To obtain these missing sequence data, the published ABC50 cDNA sequence was used in BLAST

### Table I

| p110 peptide fragment | Peptide sequence obtained |
|-----------------------|---------------------------|
| Band 1                | No data obtained          |
| Band 2                | (e/s)RLKKLVFAYXD          |
| Band 3                | Eif2/lfk                  |
| Band 4                | (S/Y/L)(Q/E/F/k)(N/T)/g/A/P/k/Q/S |
| Band 5                | YQQ/KQKELLKQ/V/C/E/QK     |

FIG. 1. Identification of p110 as a protein that co-purifies with eIF2. eIF2 was prepared from rabbit reticulocyte lysate as described under “Materials and Methods.” A, Coomassie Blue-stained SDS-polyacrylamide gel (10%) of a Mono S sample containing eIF2 and p110. The positions of the Amersham Pharmacia Biotech broad range molecular mass markers together with the three subunits of eIF2 and p110 are indicated. B, Coomassie Blue staining of consecutive fractions from the final Mono S ion exchange column in the purification procedure resolved by SDS-PAGE (15%).
Performance of BLAST searches of the public databases at the NCBI web site led to the identification of potential homologues of p110 as listed. The percentage of identity/percentage of similarity scores were determined using the BLAST 2 sequences program at the NCBI web site and represent the highest score obtained for each sequence.

| Species           | GenBank or GenProt Accession Number (Name if known) | Identity to the rat p110 protein sequence. | Similarity to the rat p110 protein sequence. |
|-------------------|-----------------------------------------------------|------------------------------------------|-------------------------------------------|
| Rattus norvegicus | AF293383 (Homo sapiens)                             | 88                                       | 91                                        |
| Homo sapiens      | AAC70891 (ABC50)                                    | 93                                       | 96                                        |
| Mus musculus      | AAF48069.1                                         | 95                                       | 97                                        |
| D. melanogaster   | AA99835.1                                           | 53                                       | 67                                        |
| A. thaliana       | CAB77574.1                                          | 43                                       | 62                                        |
| C. elegans        | AAA19672.1                                          | 43                                       | 60                                        |
|                  | AA914138, AI264984                                  | 36                                       | 56                                        |
|                  | S56147 (GCN20–2)                                   | 35                                       | 55                                        |
| S. pombe          | CAA18386                                           | 35                                       | 56                                        |
| S. cerevisiae     | P43535 (GCN20)                                     | 35                                       | 57                                        |
|                   | P40024 (YER036c)                                   | 38                                       | 59                                        |

* The human EST sequences with GenBank™ accession numbers AA083604 and AA587878 were translated and inserted into the ABC50 protein sequence at the appropriate location to obtain the percentage of identity/percentage of similarity of a potential second isoform of the human ABC50 protein relative to the rat sequence.

**p110/ABC50** is homologous to the S. cerevisiae GCN20 and YER036c proteins (summarized in Table II and also see below). ABC50 had been identified by Richard et al. (25) as a member of the ABC family of proteins by virtue of the presence of two ABC motifs within its sequence (25). The proteins that show high similarity to p110/ABC50 also each contain two ABC motifs, and the arm of the ABC family that contains ABC50 is that of the eEF3 subfamily (25). This subfamily includes eEF3 and GCN20, which are yeast proteins involved in the process of translation. p110/ABC50 is homologous to the S. cerevisiae GCN20 and YER036c proteins (Fig. 2 and Table II). GCN20 is implicated in the regulation of the eIF2α kinase GCN2 in response to amino acid starvation in yeast (16, 17), whereas YER036c has no known function (33). The similarity between the yeast proteins and p110/ABC50 is greatest in the C-termini two-thirds of the mammalian proteins. The N-terminal one-third of p110/ABC50 only shows approximately 20% identity and 30% similarity to the equivalent region of GCN20 and is significantly longer (between 54 and 88 residues depending on the presence of the 34-amino acid insert). The N-terminus of p110/ABC50 exhibits a high degree of hydrophilicity as determined using the Kyte-Doolittle algorithm (data not shown), and the entire sequence consists of a high proportion of charged residues (approximately 36% KRED residues). No transmembrane domains could be identified in the p110 or ABC50 sequences, and this together with the other features described above suggested that p110/ABC50 was not a membrane-associated protein but was more likely to be involved in the process of translation.
ABC50 Interacts with eIF2 and Associates with the Ribosome

p110/ABC50 Associates with Ribosomes—Because of the similarity of p110/ABC50 to the members of the eEF3 ABC subfamily and its co-purification with eIF2, we investigated whether p110/ABC50 associated with ribosomes. Ribosomes from HEK 293 cell extracts were sedimented through a sucrose cushion and then analyzed for the presence of p110/ABC50 by SDS-PAGE/immunoblotting (Fig. 4). p110/ABC50 was found associated with the pelleted ribosomes and also in the postribosomal supernatant. This was also the case for eIF2. After the ribosomes had been resuspended in buffer containing 0.5 M KCl and resedimented, p110/ABC50 and eIF2 were no longer associated with the ribosome but are not an intrinsic ribosomal protein, unlike L5 and S26, which remained in the pellet material. This behavior is typical of many translation factors including eIF2, eIF4F, and eIF3 (34–38). To study further this association with the ribosome, sucrose density gradient analysis was performed as described under “Materials and Methods” (Fig. 5). Immunoblotting analysis of the gradient fractions revealed that p110/ABC50 can associate with the ribosome but is not an intrinsic ribosomal protein, unlike L5 and S26, which remained in the pellet material. This behavior is typical of many translation factors including eIF2, eIF4F, and eIF3 (34–38).

Because p110/ABC50 possesses binding motifs for adenine nucleotides, we also studied the effect of the presence of ADP and ATP on the association of the protein with ribosomes. Addition of ADP to the lysate and gradient buffer caused a marked decrease in the amount of p110/ABC50 associated with the 40 and 60 S subunits (compare Control and + ADP panels in Fig. 5B), whereas addition of ATP caused a marked increase in the level of association (compare Control and + ATP panels in Fig. 5B). These effects were not due to any changes in the levels of 40 and 60 S subunits or in the association or distribution of eIF2, all of which were apparently identical under each of the conditions studied (data not shown).

Co-purification of p110/ABC50 with eIF2—To investigate further the co-purification of eIF2 and p110/ABC50, the distribution of the two proteins during the purification of the translation factors from rabbit reticulocyte lysate was determined by immunoblotting using the anti-ABC50 antibody in conjunction with antibodies to each of the three eIF2 subunits and to the eIF2α subunit. It was found that p110/ABC50 was present in all samples containing eIF2 throughout the purification procedure, except when eIF2 was complexed with eIF2B (data not shown). However, when the purification procedure was applied to HeLa cell extracts, a small fraction of the eIF2 was recovered that was deficient in p110/ABC50 (see below and Fig. 8A).

Interaction of p110/ABC50 with eIF2—The co-purification of eIF2 and p110/ABC50 was consistent with the notion that eIF2 and p110/ABC50 form a stable interaction, although it is also theoretically possible that they share identical chromatographic properties on all four column matrices used. Therefore, to investigate whether p110/ABC50 and eIF2 do interact, Mono S fractions of purified eIF2 containing p110/ABC50 were subjected to immunoprecipitation using anti-eIF2α and anti-p110 antiserum (prepared as described under “Materials and Methods”; note that anti-ABC50 antiserum did not efficiently immunoprecipitate the p110/ABC50 protein (data not shown)). Initial studies were hampered by high levels of nonspecific
binding of both eIF2 and p110/ABC50 to the PANSORBlin® matrix in the absence of any antisera (Fig. 6; note the appearance of eIF2 and p110/ABC50 in the No Ab lane). This high degree of nonspecific binding was also observed when protein A-Sepharose was used as the matrix (data not shown). To reduce this nonspecific binding it was necessary to use high stringency buffers containing nonionic detergents in the reactions and the washes (Fig. 7, compare No Ab and No Ab (lanes). When such buffers were used, the anti-p110 or anti-eIF2α precipitated material was found to contain both p110/ABC50 and eIF2, whereas the control reactions did not (Fig. 6). However, when similar immunoprecipitation reactions were performed using rabbit reticulocyte or HeLa cell lysate in place of the purified proteins, co-immunoprecipitation of the proteins could not be observed above the level of nonspecifically bound proteins (data not shown).

As an alternative strategy to analyze the interaction between the two proteins, Mono S column fractions containing the purified eIF2 and p110/ABC50 were subjected to analytical gel filtration on a Superose 6 HR 10/30 column (Fig. 7A). The two proteins co-eluted from this column at a retention time equivalent to a molecular mass of about 400 kDa. This is well in excess of the expected masses of the individual proteins (approximately 150 kDa for eIF2 and 110 kDa for p110/ABC50), which suggests that they form a complex. Repetition of the gel filtration with running buffer containing 1 M KCl did not significantly alter this retention time (Fig. 7B), whereas use of buffer containing 20% (v/v) Triton X-100 led to a shift in the retention times of both proteins to a position equivalent to that of the individual molecular masses of the two proteins (Fig. 7C). The apparent dissociation of eIF2 and p110/ABC50 by detergents and not by high salt suggests that the binding of the two proteins to each other involves hydrophobic interactions.

ABC50 Enhances the Formation of Ternary Complexes but Has No Effect on GDP Binding by eIF2—Purification of eIF2 from HeLa cell extract, rather than rabbit reticulocyte lysate, yielded a small amount of eIF2 that was deficient in ABC50 as determined by SDS-PAGE and by immunoblotting (Fig. 8A; the level of eIF2α is similar in each sample, whereas the ABC50 sample is evidently deficient in p110/ABC50). The availability of this material enabled us to test two of the known activities of eIF2, guanine nucleotide binding and ternary complex formation, in the presence and absence of p110/ABC50. Binding of GDP to the ABC50-deficient eIF2 was not significantly different from binding to the eIF2/ABC50 complex (compare −ABC50 and + ABC50 in Fig. 8B). In contrast, binding of
translation initiation factors including eIF2, increase very markedly following stimulation (34–36, 38). Primary human T-lymphocytes were stimulated for up to 24 h with phorbol myristate acetate and ionomycin. The cell extracts were analyzed by immunoblotting for eIF2 and ABC50 (Fig. 9). The samples exhibited increased expression of both p110/ABC50 and eIF2α following 8–16 h of stimulation. Similar induction of eIF2 and ABC50 proteins was observed after stimulation of resting T-cells by the addition of anti-CD3 and anti-CD28 antibodies (data not shown).

**DISCUSSION**

The purification of translation factors from rabbit reticulocyte lysate by multiple ion exchange chromatographic steps consistently led to the isolation of eIF2 in association with a 110-kDa protein. Here we identify this protein as the rabbit homologue of the human ABC50 protein. ABC50 was previously identified as a protein of no known function that was induced in tumor necrosis factor α-stimulated synoviocytes (25). For reasons of consistency, we suggest that the name ABC50 be used to refer to the mammalian homologues of the protein in the future. Rabbit ABC50 was shown by immunoblotting analysis to co-purify with eIF2 throughout our entire purification procedure, which suggested that the two proteins form a stable complex. The co-purification of ABC50 with eIF2 has not previously been noted. Examination of the data in previous publications indicates that eIF2 can be purified from mammalian sources in the absence of ABC50 (e.g. see figures in Refs. 39 and 40). However, eIF2 may have been purified with ABC50 in others, on the basis that a protein at the expected position for ABC50 is observed in the Coomassie-stained gels shown in Refs. 41–45. The absence of ABC50 from some eIF2 samples could be due to differences in the method of purification or the absence of a particular protease inhibitor in the buffers used during our purification procedure. We have confirmed that our protocol also leads to co-purification of ABC50 and eIF2 from HeLa cells and Hek 293 cells, which shows that it is not a peculiarity of the rabbit proteins or of rabbit reticulocyte lysates. However, a small amount of ABC50-deficient eIF2 sample was obtained from the HeLa cell extract, possibly because of degradation of the ABC50 or because of lower levels of the protein within this cell line.

An interaction between the two proteins was confirmed by immunoprecipitation analysis whereby anti-eIF2α and purified anti-p110 antibodies each co-immunoprecipitated rabbit ABC50 or eIF2, respectively, from the purified samples. However, similar reactions using rabbit reticulocyte or HeLa cell lysates did not lead to detectable co-immunoprecipitation of the proteins. In this case, the anti-p110 antibody did not immunoprecipitate significant amounts of ABC50 from these extracts and the anti-eIF2α antibody only immunoprecipitated a proportion of the eIF2 (which did not contain detectable ABC50), and a substantial amount of the eIF2 remained in the super-

2 J. K. Tyzack, X. Wang, and C. G. Proud, unpublished observations.
ABC50 Interacts with eIF2 and Associates with the Ribosome

J. K. Tyzack, G. Pavitt, and C. G. Proud, unpublished observations.

The association of ABC50 with the ribosome was markedly altered by addition of adenine nucleotides to the gradients. ADP caused a decrease in the association and ATP an increase that may indicate a role for the nucleotide binding motifs in the ABC50 sequence in regulating its interaction with ribosomes. GCN20 has also been shown by Marton et al. (17) to interact with the ribosome, and this interaction was also stimulated by ATP. However, GCN20 associates with the polysomes and not with the 40 or 60 S subunits. Initial studies performed by us have shown that expression of ABC50 in a GCN20 deficient strain of S. cerevisiae does not complement the function of GCN20.4 However, if ABC50 is the mammalian homologue of GCN20, this inability to complement the GCN20 strain probably reflects the lack of similarity between the N-terminal sequences of the two proteins; it has been shown that it is the N-terminal one-third of GCN20 that interacts with GCN1 and that this is the only region of the protein required for complementation of a GCN20 deletion strain of S. cerevisiae (17).

The possible functional effect of the eIF2/ABC50 interaction on the activity of eIF2 was studied by employing the ABC50-deficient eIF2 sample obtained from HeLa cell lysate in GDP binding and ternary complex assays. The presence of ABC50 was found to enhance the binding of Met-tRNA to eIF2 but not the binding of GDP. This increased ternary complex formation could be due to the stabilization of a particular conformation of eIF2, which has higher affinity for Met-tRNA, as a consequence of its interaction with ABC50. This suggests a potential in vivo function for ABC50 during the initiation of translation. Initial studies indicate that phosphorylation of eIF2α (by recombinant RNA-dependent protein kinase) is unaffected by the presence or absence of ABC50,3 indicating that ABC50 is unlikely to have a role in modulating the phosphorylation reaction.

The sequence of ABC50 showed it to be a member of the ABC family of proteins. These are generally membrane-associated transporters dependent upon ATP hydrolysis for their activity (46). Two known exceptions to this generalization are the S. cerevisiae proteins eEF3 and GCN20, both of which have been identified as proteins involved in the process of translation (although their precise functions remain obscure). eEF3 is thought to monitor the fidelity of the translational elongation process in an ATP-dependent fashion (47, 48), whereas GCN20, together with GCN1, is involved in the modulation of the GCN2 eIF2α kinase activity in response to amino acid starvation (17, 49). eEF3 and GCN20 each associate with the translational machinery, and the similarity exhibited by ABC50 to these members of the eEF3 subfamily of the ABC transporters suggested that it was also likely to be involved in the process of translation. Here we have shown by means of sucrose cushion sedimentation and sucrose density gradient analyses that ABC50 also associates with the ribosome. This association was with the 40 and 60 S subunits and not with the polysomal material. This strongly indicates a role for ABC50 in translation that is probably at the stage of initiation, and it is interesting to note that in activated T-cells, the expression of ABC50 increases after a period of 8–16 h that parallels the increased expression of other initiation factors, e.g. eIF2, as demonstrated here and elsewhere (34–36, 95, 50).

REFERENCES
1. Pain, V. M. (1996) Eur. J. Biochem. 236, 747–771
2. Price, N. T., and Proud, C. G. (1994) Biochimica (Paris) 76, 748–760
3. Proud, C. G. (1992) Curr. Top. Cell Regul. 32, 543–569
4. Hannig, E. M., Cigan, A. M., Freeman, B. A., and Kinzy, T. G. (1993) Mol. Cell. Biol. 13, 506–520
5. Flynn, A., Oldfield, S., and Proud, C. G. (1993) Biochim. Biophys. Acts 1174, 117–121
6. Donahue, T. F., Cigan, A. M., Palsch, E. K., and Valavicus, B. C. (1988) Cell 54, 621–632
7. Das, S., Maiti, T., Das, K., and Maitra, U. (1997) J. Biol. Chem. 272, 3172–3178
8. Kimball, S. R., Heinzinger, N. K., Horetsky, R. L., and Jefferson, L. S. (1998) J. Biol. Chem. 273, 3039–3044
9. Clemens, M. J. (1996) in Translational Control (Hersh, J. W. B., Mathews, M. B., and Sonenberg, N., eds) pp. 139–172 Cold Spring Harbor Labora-

Acknowledgments—We thank Andrè Beaulieu and Manon Richard (Center Hospitalier de L’Université Laval, Canada) for sharing data with us prior to publication and for providing an antibody to ABC50. We thank Kevin Howland (University of Kent at Canterbury) for protein sequence analysis and peptide synthesis; Andrew Cassidy (University of Kent at Canterbury) for valuable assistance with protein sequencing; and Graham Pavitt (University of Dundee) for help and advice.

3 J. K. Tyzack, X. Wang, and C. G. Proud, unpublished observations.
4 J. K. Tyzack, G. Pavitt, and C. G. Proud, unpublished observations.
