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The translation initiation factor eIF2β is an interactor of protein phosphatase-1

Paulina Wakula, Monique Beullens, Aleyde Van Eynde, Hugo Ceulemans, Willy Stalmans and Mathieu Bollen

Division of Biochemistry, Department of Molecular Cell Biology, Faculty of Medicine, KULeuven, B3000 Leuven, Belgium

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Corresponding author: M. Beullens,
Afdeling Biochemie
Campus Gasthuisberg
Herestraat 49, B-3000 Leuven,
Belgium
Phone: +32-16-34 57 01
Fax: +32-16-34 59 95
E-mail: Monique.Beullens@med.kuleuven.be

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ABSTRACT

It is reasonably well understood how the initiation of translation is controlled by reversible phosphorylation of the translation initiation factors eIF2α, eIF2Bε and eIF4E. Other initiation factors, including eIF2β, 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 the eukaryotic translation initiation factor eIF2β contains a putative PP1-binding RVxF motif. The predicted eIF2β-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 eIF2β contains, in addition to an RVxF-motif, at least one other PP1-binding site in its C-terminal half. eIF2β functioned as an inhibitor for the dephosphorylation of glycogen phosphorylase and eIF2α (Ser51) by PP1, but did not affect the dephosphorylation of eIF2Bε (Ser464) by this phosphatase. Strikingly, eIF2β emerged as an activator of its own dephosphorylation (Ser2, Ser67, Ser218) by associated PP1, since the substrate quality of eIF2β was decreased by the mere mutation of its RVxF-motif. These data make eIF2β an attractive in vivo candidate substrate for associated PP1. The overexpression of wild-type eIF2β or eIF2β 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 circumstances.

INTRODUCTION

The pre-initiation complex of translation contains eIF1, GTP-bound eukaryotic-initiation-factor (eIF) 2, Met-tRNA$_i^{Met}$ and the 40S ribosomal subunit, and is converted to the 43S preinitiation complex by association with mRNA and eIF5. A conformational change in this 43S preinitiation 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 60S ribosomal subunit [1, 2]. The recycling of the eIF2-GTPase complex for a next round of translation depends on the eIF2B-mediated exchange of eIF2-associated GDP by GTP. eIF2 consists of α,β and γ subunits [3]. eIF2γ with its

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intrinsic GTPase activity functions as the catalytic subunit and interacts with both GTP and Met-tRNAi^{Met}. eIF2α 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 initation of translation [4]. Finally, eIF2β interacts with mRNA, eIF1, eIF5 and eIF2Bε [5]. The recruitment of eIF1 prevents premature GTP hydrolysis by eIF2 [6]. The interaction between eIF2β and eIF2γ is required for the correct usage of the AUG start codon [7]. eIF2Bε is the catalytic subunit of the GTP exchange factor eIF2B and its recruitment by eIF2β requires phosphorylation by protein kinase CK2 [8]. Phosphorylation of eIF2Bε by protein kinase CK1 enhances its catalytic activity while phosphorylation by DYRK and the insulin-regulated protein kinase GSK3 has an inhibitory effect on the catalytic activity of eIF2Bε [7]. eIF5 acts as a GTPase-activating protein for the catalytic centre of eIF2γ [9, 10] and is known to be phosphorylated by protein kinase CK2. However, the role of this phosphorylation is unknown. eIF2β is also a phosphoprotein in vivo. Phosphorylation of eIF2β by protein kinase A increases the guanine-nucleotide exchange activity of associated eIF2B [11]. eIF2β 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].

Protein phosphatase-1 (PP1) 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, inhibitors and/or substrates for associated PP1 [14]. The large majority of PP1 interactors contain a degenerate, so-called RVxF-motif that actually conforms to the consensus sequence [RK]-x_{0-1}-[VI]-{P}-[FW], where x denotes any residue and {P} any residue except Pro [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 per se has 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].

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Various approaches have been used to identify novel protein interactors of PP1. Initially, interactors of PP1 were mainly identified by the purification and characterisation 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 matrix of microcystin, a cyclic heptapeptide that potently inhibits PP1 [14]. More recently, mass spectrometry 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 the eukaryotic initiation factor eIF2β is a direct interactor of PP1 in vitro and in vivo. We also report that eIF2β has at least two PP1-interaction sites and functions as a substrate-specifier of PP1.

EXPERIMENTAL

Reagents

The catalytic subunit 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, respectively. A synthetic peptide comprising the C-terminus of eIF2β (C^{321}VTGKRAQLRAKA^{332}) and coupled to keyhole limpet hemocyanin, was used to generate a rabbit polyclonal antibody. The anti-eIF2β antibodies were affinity-purified on the peptide coupled to bovine serum albumin 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 (University of Leuven). Monoclonal anti-FLAG antibodies were obtained from Stratagene. Polyclonal anti-EGFP antibodies were obtained from Santa Cruz. Polyclonal antibodies specific for eIF2α phosphorylated at Ser51 were delivered by Biosource International. Swine anti-rabbit and rabbit anti-mouse antibodies were purchased from DakoCytomation.

Plasmids

The sequence encoding rabbit PP1α was introduced between the *XhoI* and *BamHI* sites of pEGFP-C1 (Clonetech), yielding an expression vector for EGFP-PP1α. The

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sequence encoding human eIF2β was subcloned in the *XhoI-BamHI* sites of pEGFP-C1 and the EGFP sequence in this vector was replaced by an adaptor of 1 x FLAG-sequence, generating an expression vector for FLAG-eIF2β. The human cDNAs encoding eIF2β-(1-333), eIF2β-(1-333)-V131A/F133A), eIF2β-(1-247)-V131A/F133A, eIF2β-(1-215)-V131A/F133A, eIF2β-(1-145)-V131A/F133A and eIF2β-(187-314) were subcloned into the pET16b vector in frame with the polyhistidine encoding sequence, using the *NdeI-XhoI* restriction sites. The pET15b plasmid encoding polyhistidine-tagged rabbit GSK3 and the pET8c-CK1δ plasmid encoding polyhistidine-tagged protein kinase CK1, were gifts from Dr. P. Roach (Indiana University). Vectors encoding eIF2α (pGEX-2T-eIF2α), eIF5 (pGEX-KG-eIF5) and eIF2Bε (pGEX-HA-1-eIF2Bε) were gifts from Drs. T. Matsui (Kanazawa Medical University), U. Maitra (Albert Einstein College of Medicine) and C. Proud (University of Dundee). Mutations were introduced by the QuickChange site-directed mutagenesis method (Stratagene) with the appropriate primers and templates. The sequence of all DNA constructs was verified.

Preparation of recombinant proteins

The polyhistidine-tagged polypeptides and GST-fusions were produced in *Escherichia coli* BL21(DE3)pLysS cells by transformation with the pET plasmid and the pGEX plasmid, respectively. The His-tagged proteins were purified on Ni^{2+}-Sepharose™6 Fast Flow (GE Healthcare). The GST-fusion proteins were purified on glutathione-agarose (Sigma-Aldrich).

Pull-down experiments with Ni^{2+}-Sepharose

Equimolar amounts of BSA, polyhistidine-tagged eIF2β wt and eIF2β mutants were pre-incubated for 1.5 hour at 10°C with Ni^{2+}-Sepharose™6 Fast Flow (GE Healthcare) that had been pre-blocked with TBS plus BSA (1 mg/ml) and 0.1% Nonidet P-40. After washing of the beads, the suspension was incubated for 45 minutes at 10°C with 0.2 pmol PP1. Subsequently, the beads were sedimented (1 min at 1000 x *g*), washed 3 times, first with TBS buffer plus 0.25 M LiCl, then with TBS buffer that was supplemented with 0.1% Nonidet P-40, and finally with 50 mM imidazole at pH 7.4, 0.5 mM dithiothreitol and 5 mM β-mercaptoethanol. The

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washed beads were used for the assay of trypsin-revealed phosphorylase phosphatase activity.

Cell culture

HEK293T cells (human embryonic kidney cells) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum on 10-cm plates or in sterile 6-well plates (Greiner). One day after seeding, the cells were transfected with plasmid DNA, using the FuGene™6 Transfection reagent (Roche), according to the protocol of the manufacturer, or using polyethylenimine (PEI) obtained from Sigma-Aldrich. Cell transfection experiments with PEI were performed in DMEM with 5% fetal calf serum. One day after transfection, the medium was replaced with fresh DMEM supplemented with 10% fetal calf serum.

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

48 hours after transfection, the cells were lysed in PBS buffer, supplemented with 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, and 5 μM leupeptin. Cell lysates were sonicated (3 x 30 seconds) and centrifuged at 20,000 x *g* for 10 min. The supernatants were used for immunoprecipitation experiments. Within the same experiment, the total protein concentration in the various conditions was equalized.

Immunological procedures

For immunoprecipitation, cell lysates were incubated with antibodies against eIF2β, EGFP or the FLAG-peptide for 3 hours at 4°C. Subsequently, protein-A-TSK-Sepharose® (Affiland) was added, and the mixture was rotated during 30 min at 4°C. Following centrifugation (1 min at 1000 x *g*), the pellet was washed twice with TBS buffer supplemented with 0.1% Nonidet P-40, and once with 50 mM imidazole at pH 7.4, 0.5 mM dithiothreitol and 5 mM β-mercaptoethanol. The pellets were either boiled in SDS-sample buffer for immunoblotting or assayed for trypsin-revealed

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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 Polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked in 20 mM Tris-HCl at pH 7.5, 0.5 M NaCl, 3% milk powder for 3 hours at 4°C. Subsequently, the blots were incubated overnight in TBS buffer with 1 mg/ml BSA and digoxygenin-labelled PP1. After extensive washes with TBS, the retained PP1 was detected with anti-digoxygenin 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 hours at room temperature. After washing twice with TBS buffer and once with TBS buffer with 5% 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 the measurement of released acid-stable radioactivity [23]. To measure the total protein phosphatase activity of PP1 that co-sedimented with eIF2β, the samples were pretreated with trypsin (0.1 mg/ml) for 5 minutes at 30°C to release the free, fully active catalytic subunit. Subsequently, trypsin inhibitor (1mg/ml) was added and the phosphorylase phosphatase activity was measured. Other, recombinant substrates of PP1 were phosphorylated by incubation for 30-60 minutes at 30°C in 20 mM Tris-HCl at pH 7.5 plus 0.1 mM ATP (with or without 0.1 μCi/μl [γ-^{32}P]ATP), 2mM 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 or without eIF2β. 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 eIF2α phosphoserine51-specific antibodies.

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Protein synthesis measurements

Equal numbers of HEK293T cells were split into 6-well dishes and then transfected with plasmids encoding Flag-eIF2β or Flag-eIF2β-V131A/F133A. After 48 hours, the cells were labelled for 2 hours at 37°C with 50 µCi of [^{35}S]methionine (GE Healthcare)/ml in methionine-free medium (Invitrogen), supplemented with 10% fetal bovine serum. Cells were washed three times in PBS, and disrupted with lysis buffer. After sonication, the lysates were centrifuged (10,000 x *g*, 10 min). An aliquot of 5 µl of the supernatants was diluted in 2 ml scintillation liquid Lumasafe Plus (Lumac LSC) and analyzed by scintillation counting to determine the incorporation of [^{35}S]methionine per mg of proteins.

RESULTS

Identification of eIF2β 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 NIPP1 (nuclear inhibitor of protein phosphatase-1) and the translation initiation factor eIF2β (Fig. 1A). The similarities included residues 129-133 of eIF2β, KNVKF, which forms an instance of the consensus PP1-binding 'RVxF'-motif, i.e. [RK]-x_{0-1}-[VI]-{P}-[FW], as defined by Wakula et al. [16]. Moreover, as in NIPP1 and various other established PP1-interactors [16], the RVxF-instance of eIF2β is flanked N-terminally by a stretch of basic residues and C-terminally by an acidic stretch. This instance is conserved in eIF2β from mammals, birds, amphibia and some fish, but not in non-chordate eIF2β (Fig. 1B). Clearly, the acquisition of the RVxF-instance postdates the primary function of eIF2β 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 experimentally explore the predicted interaction between PP1 and eIF2β, we generated bacterially expressed His-eIF2β, affinity-purified on Ni^{2+}-Sepharose. His-eIF2β bound to digoxigenin-labelled PP1 in overlay assays (Fig. 2A). NIPP1 served as a positive control in this experiment while NIPP1-(1-329)RATA and BSA were used as negative controls. His-eIF2β also inhibited PP1 with an IC_{50} of around

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50 nM, using glycogen phosphorylase *a* as substrate (Fig. 2B). In contrast, eIF2β did not affect the phosphorylase phosphatase activity of the structurally related protein phosphatase-2A, indicating that it is a specific inhibitor of PP1. Importantly, the activity of eIF2β-inhibited PP1 could be fully recovered by the destruction of eIF2β with trypsin (not shown). Thus, unlike the PP1 interactor Inhibitor-2, eIF2β does not convert PP1 into an inactive, trypsin-sensitive conformation (25).

Subsequently, we have examined the association between eIF2β and PP1 in cell lysates. For this purpose, we performed immunoprecipitations with anti-FLAG or anti-EGFP antibodies, following the expression of FLAG-eIF2β and EGFP-PP1α in HEK293T cells. These experiments revealed a reciprocal co-immunoprecipitation of FLAG-eIF2β and EGFP-PP1α (Fig. 3A). In addition, we found that the immunoprecipitation of endogenous eIF2β resulted in the co-precipitation of endogenous PP1, as revealed by immunoblotting (Fig. 3B) and phosphorylase phosphatase assays (Fig. 3C). The latter activity was completely inhibited by the addition of NIPP1, showing that it was derived from PP1.

We have also explored whether the eIF2β-PP1 interaction is affected by treatments that are known to influence the rate of translation by affecting the phosphorylation of eIF2α, such as the addition of thapsigargin, a treatment with UV-C irradiation, or the synchronization of cells in the G_0-phase (serum starvation), M-phase (nocodazole treatment) or G1/G2-phases (addition of trichostatin A). However, none of these treatments measurably affected the association between eIF2β and PP1, but we could confirm that these treatments indeed affected the phosphorylation level of eIF2α (not illustrated).

Mapping of the eIF2β-PP1 interaction sites

To examine whether residues 129-133 of eIF2β, KNVKF, constitute a functional RVxF-instance, we mutated Val131 and Phe133 into an alanine. The resulting peptide His-eIF2β-V131A/F133A, further referred to as eIF2β-KNAKA, bound to Ni^{2+}-Sepharose, was used as an affinity matrix for the binding of purified PP1. Compared to the wild-type protein, eIF2β-KNAKA displayed a severely decreased ability to bind (Fig. 4A) and inhibit PP1 (Fig. 4B). The importance of the RVxF-instance for the inhibition of PP1 was further examined by using a synthetic RVxF-

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containing decapeptide, namely NIPP1-(197-206), as a competitor for the binding to the RVxF-binding site on PP1. The addition of 50 μM of NIPP1-(197-206), but not the corresponding RATA-peptide, abrogated the inhibition of PP1 by eIF2β (Fig. 4C), indicating that the RVxF-instance of eIF2β is indeed required for its ability to inhibit the phosphorylase phosphatase activity of PP1.

Although eIF2β-KNAKA was clearly deficient in the inhibition of PP1 (Fig. 4B), it could still retain about 25% of the amount of PP1 that interacted with wild-type eIF2β (Fig. 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 eIF2β wt, (2) PP1 still bound to the eIF2β-KNAKA in overlay assays, and (3) eIF2β-KNAKA, when expressed in HEK293T cells, also co-immunoprecipitated with EGFP-PP1 from cell lysates in a reciprocal manner (not illustrated). To gain more insight into the region of eIF2β that harbours a second PP1 binding site, we generated C-terminal deletions of eIF2β-KNAKA and examined the ability of these eIF2β fragments to bind PP1 (Fig. 5). Carboxyterminal truncation mutants of eIF2β-KNAKA clearly bound less PP1 and eIF2β-(1-145)-KNAKA barely retained any PP1 (Fig. 5) and did not bind to PP1 in overlay assays (Fig. 2A), pointing to the existence of a second, C-terminal binding site for PP1. Consistent with this view, we found that eIF2β-(187-341) bound about as much PP1 as did eIF2β-KNAKA (Fig. 5). Thus, binding of eIF2β to PP1 is mediated by the RVxF-motif and at least one additional binding site in the C-terminal half of eIF2β.

Functional analysis of the eIF2β-PP1 interaction

Mammalian eIF2β interacts with eIF5, eIF2B and eIF2γ, and the latter binds in turn to eIF2α. Since the initiation factors eIF2α, eIF2Bε and eIF5 are established phosphoproteins, we examined whether these phosphoproteins are substrates for PP1 in the absence or presence of eIF2β. Ser51 of eIF2α, phosphorylated in vitro with protein kinase MELK [26] was readily dephosphorylated by PP1. The addition of eIF2β wt, but not of eIF2β–KNAKA, inhibited the activity of PP1 towards eIF2α (Fig. 6A). Thus, eIF2β is an inhibitor of the dephosphorylation by PP1 of both glycogen phosphorylase *a* (Fig. 2B) and eIF2α (Fig. 6A). eIF2Bε is phosphorylated by protein kinases CK2 (Ser712, Ser713), DYRK1 (Ser539) and CK1 (Ser464).

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However, only CK1-phosphorylated eIF2Bε was dephosphorylated by purified PP1 (Fig. 6B and not shown) and, surprisingly, this dephosphorylation was only very mildly affected by the addition of eIF2β or eIF2β-KNAKA (Fig. 6B). eIF5 phosphorylated by protein kinase CK2 (Ser387, Ser388) was not dephosphorylated by PP1, whether or not eIF2β was added (not shown). Finally, we have examined whether eIF2β, phosphorylated by protein kinase CK2 (Ser2, Ser67) or protein kinase A (Ser218) [13], is itself a substrate for associated PP1. The phosphorylation of eIF2β by either kinase was reversed by PP1 but, surprisingly, the dephosphorylation of eIF2β-KNAKA was clearly less efficient (Fig. 6C). These results imply that the binding of PP1 to the RVxF-instance promotes the dephosphorylation of eIF2β by associated PP1.

Since the in-vivo function of the phosphorylation of eIF2β by protein kinases A and CK2 is not known, we have examined whether the recruitment of PP1 by eIF2β is a determinant of the rate of translation. For this purpose, wild-type eIF2β or eIF2β-KNAKA were transiently expressed in HEK293T cells and the rate of translation was derived from the incorporation of [^{35}S]methionine into proteins. The expression of eIF2β or the KNAKA-mutant inhibited the rate of translation by 42 ± 7 % and 53 ± 4 % (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 eIF2β-wt or eIF2β-KNAKA to reticulocyte lysates decreased the rate of translation of a reporter transcript, encoding the histone methyltransferase EZH2, to a similar extent (supplementary Fig. S1). It is worthy of note that EZH2 is not an interactor of PP1 and has no effect on translation. Collectively, these data suggest that the eIF2β-PP1 interaction is not rate-limiting for protein synthesis.

DISCUSSION

A bioinformatics approach has previously already led to the identification of new members of established families of PP1 interactors [18]. Also, Meiselbach et al. [19] recently showed that a more stringent definition of the RVxF-motif enables a reasonably specific identification of functional RVxF-instances. Here, we report the sequence-alignment assisted identification of eIF2β as an interactor of PP1. The

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sequence similarity between NIPP1 and eIF2β 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 eIF2β not only comprised the RVxF-motif but also the flanking sequences of this motif (Fig. 1A). The RVxF-instance of eIF2β is absent from non-chordates and from some fish species (Fig. 1B), indicating that PP1 binding, via its RVxF-instance, is a property that eIF2β acquired during metazoan evolution. We have demonstrated by various independent biochemical approaches that the predicted RVxF-instance of eIF2β is functional (Fig. 4). Moreover, the region of eIF2β that harbours the RVxF-motif is predicted by the secondary structure prediction programme 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 eIF2β corresponds to the more stringent definition of RVxF-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 promote thereby the dephosphorylation of Ser51 of eIF2α [29-31]. Here, we have shown that another subunit of eIF2, namely the β-subunit, directly recruits PP1 (Figs. 2 and 3) and is itself a likely substrate for dephosphorylation by associated PP1 (Fig. 6C and D). It remains to be examined whether eIF2β 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 eIF2β is not rate-limiting for translation under basal circumstances. However, it can not be excluded that eIF2β-associated PP1 only exerts a translational control for a selected group of transcripts or under specific conditions. Consistent with the role of PP1 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 but inhibited the dephosphorylation of glycogen phosphorylase and eIF2α, and did not affect the dephosphorylation of eIF2Bε (Fig. 6). Thus, like many PP1-interactors [14], eIF2β

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appears to have a substrate-specifying function. Interestingly, eIF2β not only binds to PP1 (this work) 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, and that somehow controls the initiation of translation.

In conclusion, we have shown here that PP1 is a physiological interactor of eIF2β. We have identified a functional RVxF-motif and a second PP1-binding site in the C-terminal half of eIF2β and have provided evidence that eIF2β functions as a substrate-specifier for associated PP1. Strikingly, eIF2β itself emerges as the most likely substrate for associated PP1 and its dephosphorylation depends on a functional RVxF-motif.

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Abbreviations

BSA, bovine serum albumin; eIF, eukaryotic initiation factor; PBS, phosphate buffered saline; PP1, protein phosphatase-1; TBS, Tris buffered saline

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LEGENDS TO THE FIGURES

Figure 1 Similarities between eIF2β and the central domain of NIPP1

(**A**) Alignment of the corresponding parts of human NIPP1 and eIF2β sequences by the MaxHom programme. Numbers outside the alignments indicate NIPP1 (top) and eIF2β (bottom) residues; numbers within the alignments indicate the insertions (not

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to scale) of non-conserved fragments in eIF2β. Identical residues are boxed in black. Residues conserved whithin the same group [positively charged (H,K,R); negatively charged (D,E); small residues (G,A,S,T); bulky hydrophobic residues (I,L,V,M,F,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.

Figure 2 eIF2β is a specific inhibitor of PP1

(**A**) An overlay with digoxigenin-labelled catalytic subunit of PP1 shows binding to eIF2β wt but not to the mutant eIF2β-(1-145)-KNAKA (see also Fig. 5). NIPP1 was included as a positive control and NIPP1-(1-329)-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 eIF2β on the phosphorylase phosphatase activity of PP1 and PP2A. The results represent means ± S.E.M. of three assays.

Figure 3 Co-immunoprecipitation experiments confirm the eIF2β-PP1 interaction

(**A**) Lysates from HEK293T cells co-expressing EGFP-PP1α and FLAG-eIF2β were used for immunoprecipitations with protein-A-TSK and anti-EGFP antibodies, anti-FLAG antibodies or without antibodies (control). The presence of EGFP-PP1 and FLAG-eIF2β was detected by western analysis with anti-EGFP and anti-FLAG antibodies. (**B and C**) Endogenous eIF2β from HEK293T cell lysates was immunoprecipitated and the precipitates were screened for the presence of eIF2β and PP1 by immunoblotting (**B**) and for trypsin-revealed phosphorylase phosphatase activities in the absence and presence of 1 μM NIPP1 (**C**).

Figure 4 eIF2β contains a functional RVxF-motif

(**A**) Equimolar amounts of recombinant His-eIF2β-wt and His-eIF2β-KNAKA, coupled to Ni^{2+}-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

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with trypsin. (**B**) Effect of eIF2β-wt 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 NIPP1-(197-206) (RVTF) or 50 μM of a variant peptide in which the RVTF-sequence was replaced by the RATA-sequence, on the inhibition of PP1 by eIF2β. All results are means ± S.E.M. for three phosphatase assays.

Figure 5 Mapping of a second PP1 interaction site in eIF2β

The figure shows the His-tagged eIF2β mutants/fragments that were used for PP1 binding assays. Equimolar concentrations of the fusions, linked to Ni^{2+}-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 means ± S.E.M. for 4-8 phosphatase assays.

Figure 6 In vitro dephosphorylation of eIF2α, eIF2Bε 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 eIF2β-wt 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-phospho-Ser51 antibodies. (**B**) Dephosphorylation of eIF2Bε phosphorylated by protein kinase CK1, as visualized by autoradiography. (**C** and **D**). An autoradiogram of the dephosphorylation of eIF2β and eIF2β-KNAKA, phosphorylated by protein kinase CK2 (**C**) or protein kinase A (**D**).

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Fig. 1

A.

```
Human NIPP1   90 STHGTFLGHIRLEPHKPQQIPIDSTVSFGASTRAYTLREKPQTLPSAVKG 139
Human eIF2β    2 SGDEMIFDPTMSKKKKKKKPFMLDEEGDTQTEETQPSETKEVEPEPTED  51

Human NIPP1  140 DEKMGGEDDELKGLLGLPEEETELDNLTEFNTAHNKR---------ISTL 180
Human eIF2β   52 KDLEADEEDTRKK-----DASDDLDDLNFFNQKKKKK - 23 aa-VQEP 110

Human NIPP1  181 TIEEGNLDIQRPKRKRKNSRVTESEDDEIINPED-----------VDPSV 219
Human eIF2β  111 TEPEDDLDIMLGNKKKKKKNVKEPDEDEILEKDE - 35 aa - LNRVF 184

Human NIPP1  220 GRFRNMVQTAVVPVKKKRVEGPGSLGLEESGSRRMQNFAFSGGLYGGLPP 269
Human eIF2β  185 NIMREKNPDMVAGEKRKFVMKPPQVVRVGTKKTSFVNFTDICKLLHRQPK 234

Human NIPP1  270 THSEAGSQPHGIHGT 284
Human eIF2β  235 HLLAFLLAELGTSGS 249
```

B.

Species	Residues	Sequence
Homo sapiens	124-143	KKKKK**KNVKF**PDEDEILEKD
Gallus gallus	120-139	KKKKK**KFVKF**PDEDEILEKD
Xenopus tropicalis	125-144	KKKKK**KNVKF**PEEDDTLDRD
Takifugu rubripes	119-137	KKKKSKKVEI-DEGDALDKD
Tetraodon nigroviridis (a puffer fish)	124-142	KKKKT**KKVDF**-DEGDTLDKD
Danio rerio	120-137	KKKKPK--KVKEDTDSQSKE
Drosophila melanogaster	111-130	KKKKSKKKELDELFADQADD
Ciona intestinalis (a sea squirt)	119-138	KKKKKHKILPDDSE**KKVSF**A
Saccharomyces cerevisiae	84-103	KKKKTKDSSVDAFEKELAKA

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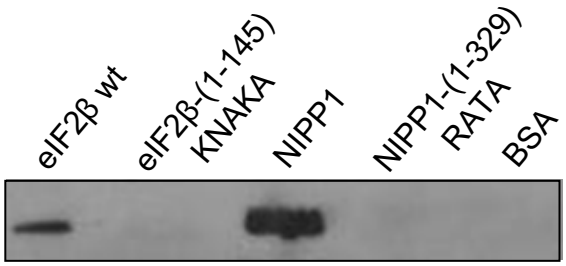
Fig. 2

A.



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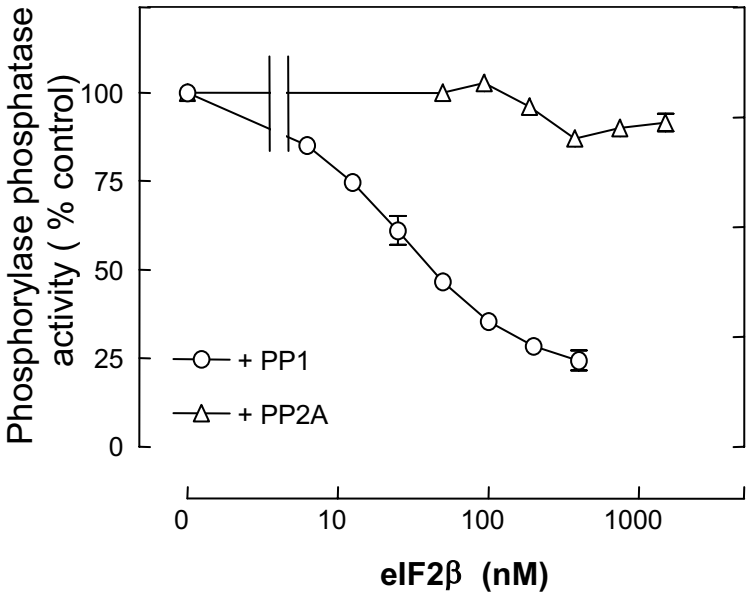

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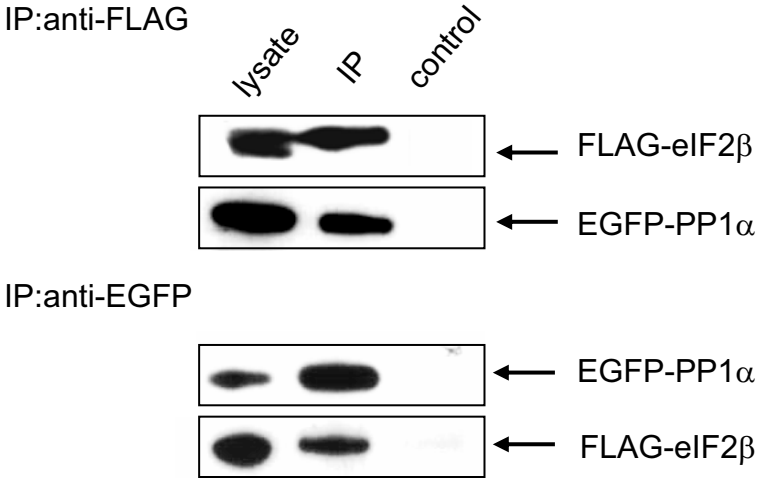
Fig. 3

A.



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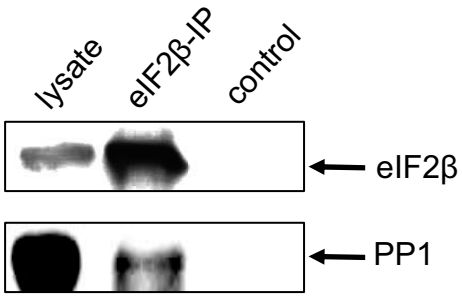

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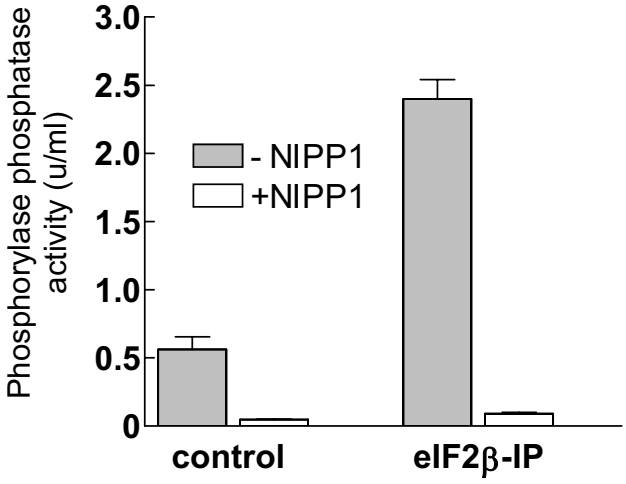

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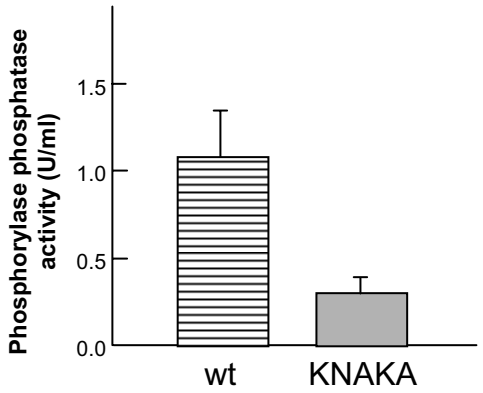
Fig. 4

A.



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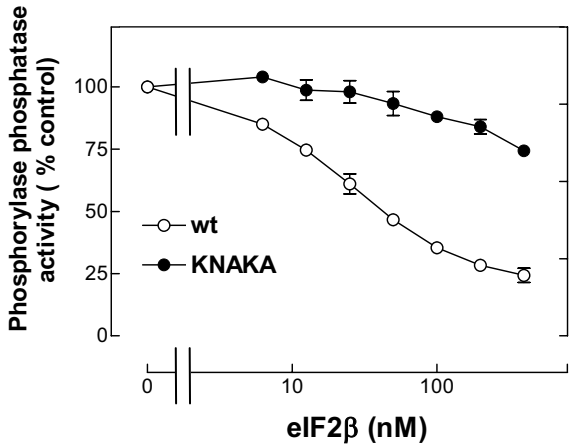

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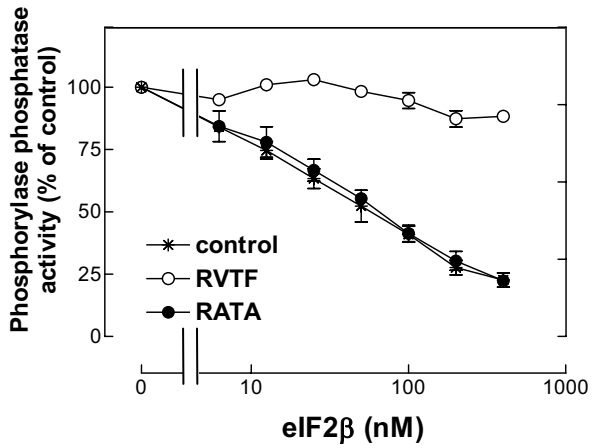

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Fig. 5

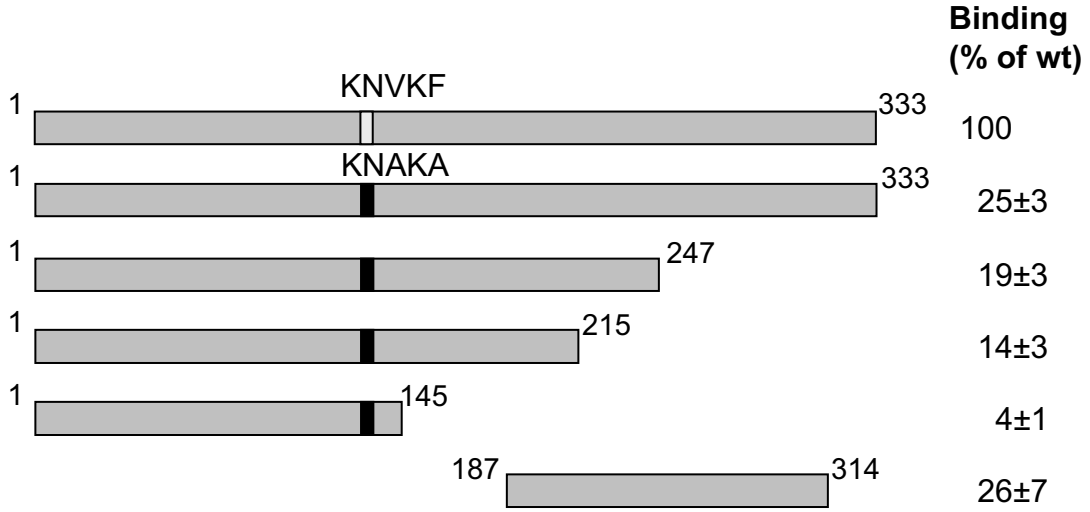

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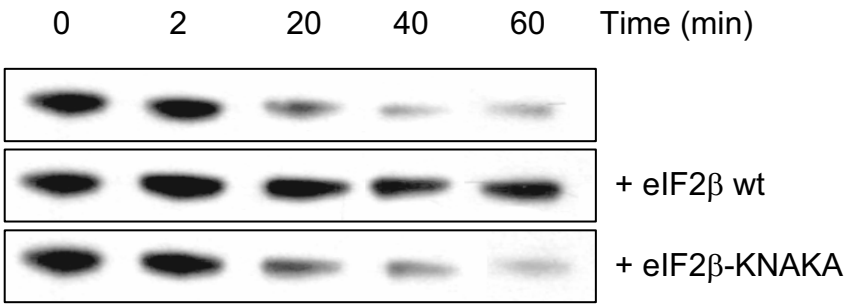
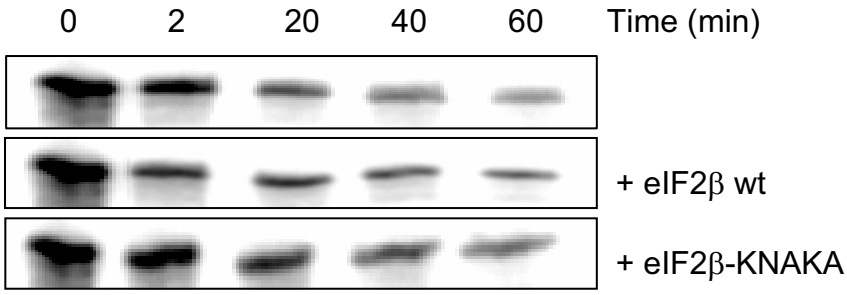
Fig. 6

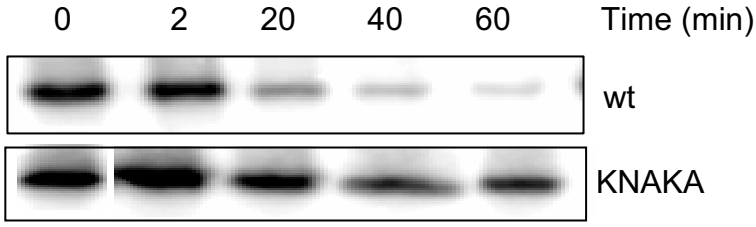
A. Dephosphorylation of eIF2α (Ser51)



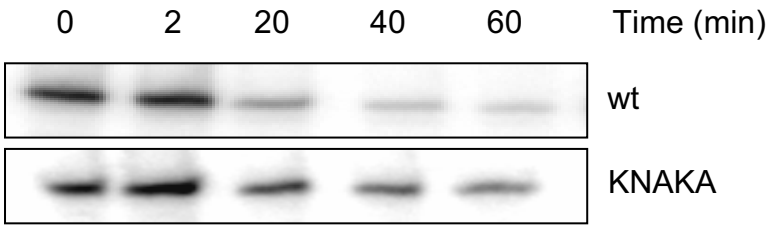
B. Dephosphorylation of eIF2Bε (Ser464)



C. Dephosphorylation of eIF2β (Ser2/Ser67)



D. Dephosphorylation of eIF2β (Ser218)



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