The translation initiation factor eIF2 β is an interactor of protein phosphatase-1

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It is reasonably well understood how the initiation of translation is controlled by reversible phosphorylation of the eukaryotic translation initiation factors eIF2 α , eIF2 β and eIF4E. Other initiation factors, including eIF2 β , are also established phosphoproteins but the physiological impact of their phosphorylation is not known. Using a sequence homology search we found that the central region of eIF2 β contains a putative PP1-(protein phosphatase-1) binding RVxF-motif. The predicted eIF2 β -PP1 interaction was confirmed by PP1 binding and co-immunoprecipitation assays on cell lysates as well as with the purified components. Site-directed mutagenesis showed that eIF2 β contains, in addition to an RVxF-motif, at least one other PP1-binding site in its C-terminal half. eIF2 β functioned as an inhibitor for the dephosphorylation of gly-

cogen phosphorylase and Ser⁵¹ of eIF2 α by PP1, but did not affect the dephosphorylation of Ser⁴⁶⁴ of eIF2 β by this phosphatase. Strikingly, eIF2 β emerged as an activator of its own dephosphorylation (Ser², Ser⁶⁷, Ser²¹⁸) by associated PP1, since the substrate quality of eIF2 β was decreased by the mere mutation of its RVxF-motif. These results make eIF2 β an attractive candidate substrate for associated PP1 *in vivo*. The overexpression of wild-type eIF2 β or eIF2 β with a mutated RVxF-motif did not differentially affect the rate of translation, indicating that the binding of PP1 is not rate-limiting for translation under basal conditions.

Key words: protein (de)phosphorylation, eIF2 β , protein phosphatase-1, translation.

INTRODUCTION

The pre-initiation complex of translation contains eIF1 (eukaryotic initiation factor 1), GTP-bound eIF2, Met-tRNA^{Met} and the 40 S ribosomal subunit, and is converted into the 43 S pre-initiation complex by association with mRNA and eIF5. A conformational change in this 43 S pre-initiation complex activates the hydrolysis of GTP. Subsequently, the recognition of the AUG start codon triggers the release of the resulting inorganic phosphate from eIF2 and the dissociation of eIF1 from the complex. Next, the release of eIF2-GDP from the complex enables the recruitment of the 60 S ribosomal subunit [1,2]. The recycling of the eIF2-GTPase complex for a subsequent round of translation depends on the eIF2B-mediated exchange of eIF2-associated GDP by GTP. eIF2 consists of α , β and γ subunits [3]. eIF2 γ with its intrinsic GTPase activity functions as the catalytic subunit and interacts with both GTP and Met-tRNA^{Met}. eIF2 α is a regulatory subunit and its phosphorylation on Ser⁵¹ by one of at least four stress-induced protein kinases turns it into an inhibitor of the GTP exchange factor eIF2B and thus into an inhibitor of the initiation of translation [4]. Finally, eIF2 β interacts with mRNA, eIF1, eIF5 and eIF2 β [5]. The recruitment of eIF1 prevents premature GTP hydrolysis by eIF2 [6]. The interaction between eIF2 β and eIF2 γ is required for the correct usage of the AUG start codon [7]. eIF2 β is the catalytic subunit of the GTP exchange factor eIF2B and its recruitment by eIF2 β requires phosphorylation by protein kinase CK2 [8]. Phosphorylation of eIF2 β by protein kinase CK1 enhances its catalytic activity, whereas phosphorylation by DYRK [dual-specificity tyrosine-(Y)-phosphorylation regulated kinase] and the insulin-regulated protein kinase GSK3 (glycogen synthase kinase 3) have an inhibitory effect on the catalytic activity of eIF2 β [7]. eIF5 acts as a GTPase-

activating protein for the catalytic centre of eIF2 γ [9,10] and is phosphorylated by protein kinase CK2. However, the role of this phosphorylation is unknown. eIF2 β is also a phosphoprotein *in vivo*. Phosphorylation of eIF2 β by protein kinase A increases the guanine-nucleotide exchange activity of associated eIF2B [11]. eIF2 β is also phosphorylated by the DNA-dependent protein kinase as well as by protein kinases C and CK2, but the significance of phosphorylation by these kinases is not understood [12,13].

PP1 (Protein phosphatase-1) is a major protein Ser/Thr phosphatase that regulates numerous cellular processes by its ability to dephosphorylate key regulatory proteins. The catalytic subunit of PP1 is present in all eukaryotic cells and forms dozens of different PP1 holoenzymes by association with a host of regulatory polypeptides. These protein interactors function as targeting subunits, substrate-specifiers, and inhibitors and/or substrates for associated PP1 [14]. The large majority of PP1 interactors contain a degenerate, so-called RVxF-motif that conforms to the consensus sequence: [RK]-x₀₋₁-[VI]-{P}-[FW], where x denotes any residue and {P} any residue except proline [15,16]. This motif binds with high affinity to a hydrophobic channel that is remote from the catalytic site of PP1. The binding of the RVxF-motif has, itself, no major effects on the conformation or activity of PP1 [17]. However, the RVxF-mediated anchoring of PP1 promotes the occupation of secondary, lower-affinity binding sites and this often does affect the activity and/or substrate specificity of PP1 [15].

Various approaches have been used to identify novel protein interactors of PP1. Initially, interactors of PP1 were mainly identified by the purification and characterization of newly purified PP1 holoenzymes. Later, the identification of new PP1 interactors was mainly driven by yeast two-hybrid screens. Other PP1 interacting proteins were identified by affinity chromatography on an affinity

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; DYRK, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase; EGFP, enhanced green fluorescent protein; eIF, eukaryotic initiation factor; FCS, foetal calf serum; GSK3, glycogen synthase kinase 3; HEK-293T cells, human embryonic kidney 293 cells expressing the large T-antigen of simian virus 40; MELK, maternal embryonic leucine zipper kinase; NIPP1, nuclear inhibitor of protein phosphatase-1; PEI, polyethylenimine; PP1, protein phosphatase-1; TBS, Tris-buffered saline; wt, wild-type.

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matrix of microcystin, a cyclic heptapeptide that potently inhibits PP1 [14]. More recently, MS and tools of bioinformatics have been used to extend our knowledge of the PP1 interactome [18,19]. Using a combination of sequence alignment and biochemical data we show here that eIF2 β is a direct interactor of PP1 both *in vitro* and *in vivo*. We also report that eIF2 β has at least two PP1-interaction sites and functions as a substrate-specifier for PP1.

EXPERIMENTAL

Reagents

The catalytic subunits of PP1 and glycogen phosphorylase *b* were purified from rabbit skeletal muscle [20,21]. Protein kinase CK2 was isolated from pig spleen [22]. The catalytic subunit of beef protein kinase A and recombinant rat DYRK1A were obtained from Sigma-Aldrich and Upstate (Millipore) respectively. A synthetic peptide comprising the C-terminus of eIF2 β (C³²¹VTGK-RAQLRAKA³³²) coupled to keyhole limpet haemocyanin was used to generate a rabbit polyclonal antibody. The anti-eIF2 β antibodies were affinity-purified on the peptide coupled to BSA and linked to CNBr-activated Sepharose 4B (GE Healthcare). Monoclonal anti-PP1 antibodies were purified on protein-A Sepharose CL-4B (GE Healthcare). The hybridoma clone producing these antibodies was a gift from Dr J. Vandenheede (Afdeling Biochemie, University of Leuven, Leuven, Belgium). Monoclonal anti-FLAG antibodies were obtained from Stratagene. Polyclonal anti-EGFP (enhanced green fluorescent protein) antibodies were obtained from Santa Cruz Biotechnology, Inc. Polyclonal antibodies specific for eIF2 α phosphorylated at Ser⁵¹ were from Biosource International. Swine anti-rabbit and rabbit anti-mouse antibodies were purchased from DakoCytomation.

Plasmids

The sequence encoding rabbit PP1 α was cloned in between the XhoI and BamHI sites of pEGFP-C1 (Clontech), yielding an expression vector for EGFP-PP1 α . The sequence encoding human eIF2 β was subcloned between the XhoI and BamHI sites of pEGFP-C1 and the EGFP sequence in this vector was replaced by the FLAG-sequence, generating an expression vector for FLAG-eIF2 β . The human cDNAs encoding eIF2 β ^{1–333}, eIF2 β ^{1–333(V131A/F133A)}, eIF2 β ^{1–247(V131A/F133A)}, eIF2 β ^{1–215(V131A/F133A)}, eIF2 β ^{1–145(V131A/F133A)} and eIF2 β ^{187–314} were subcloned into the pET16b vector in frame with the His₆-tag, in between the NdeI and XhoI restriction sites. The pET15b plasmid encoding His₆-tagged rabbit GSK3 and the pET8c-CK1 δ plasmid encoding His₆-tagged protein kinase CK1, were gifts from Dr P. Roach (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IA, U.S.A.). Vectors encoding GST (glutathione S-transferase)-fused to eIF2 α (pGEX-2T-eIF2 α), eIF5 (pGEX-KG-eIF5) and eIF2B ϵ (pGEX-HA-1-eIF2B ϵ) were gifts from Dr T. Matsui (Department of Biochemistry, Kanazawa Medical University, Uchinada-cho, Ishikawa, Japan), Dr U. Maitra (Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, NY, U.S.A.) and Dr C. Proud (Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada) respectively. Mutations were introduced by the QuikChange[®] (Stratagene) site-directed mutagenesis method, with the appropriate primers and templates. The sequences of all DNA constructs were verified.

Preparation of recombinant proteins

The His₆-tagged polypeptides and GST-fusions were produced in *Escherichia coli* BL21(DE3)pLysS cells by transformation with

the pET plasmid or the pGEX plasmid respectively. The His₆-tagged proteins were purified on Ni²⁺-Sepharose[™] 6 Fast Flow (GE Healthcare). The GST-fusion proteins were purified on glutathione-agarose (Sigma-Aldrich).

Pull-down experiments with Ni²⁺-Sepharose

Equimolar amounts of BSA, His₆-tagged eIF2 β wt (wild-type) and eIF2 β mutants were pre-incubated for 1.5 h at 10°C with Ni²⁺-Sepharose[™] 6 Fast Flow that had been pre-blocked with TBS (Tris-buffered saline) containing 1 mg/ml BSA and 0.1 % Nonidet P40. After washing of the beads, the suspension was incubated for 45 min at 10°C with 0.2 pmol of PP1. Next the beads were sedimented by centrifugation for 1 min at 1000 *g*, and then washed 3 times, first with TBS containing 0.25 M LiCl, then with TBS plus 0.1 % Nonidet P40, and finally with buffer containing 50 mM imidazole, pH 7.4, 0.5 mM DTT (dithiothreitol) and 5 mM 2-mercaptoethanol. The washed beads were used for the assay of trypsin-revealed phosphorylase phosphatase activity.

Cell culture

HEK-293T cells [human embryonic kidney 293 cells expressing the large T-antigen of SV40 (simian virus 40)] were grown in DMEM (Dulbecco's modified Eagle's medium) with 10 % (v/v) FCS (foetal calf serum) on 10 cm diameter plates or in sterile 6-well plates (Greiner). One day after seeding, the cells were transfected with plasmid DNA using the FuGene6[™] transfection reagent (Roche), according to the manufacturers protocol, or using PEI (polyethylenimine) obtained from Sigma-Aldrich. Cell transfection experiments with PEI were performed in DMEM with 5 % (v/v) FCS. One day after transfection, the medium was replaced with fresh DMEM with 10 % (v/v) FCS.

Cells were blocked in mitosis by the addition of 500 ng/ml Nocodazole (Sigma-Aldrich) for 17 h. Cells were blocked in the G1/G2-phases by the addition of 1 μ M Trichostatin A (BioMol) for 17 h. To induce endoplasmic reticulum stress, cells were incubated for 1 h with 100 μ M thapsigargin (Alexis Biochemicals). UV-C irradiation at 254 nm of cells was carried out using a UV Stratalinker 1800 (Stratagene).

The cells were lysed 48 h after transfection, in PBS supplemented with 0.5 mM PMSF, 0.5 mM benzamidine and 5 μ M leupeptin. Cell lysates were sonicated (3 $\times$ 30 s) and centrifuged at 20000 *g* for 10 min. The supernatants were used for immunoprecipitation experiments. Within the same experiment, the total protein concentration under the various conditions was equalized.

Immunological procedures

For immunoprecipitation, cell lysates were incubated with antibodies against eIF2 β , EGFP or the FLAG-peptide for 3 h at 4°C. Subsequently, protein-A-TSK-Sepharose[®] (Affiland) was added, and the mixture was rotated for 30 min at 4°C. Following centrifugation (1 min at 1000 *g*), the pellet was washed twice with TBS supplemented with 0.1 % Nonidet P40, and once with buffer containing 50 mM imidazole (pH 7.4), 0.5 mM DTT and 5 mM 2-mercaptoethanol. The pellets were either boiled in SDS/sample buffer for immunoblotting or assayed for trypsin-revealed phosphorylase phosphatase activity. For immunoblotting, the retained antibodies were visualized by enhanced chemiluminescence (Western Lightning, Perkin Elmer).

For overlay assays, proteins were directly spotted on a PVDF membrane, and the membrane was blocked in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 3 % (w/v) dried milk powder for 3 h at 4°C. Subsequently, the blots were incubated overnight in TBS with

1 mg/ml BSA and digoxigenin-labelled PP1. After extensive washes with TBS, the retained PP1 was detected by anti-digoxigenin antibodies (Roche Diagnostics), according to the manufacturer's protocol. For far-Western overlays, the blocked blots were incubated in TBS with 1 mg/ml BSA and PP1 for 5 h at room temperature. After washing twice with TBS and once with TBS containing 5 % (w/v) dried milk powder and 0.2 % Triton X-100, PP1 was visualized by immunoblotting.

Assays

Protein phosphatase activities were assayed at 30 °C with phosphorylase *a* as a substrate, by measuring the released acid-stable radioactivity [23]. To measure the total protein phosphatase activity of PP1 that co-sedimented with eIF2 β , the samples were pretreated with 0.1 mg/ml trypsin for 5 min at 30 °C to release the free, fully active catalytic subunit. Subsequently, 1 mg/ml of trypsin inhibitor was added and the phosphorylase phosphatase activity was measured. Other, recombinant substrates of PP1 were phosphorylated by incubation for 30–60 min at 30 °C in 20 mM Tris/HCl, pH 7.5, plus 0.1 mM ATP (with 0.1 μ Ci/ μ l [γ -³²P]ATP or without), 2 mM magnesium acetate and the indicated protein kinase. Phosphorylation reactions were terminated by the addition of 6.6 mM EDTA. Subsequently, the mixtures were incubated with PP1 with eIF2 β or without. At the indicated times aliquots were boiled in SDS/sample buffer and the extent of phosphorylation was visualized by autoradiography after SDS/PAGE or by immunoblotting with eIF2 α phosphoSer⁵¹-specific antibodies.

Protein synthesis measurements

Equal numbers of HEK-293T cells were plated onto 6-well dishes and then transfected with plasmids encoding FLAG-eIF2 β or FLAG-eIF2 β ^{V131A/F133A}. After 48 h, the cells were labelled with 50 μ Ci of [³⁵S]Met (GE Healthcare) per ml in methionine-free culture medium (Invitrogen), supplemented with 10 % foetal bovine serum for 2 h at 37 °C. Cells were washed three times in PBS and then disrupted with lysis buffer. After sonication, the lysates were centrifuged (10 000 *g* for 10 min). An aliquot of 5 μ l of the supernatants was diluted in 2 ml of scintillation liquid Lumasafe Plus (Lumac LSC) and analysed by scintillation counting to determine the incorporation of [³⁵S]Met per mg of protein.

RESULTS

Identification of eIF2 β as a novel interactor of PP1

The sequence-profile alignment method MaxHom (<http://www.cbi.pku.edu.cn/mirror/predictprotein/>) [24] suggested structural similarities between the established PP1 interactor NIPPI1 (nuclear inhibitor of protein phosphatase-1) and the translation initiation factor eIF2 β (Figure 1A). The similarities included residues K¹²⁹NVKF¹³³ of eIF2 β , which form a consensus PP1-binding 'RVxF'-motif, i.e. [RK]-x₀₋₁-[VI]-{P}-[FW], as defined by Wakula et al. [16]. Moreover, as in NIPPI1 and various other established PP1-interactors [16], the RVxF-motif of eIF2 β is flanked N-terminally by a stretch of basic residues and C-terminally by an acidic stretch. This instance is conserved in eIF2 β from mammals, birds, amphibians and some fish, but not in non-chordate eIF2 β (Figure 1B). Clearly, the acquisition of the RVxF-instance postdates the primary function of eIF2 β in translation, which qualifies the protein as a 'secondary' interactor of PP1, i.e. a polypeptide that acquired its PP1-binding site later in evolution [18].

To explore experimentally the predicted interaction between PP1 and eIF2 β , we generated bacterially expressed His₆-eIF2 β ,

affinity-purified on Ni²⁺-Sephacrose. His₆-eIF2 β bound to digoxigenin-labelled PP1 in overlay assays is shown in Figure 2(A). NIPPI1 served as a positive control in this experiment while NIPPI1¹⁻³²⁹-RATA and BSA were used as negative controls. His₆-eIF2 β also inhibited PP1 with an IC₅₀ of around 50 nM, using glycogen phosphorylase *a* as substrate (Figure 2B). In contrast, eIF2 β did not affect the phosphorylase phosphatase activity of the structurally related PP2A, indicating that it is a specific inhibitor of PP1. Importantly, the activity of PP1 inhibited by eIF2 β could be fully recovered by the destruction of eIF2 β with trypsin (results not shown). Thus, unlike the PP1 interactor inhibitor-2, eIF2 β does not convert PP1 into an inactive, trypsin-sensitive conformation [25].

Subsequently, we examined the association between eIF2 β and PP1 in cell lysates. For this purpose, we performed immunoprecipitations with anti-FLAG or anti-EGFP antibodies, following the expression of FLAG-eIF2 β and EGFP-PP1 α in HEK-293T cells. These experiments revealed a reciprocal co-immunoprecipitation of FLAG-eIF2 β and EGFP-PP1 α (Figure 3A). In addition, we found that the immunoprecipitation of endogenous eIF2 β resulted in the co-precipitation of endogenous PP1, as revealed by immunoblotting (Figure 3B) and phosphorylase phosphatase assays (Figure 3C). The latter activity was completely inhibited by the addition of NIPPI1, showing that it was derived from PP1.

We have also explored whether the eIF2 β -PP1 interaction is affected by treatments that are known to influence the rate of translation by affecting the phosphorylation of eIF2 α , such as the addition of thapsigargin, treatment with UV-C irradiation or synchronization of cells in the G₀-phase (by serum starvation), M-phase (by nocodazole treatment) or G1/G2-phases (by the addition of trichostatin A). However, none of these treatments measurably affected the association between eIF2 β and PP1. However, we were able to confirm that these treatments indeed affected the phosphorylation level of eIF2 α (results not shown).

Mapping of the eIF2 β -PP1 interaction sites

To examine whether residues K¹²⁹NVKF¹³³ of eIF2 β constituted a functional RVxF-motif, we mutated Val¹³¹ and Phe¹³³ to alanine. The resulting peptide His₆-eIF2 β ^{V131A/F133A}, henceforth referred to as eIF2 β -KNAKA, bound to Ni²⁺-Sephacrose, was used as an affinity matrix for the binding of purified PP1. Compared with the wt protein, eIF2 β -KNAKA displayed a severely decreased ability to bind (Figure 4A) and inhibit PP1 (Figure 4B). The importance of the RVxF-motif for the inhibition of PP1 was further examined using a synthetic RVxF-containing decapeptide, namely NIPPI1¹⁹⁷⁻²⁰⁶, as a competitor for the binding to the RVxF-binding site on PP1. The addition of 50 μ M NIPPI1¹⁹⁷⁻²⁰⁶, but not the corresponding RATA-peptide, abrogated the inhibition of PP1 by eIF2 β (Figure 4C), indicating that the RVxF-motif of eIF2 β is indeed required for its ability to inhibit the phosphorylase phosphatase activity of PP1.

Although eIF2 β -KNAKA was clearly deficient in the inhibition of PP1 (Figure 4B), it could still retain approx. 25 % of the amount of PP1 that interacted with wt eIF2 β (Figure 4A). Consistent with the presence of a second PP1-interaction site, we found that: (1) RVxF-containing peptides could only partially disrupt the binding between PP1 and wt eIF2 β ; (2) PP1 still bound to the eIF2 β -KNAKA in overlay assays, and; (3) eIF2 β -KNAKA, when expressed in HEK-293T cells, also co-immunoprecipitated with EGFP-PP1 from cell lysates in a reciprocal manner (results not shown). To gain more insight into the region of eIF2 β that harbours a second PP1 binding site, we generated C-terminal deletions of eIF2 β -KNAKA and examined the ability of these

Figure 1 Similarities between eIF2 β and the central domain of NIPP1

(A) Alignment of the corresponding parts of human NIPP1 and eIF2 β sequences by the MaxHom programme. Numbers outside the alignments indicate NIPP1 (top) and eIF2 β (bottom) residues; numbers within the alignments indicate the insertions (not to scale) of non-conserved fragments in eIF2 β . Identical residues are boxed in black. Residues conserved within the same group [positively charged (H, K and R); negatively charged (D and E); small residues (G, A, S and T); bulky hydrophobic residues (I, L, V, M, F and Y)] are boxed in grey. (B) The Table shows the sequence of RVxF-motifs with respect to a conserved poly-lysine tract. The residues of the RVxF-motif are in bold. The N-terminal basic residues and the C-terminal acidic residues are underlined.

eIF2 β fragments to bind PP1 (Figure 5). C-terminal truncation mutants of eIF2 β -KNAKA clearly bound less PP1, and eIF2 β ¹⁻¹⁴⁵-KNAKA barely retained any PP1 (Figure 5) and did not bind to PP1 in overlay assays (Figure 2A), which points to the existence of a second, C-terminal binding site for PP1. Consistent with this view, we found that eIF2 β ¹⁸⁷⁻³⁴¹ bound about as much PP1 compared with eIF2 β -KNAKA (Figure 5). Thus, binding of eIF2 β to PP1 is mediated by the RVxF-motif and at least one additional binding site in the C-terminal half of eIF2 β .

Functional analysis of the eIF2 β -PP1 interaction

Mammalian eIF2 β interacts with eIF5, eIF2B and eIF2 γ , and the latter binds in turn to eIF2 α . Since the initiation factors eIF2 α , eIF2B ϵ and eIF5 are established phosphoproteins, we examined whether these phosphoproteins are substrates for PP1 in the absence or presence of eIF2 β . Ser⁵¹ of eIF2 α , phosphorylated *in vitro* with protein kinase MELK (maternal embryonic leucine zipper kinase) [26] was readily dephosphorylated by PP1. The addition of wt eIF2 β , but not of eIF2 β -KNAKA, inhibited the activity of PP1 towards eIF2 α (Figure 6A). Thus, eIF2 β is an inhibitor of the dephosphorylation by PP1 of both glycogen phosphorylase α (Figure 2B) and eIF2 α (Figure 6A). eIF2B ϵ is phosphorylated

by protein kinases CK2 (Ser⁷¹² and Ser⁷¹³), DYRK1 (Ser⁵³⁹) and CK1 (Ser⁴⁶⁴). However, only CK1-phosphorylated eIF2B ϵ was dephosphorylated by purified PP1 (Figure 6B and results not shown) and, surprisingly, this dephosphorylation was only very mildly affected by the addition of eIF2 β or eIF2 β -KNAKA (Figure 6B). eIF5 phosphorylated by protein kinase CK2 (Ser³⁸⁷ and Ser³⁸⁸) was not dephosphorylated by PP1, whether or not eIF2 β was added (results not shown). Finally, we have examined whether eIF2 β , phosphorylated by protein kinase CK2 (Ser² and Ser⁶⁷) or protein kinase A (Ser²¹⁸) [13], is itself a substrate for associated PP1. The phosphorylation of eIF2 β by either kinase was reversed by PP1 but, surprisingly, the dephosphorylation of eIF2 β -KNAKA was clearly less efficient (Figure 6C). These results imply that the binding of PP1 to the RVxF-instance promotes the dephosphorylation of eIF2 β by associated PP1.

Since the *in vivo* function of the phosphorylation of eIF2 β by protein kinases A and CK2 is not known, we have examined whether the recruitment of PP1 by eIF2 β is a determinant of the rate of translation. For this purpose, wt eIF2 β or eIF2 β -KNAKA were transiently expressed in HEK-293T cells and the rate of translation was derived from the incorporation of [³⁵S]Met into proteins. The expression of wt eIF2 β or the eIF2 β -KNAKA mutant inhibited the rate of translation by 42 $\pm$ 7 % and 53 $\pm$ 4 %

Figure 2 eIF2 β is a specific inhibitor of PP1

(A) An overlay with digoxigenin-labelled catalytic subunit of PP1 shows binding to wt eIF2 β but not to eIF2 β -KNAKA (see also Figure 5). NIPP1 was included as a positive control, and NIPP1¹⁻³²⁹-RATA (a NIPP1-mutant that does not bind to PP1 [34]) and BSA were used as negative controls; 380 pmol of each protein was used. (B) The effect of the indicated concentrations of eIF2 β on the phosphorylase phosphatase activity of PP1 and PP2A. The results are presented as means $\pm$ S.E.M. of three assays.

($n = 10$) respectively. Thus, the severely decreased ability of the KNAKA-mutant to interact with PP1 did not differentially affect the rate of translation. Consistent with this observation, we found that the addition of equimolar concentrations of wt eIF2 β or eIF2 β -KNAKA to reticulocyte lysates decreased the rate of translation of a reporter transcript, encoding the histone methyltransferase EZH2, to a similar extent (see supplementary Figure S1 at <http://www.BiochemJ.org/bj/400/bj4000377add.htm>). It is noteworthy that EZH2 is not an interactor of PP1 and has no effect on translation. Collectively, these results suggest that the eIF2 β -PP1 interaction is not rate-limiting for protein synthesis.

DISCUSSION

A bioinformatics approach has already previously led to the identification of new members of established families of PP1 interactors [18]. Also, Meiselbach et al. [19] showed recently that a more stringent definition of the RVxF-motif enables a reasonably specific identification of functional RVxF-instances. In the present paper, we report the sequence-alignment-assisted identification of eIF2 β as an interactor of PP1. The sequence similarity between NIPP1 and eIF2 β was not expected since these proteins are functionally unrelated. Indeed, NIPP1 has been implicated in transcription and pre-mRNA splicing but not in translation [27,28]. The structural similarities between NIPP1 and eIF2 β not only comprised the RVxF-motif but also the flanking sequences of this motif (Figure 1A). The RVxF-instance of eIF2 β is absent from non-chordates and from some fish species (Figure 1B), indicating that PP1 binding, via its RVxF-instance, is a property that eIF2 β acquired during metazoan evolution. We have demonstrated by various independent biochemical approaches that the predicted RVxF-instance of eIF2 β is functional (Figure 4). Moreover, the region of eIF2 β that harbours the RVxF-motif is predicted by the secondary structure prediction programme

Figure 3 Co-immunoprecipitation experiments confirm the eIF2 β -PP1 interaction

(A) Lysates from HEK-293T cells co-expressing EGFP-PP1 α and FLAG-eIF2 β were used for immunoprecipitations with protein-A-TSK and anti-EGFP antibodies, anti-FLAG antibodies or without antibodies (control). The presence of EGFP-PP1 α and FLAG-eIF2 β was detected by Western analysis with anti-EGFP and anti-FLAG antibodies. Endogenous eIF2 β from HEK-293T cell lysates was immunoprecipitated and the precipitates were screened for the presence of eIF2 β and PP1 by immunoblotting (B) and for trypsin-revealed phosphorylase phosphatase activities in the absence and presence of 1 μ M NIPP1 (C).

SCRATCH (<http://www.igb.uci.edu/tools/scratch>) to form an exposed loop, which is also true for RVxF-motifs in other established PP1 interactors [18]. In addition the RVxF-instance of eIF2 β corresponds to the more stringent definition of these motifs described by Meiselbach et al. [19].

Two PP1 interactors, namely GADD34 and CreP, were already known to target PP1 to the eIF2 complex and to thereby promote the dephosphorylation of Ser⁵¹ of eIF2 α [29–31]. In the present paper, we have shown that another subunit of eIF2, namely the β -subunit, directly recruits PP1 (Figures 2 and 3) and is itself a likely substrate for dephosphorylation by associated PP1 (Figure 6C and 6D). It remains to be examined whether eIF2 β is also an *in vivo* substrate of associated PP1 and whether this somehow affects translation. Our preliminary data show that the binding of PP1 to eIF2 β is not rate-limiting for translation under basal circumstances. However, it cannot be excluded that eIF2 β -associated PP1 only exerts a translational control for a selected group of transcripts or under specific conditions. Consistent with the role of PP1

Figure 4 eIF2β contains a functional RVxF-motif

(A) Equimolar amounts of recombinant His₆-eIF2β and His₆-eIF2β-KNAKA, coupled to Ni²⁺-Sepharose, were incubated with the catalytic subunit of PP1, purified from rabbit skeletal muscle. The retained phosphorylase phosphatase activities were quantified after the release of PP1 from the beads by pre-incubation with trypsin. (B) Effect of wt eIF2β and eIF2β-KNAKA on the phosphorylase phosphatase activities of the catalytic subunit of PP1. (C) Effect of the addition of buffer (control), 50 μM of synthetic decapeptide NIPP¹⁹⁷⁻²⁰⁶-RVTF or 50 μM of a variant peptide in which the RVTF residues were replaced by RATA, on the inhibition of PP1 by eIF2β. Results are presented as means ± S.E.M. for three phosphatase assays.

as a cellular 'reset' button [14], it can also be envisaged that the dephosphorylation of eIF2β by associated PP1 is implicated in the recycling of eIF2β for a next round of translation. Also, since the addition of leptomycin-B, an inhibitor of nuclear export, causes the nuclear accumulation of eIF2β [32], PP1 may play a regulatory role in the poorly studied nuclear functions of eIF2β. Intriguingly, the binding of PP1 to eIF2β promoted the dephosphorylation of eIF2β, while it inhibited the dephosphorylation of glycogen phosphorylase and eIF2α, but did not affect the dephosphorylation of eIF2β_E (Figure 6). Thus, like many PP1-interactors [14], eIF2β appears to have a substrate-specifying function. Interestingly, eIF2β not only binds to PP1 but also interacts directly with protein kinase CK2 [33]. Thus, eIF2β may function as a scaffold for the assembly of a signalling complex that includes CK2 and PP1, which somehow controls the initiation of translation.

Figure 5 Mapping of a second PP1 interaction site in eIF2β

The Figure shows the His₆-tagged eIF2β mutants/fragments that were used for PP1 binding assays. Equimolar concentrations of the fusions, linked to Ni²⁺-Sepharose, were incubated with an equal amount of purified PP1. The right column shows the amount of retained PP1, as derived from the trypsin-revealed phosphorylase phosphatase activity and expressed as a percentage of binding to wild-type eIF2β. The results are presented as means ± S.E.M. for 4–8 phosphatase assays.

A. Dephosphorylation of eIF2α (Ser51)

B. Dephosphorylation of eIF2β_E (Ser464)

C. Dephosphorylation of eIF2β (Ser2/Ser67)

D. Dephosphorylation of eIF2β (Ser218)

Figure 6 *In vitro* dephosphorylation of eIF2α, eIF2β_E and eIF2β by PP1

The panels show time courses of dephosphorylation of the indicated substrates by 88 nM PP1 in the presence of buffer, 1 μM wt eIF2β or eIF2β-KNAKA. Prior to the addition of PP1, phosphorylation reactions were terminated by the addition of EDTA. (A) Dephosphorylation of MELK-phosphorylated Ser⁵¹ in eIF2α, as visualized by anti-phosphoSer51 antibodies. (B) Dephosphorylation of eIF2β_E phosphorylated by protein kinase CK1, as visualized by autoradiography. An autoradiogram of the dephosphorylation of wt eIF2β and eIF2β-KNAKA, phosphorylated by protein kinase CK2 (C) or protein kinase A (D).

In conclusion, we have shown, in the present paper, that PP1 is a physiological interactor of eIF2 β . We have identified a functional RVxF-motif and a second PP1-binding site in the C-terminal half of eIF2 β and have provided evidence that eIF2 β functions as a substrate-specifier for associated PP1. Strikingly, eIF2 β itself emerges as the most likely substrate for associated PP1 and its dephosphorylation depends on a functional RVxF-motif.

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REFERENCES

- Preiss, T. and Hentze, W. M. (2003) Starting the protein synthesis machine: eukaryotic translation initiation. *Bioessays* **25**, 1201–1211
- Algire, M. A., Maag, D. and Lorsch, J. R. (2005) P_i release from eIF2, not GTP hydrolysis is the step controlled by start-site selection during eukaryotic translation initiation. *Mol. Cell* **20**, 251–262
- Kimball, S. R. (1999) Eukaryotic initiation factor eIF2. *Int. J. Biochem. Cell B.* **31**, 25–29
- Holcik, M. and Sonenberg, N. (2005) Translational control in stress and apoptosis. *Nat. Rev. Mol. Cell Biol.* **6**, 318–327
- Kapp, L. D. and Lorsch, J. R. (2004) The molecular mechanics of eukaryotic translation. *Annu. Rev. Biochem.* **73**, 657–704
- Unbehauen, A., Borukhov, S. I., Hellen, C. U. and Pestova, T. V. (2004) Release of initiation factors from 48 S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP. *Genes Dev.* **18**, 3078–3093
- Hashimoto, N. N., Carnevali, L. S. and Castilho, B. A. (2002) Translation initiation at non-AUG codons mediated by weakened association of eukaryotic initiation factor (eIF) 2 subunits. *Biochem. J.* **367**, 359–368
- Wang, X., Paulin, F. E., Campbell, L. E., Gomez, E., O'Brien, K., Morrice, N. and Proud, C. G. (2001) Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the epsilon-subunit and their functions *in vivo*. *EMBO J.* **20**, 4349–4359
- Paulin, F. E., Campbell, L. E., O'Brien, K., Loughlin, J. and Proud, C. G. (2001) Eukaryotic translation initiation factor 5 (eIF5) acts as a classical GTPase activator protein. *Curr. Biol.* **11**, 55–59
- Das, S., Ghosh, R. and Maitra, U. (2001) Eukaryotic translation initiation factor 5 functions as a GTPase activating protein. *J. Biol. Chem.* **276**, 6720–6726
- Kimball, S. R., Heinzinger, N. K., Horetsky, R. L. and Jefferson, L. S. (1998) Identification of interprotein interactions between the subunits of eukaryotic initiation factors eIF2 and eIF2B. *J. Biol. Chem.* **273**, 3039–3044
- Ting, N. S., Kao, P. N., Chan, D. W., Lintott, L. G. and Lees-Miller, S. P. (1998) DNA-dependent protein kinase interacts with antigen receptor response element binding proteins NF90 and NF45. *J. Biol. Chem.* **273**, 2136–2145
- Welsh, G., Price, N. T., Bladergroen, B. A., Bloomberg, G. and Proud, C. G. (1994) Identification of novel phosphorylation sites in the β -subunit of translation initiation factor eIF-2. *Biochem. Biophys. Res. Commun.* **201**, 1279–1288
- Ceulemans, H. and Bollen, M. (2004) Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev.* **84**, 1–39
- Bollen, M. (2001) Combinatorial control of protein phosphatase-1. *Trends Biochem. Sci.* **26**, 426–431
- Wakula, P., Beullens, M., Ceulemans, H., Stalmans, W. and Bollen, M. (2003) Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. *J. Biol. Chem.* **278**, 18817–18823
- Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T., Cohen, P. and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* **16**, 1876–1887
- Ceulemans, H., Stalmans, W. and Bollen, M. (2002) Regulator-driven functional diversification of protein phosphatase-1 in eukaryotic evolution. *Bioessays* **24**, 371–381
- Meiselbach, H., Sticht, H. and Enz, R. (2006) Structural analysis of the protein phosphatase 1 docking motif: molecular description of binding specificities identifies interacting proteins. *Chem Biol.* **13**, 49–59
- DeGuzman, A. and Lee, E. Y. C. (1988) Preparation of low-molecular-weight forms of rabbit muscle protein phosphatase. *Methods Enzymol.* **159**, 356–368
- Fischer, E. H. and Krebs, E. G. (1958) The isolation and crystallization of rabbit skeletal muscle phosphorylase *b*. *J. Biol. Chem.* **231**, 65–71
- Agostinis, P., Derau, R., Sarno, S., Goris, J. and Merlevede, W. (1992) Phosphorylation of the phosphatase modulator subunit (inhibitor-2) by casein kinase-1. *FEBS Lett.* **305**, 121–124
- Beullens, M., Van Eynde, A., Stalmans, W. and Bollen, M. (1992) The isolation of novel inhibitory polypeptides of protein phosphatase 1 from bovine thymus nuclei. *J. Biol. Chem.* **267**, 16538–16544
- Sander, C. and Schneider, R. (1991) Database of homology-derived structures and the structural meaning of sequence alignment. *Proteins* **9**, 56–58
- Bollen, M. and Stalmans, W. (1992) The structure, role, and regulation of type 1 protein phosphatases. *Crit. Rev. Biochem. Mol.* **27**, 227–281
- Beullens, M., Vancauwenbergh, S., Morrice, N., Derau, R., Ceulemans, H., Waelkens, E. and Bollen, M. (2005) Substrate specificity and activity regulation of protein kinase MELK. *J. Biol. Chem.* **280**, 40003–40011
- Jin, Q., Van Eynde, A., Beullens, M., Roy, N., Thiel, G., Stalmans, W. and Bollen, M. (2003) The protein phosphatase-1 (PP1) regulator, nuclear inhibitor of PP1 (NIPP1), interacts with the polycomb group protein, embryonic development (EED), and functions as a transcriptional repressor. *J. Biol. Chem.* **278**, 30677–30685
- Beullens, M. and Bollen, M. (2002) The protein phosphatase-1 regulator NIPP1 is also a splicing factor involved in a late step of spliceosome assembly. *J. Biol. Chem.* **277**, 19855–19860
- Novoa, I., Zheng, H., Harding, H. P. and Ron, D. (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 α . *J. Cell Biol.* **153**, 1011–1022
- Connor, J. H., Weiser, D. C., Li, S., Hallenbeck, J. M. and Shenolikar, S. (2001) Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. *Mol. Cell Biol.* **21**, 6841–6850
- Jousse, C., Oyadomari, S., Novoa, I., Lu, P., Zhang, Y., Harding, H. P. and Ron, D. (2003) Inhibition of a constitutive translation initiation factor 2 α phosphatase, CreP, promotes survival of stressed cells. *J. Cell Biol.* **163**, 767–775
- Bohnsack, M. T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E. and Gorlich, D. (2002) Exp5 exports eEF1A via tRNA from nuclei and synergizes other transport pathways to confine translation to the cytoplasm. *EMBO J.* **21**, 6205–6215
- Llorens, F., Roher, N., Miro, F. A., Sarno, S., Ruiz, F. X., Meggio, F., Plana, M., Pinna, L. and Itarte, E. (2003) Eukaryotic translation initiation factor eIF2 β binds to protein kinase CK2: effects on CK2 α activity. *Biochem. J.* **375**, 623–631
- Beullens, M., Vulsteke, V., Van Eynde, A., Jagiello, I., Stalmans, W. and Bollen, M. (2000) The C-terminus of NIPP1 (nuclear inhibitor of protein phosphatase-1) contains a novel binding site for protein phosphatase-1 that is controlled by tyrosine phosphorylation and RNA binding. *Biochem. J.* **352**, 651–658

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