The EMBO Journal vol.15 no.24 pp.7156–7167, 1996

The catalytic subunit of protein phosphatase 2A associates with the translation termination factor eRF1

Nataša Andjelković, Stanislaw Zolnierowicz1, Christine Van Hoof2, Jozef Goris2 and Brian A. Hemmings3

Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland and 2Afdeling Biochemie, Faculteit der Geneeskunde, Katholieke Universiteit te Leuven, Hertiestraat 49, 3000 Leuven, Belgium

1Present address: Department of Biochemistry, Faculty of Biotechnology, Medical University of Gdańsk, Debinki 1, 80-211 Gdańsk, Poland

2Corresponding author

By a number of criteria, we have demonstrated that the translation termination factor eRF1 (eukaryotic release factor 1) associates with protein phosphatase 2A (PP2A). Trimeric PP2A1 was purified from rabbit skeletal muscle using an affinity purification step. In addition to the 36 kDa catalytic subunit (PP2Ac) and established regulatory subunits of 65 kDa (PR65) and 55 kDa (PR55), purified preparations contained two proteins with apparent Ms of 54 and 55 kDa. Protein microsequencing revealed that the 55 kDa component is a novel protein, whereas the 54 kDa protein was identified as eRF1, a protein that functions in translational termination as a polypeptide chain release factor. Using the yeast two-hybrid system, human eRF1 was shown to interact specifically with PP2Ac, but not with the PR65 or PR55 subunits. By deletion analysis, the binding domains were found to be located within the 50 N-terminal amino acids of PP2Ac, and between amino acid residues 338 and 381 in the C-terminal part of human eRF1. This association also occurs in vivo, since PP2A can be co-immunoprecipitated with eRF1 from mammalian cells. We observed a significant increase in the amount of PP2A associated with the polysomes when eRF1 was transiently expressed in COS1 cells, and eRF1 immunoprecipitated from those fractions contained associated PP2A. Since we did not observe any dramatic effects of PP2A on the polypeptide chain release activity of eRF1 (or vice versa), we postulate that eRF1 also functions to recruit PP2A into polysomes, thus bringing the phosphatase into contact with putative targets among the components of the translational apparatus.

Keywords: eRF1/protein phosphatase 2A/signal transduction/translational termination

Introduction

Protein phosphatase 2A (PP2A) is implicated in the regulation of many cellular processes including metabolism, signal transduction, growth, development, cell cycle progression and transformation (reviewed in Mumby and Walter, 1993; Mayer-Jaekel and Hemmings, 1994). PP2A encompasses a family of trimeric holoenzymes which consist of a 36 kDa catalytic subunit (PP2Ac) bound to the constant regulatory subunit of 65 kDa (PR65/A) which then associate further with the third, variable regulatory subunit. Several trimeric PP2A holoenzymes have been purified which contain different variable subunits of either 54, 55, 72 or 74 kDa (reviewed in Kamibayashi and Mumbly, 1995; Wera and Hemmings, 1995).

As documented by in vitro reconstitution assays and by analyzing yeast and Drosophila mutants deficient in regulatory proteins, both the constant and variable subunits are important for controlling PP2A activity and substrate specificity (reviewed in Mayer-Jaekel and Hemmings, 1994; Wera and Hemmings, 1995). For instance, PP2A activity from brain extracts of Drosophila aar1 mutants, in which the gene encoding PR55 is disrupted by P-element insertion, is several fold lower towards histone H1 and caldesmon phosphorylated by p34cdc2 as compared with wild-type flies (Mayer-Jaekel et al., 1994). In contrast, phosphorylase phosphatase activity of PP2A is similar in aar1 and control flies. The variable regulatory subunits also represent targets for potential second messengers and viral proteins. Dobrowsky et al. (1993) demonstrated that ceramide activates only trimeric PP2A containing the PR55 subunit whereas the PP2Ac–PR65 dimer is unaffected. Recent data, however, show that neither the constant nor variable regulatory subunits are required for ceramide stimulation of PP2A activity, since both PP2Ac and PP2Ac–PR65 dimer can be stimulated by ceramide in a manner similar to that of the trimeric holoenzyme, suggesting that PP2Ac itself is a target of ceramide action (Law and Rossie, 1995). Furthermore, PP2A associates with transforming antigens of certain DNA tumor viruses, such as polyomavirus small t and middle T, and SV40 small t (Pallas et al., 1990). It is believed that these oncproteins act to alter PP2A activity by displacing the normal cellular variable regulatory subunits from the trimeric holoenzyme. Some viral proteins interact only with specific forms of PP2A holoenzymes, e.g. SV40 small t antigen is able to replace only the B subunit (PR55), but not the B' subunit (PR61) from trimeric PP2A (Sontag et al., 1994). It was also shown that adenovirus E4orf4 binds to the trimeric PP2A holoenzyme that contains PR55 (Kleinberger and Shenk, 1993). Taken together, these examples illustrate that the activity of PP2Ac is tightly controlled in vivo by regulatory proteins.

We developed a strategy for simultaneous purification of different PP2A holoenzymes from rabbit skeletal muscle in order to analyze their subunit structure further. This approach resulted in the purification of two heterotrimeric forms of PP2A0 containing different isoforms of a novel type of variable regulatory subunit (termed PR61) that
probably function to target PP2A to nuclear substrates (Tehran et al., 1996; Zolnierowicz et al., 1996). We also found two novel proteins of 54 and 55 kDa that apparently co-purify with the trimeric PP2A1 holoenzyme following affinity purification. Here we report the identification of the 54 kDa protein as a member of the eRF1 family of proteins involved in termination of protein synthesis as well as further examine the functional consequences of its interaction with constituent components of the PP2A holoenzyme.

Results

Co-purification of PP2A1 holoenzyme with two proteins of 54 and 55 kDa

A modified protocol for PP2A purification from rabbit skeletal muscle (see Figure 1 and Materials and methods) was used to identify novel regulatory and/or associated proteins. The partially purified material obtained from DEAE–Sepharose pools 1, 2 and 3 was analyzed using several antisera developed against the constituent subunits of PP2A reported in earlier publications (Hendrix et al., 1993a, b; Turowski et al., 1995). MonoQ FPLC of fractions corresponding to pool 1 (eluted from DEAE–Sepharose between 0.27 and 0.32 M NaCl) revealed the existence of a trimeric PP2A holoenzyme containing 36 kDa catalytic (PP2Ac) and 65 kDa regulatory (PR65) subunits and a novel type of variable regulatory subunits with apparent Mr ranging from 56 to 61 kDa. This trimeric holoenzyme apparently corresponds to PP2A0 (Zolnierowicz et al., 1996) according to the classification established previously by Tung et al. (1985).

SDS–PAGE analysis of pool 2 (fractions eluted from DEAE–Sepharose between 0.34 and 0.38 M NaCl) after the thiophosphorylase α-Sepharose purification step revealed that this preparation contained the 36 kDa catalytic and 65 kDa regulatory subunits and several proteins in the range of 54–55 kDa (Figure 2B). Further fractionation by MonoQ FPLC resulted in the separation of PP2A1 (trimer containing the 55 kDa regulatory subunit, PR55) and PP2A2 (the dimeric form of the enzyme) from two proteins of 54 and 55 kDa (Figure 2A and C). These two proteins appear to be unrelated to PR55 since they did not cross-react with anti-PR55 antibodies (data not shown). In addition, we identified free PR55α in this preparation by SDS–PAGE and immunoblot analysis (fractions 37 and 38). This suggests that the PP2A2 identified probably results from the dissociation of PR55, and possibly the 54 and 55 kDa proteins, from the complex (see below).

54 kDa protein that co-purifies with PP2A1 is a member of the eRF1 family of proteins with polypeptide chain release factor activity

The 54 and 55 kDa proteins purified by MonoQ FPLC chromatography were used to obtain protein sequence data as described in Materials and methods. Sequences of three tryptic peptides comprising 30 amino acids derived from the 54 kDa protein were obtained: peptide 3, YTFEDISQDTGK, peptide 9/10, ILYLTEQEK and peptide 25/26, S/GFGGGIGGIL. Comparison of these sequences using the FASTA program (Pearson and Lipman, 1988) revealed 76% homology to the predicted protein sequence encoded by the Saccharomyces cerevisiae SUP45 gene (Breining and Piersberg, 1986). Human cDNAs corresponding to this protein were isolated using a reverse transcription–PCR approach (see Materials and methods). Among several clones isolated from human fetal brain library and analyzed by sequencing, only one, termed BBZ.eRF1-4b, contained a full-length open reading frame (1311 bp) as well as 231 bp of the 5'-non-coding region and ~2.22 kb of the 3'-non-coding region. This cDNA is identical to the TB3-1 cDNA, originally identified by Grenet et al. (1992) and resequenced by Frolova et al. (1994). cDNAs encoding homologous proteins have been identified recently from Arabidopsis thaliana (Quigley et al., 1994) and Xenopus laevis (Tassan et al., 1993). The report by Frolova et al. (1994) demonstrated that human and Xenopus proteins possess polypeptide chain release factor activity and termed this factor eRF1. Sequence comparison revealed that eRF1 protein is highly conserved between species, with yeast and human protein being 67.5% identical (Figure 3).

Partial amino acid sequence analysis of seven peptides comprising 71 amino acids derived from the 55 kDa protein did not display any homologies to the sequences present in currently available databases (GenBank™, release number 95). Currently we are attempting to isolate cDNAs corresponding to this protein to establish its relationship to PP2A and eRF1.

mRNA encoding human eRF1 is ubiquitously expressed

The levels of transcripts encoding human eRF1 were analyzed in poly(A)+ RNA isolated from different human tissues. With the probe corresponding to the complete
human eRF1 cDNA (BBZ.eRF1-4b) multiple transcripts were detected of ~2, 2.5 and 4 kb (Figure 4). mRNA encoding human eRF1 appears to be ubiquitously expressed, with the highest transcript levels found in lung, skeletal muscle and placental tissues. Of the three classes of transcripts detected, the 4 kb species showed the highest level of expression in all tissues. Quantitation by ImageQuant software showed that 2.5 and 2 kb classes of transcripts were expressed ~2- to 4-fold less than the high molecular weight transcript. This mRNA distribution is different from that reported for CII, the X.laevis eRF1 homolog, where a much more restricted pattern of expression was found, with both mRNA and the protein being completely absent in liver (Tassan et al., 1993).

**Human eRF1 interacts with the catalytic subunit of PP2A in the yeast two-hybrid system**

In order to assess which subunit of PP2A associates with human eRF1, we used the yeast two-hybrid system (Fields and Song, 1989). Another protein, termed eRF3, was previously shown to bind to eRF1 and stimulate its activity in polypeptide chain termination in *S.cerevisiae* and *X.laevis* (Stansfield et al., 1995; Zhourovleva et al., 1995). Therefore, we extended the two-hybrid analysis to see whether eRF3 could be an interaction partner as well. Human PP2Acα, PR65α, PR55α, eRF1 and eRF3 cDNAs were fused with the yeast transcriptional activator GAL4 DNA binding or transactivation domains (as described in Materials and methods). Expression of fusion proteins was checked in double transformants, and the interaction of PP2A subunits with human eRF1 and eRF3 evaluated by monitoring the expression of two different reporter genes, lacZ and HIS3. Transcriptional activation of reporter genes driven by wild-type GAL4 protein, or brought about by the interaction between SV40 large T and p53, were used as positive controls. To exclude intrinsic transcriptional activation capacity or non-specific binding of either molecule to unrelated proteins, co-transformations with the empty vector or vector encoding human lamin C fused to the opposite domain of GAL4 were used as negative controls. These experiments showed that human eRF1 binds to eRF3 (Figure 6), as one would expect based on the previous reports from *S.cerevisiae* and *X.laevis* studies (Stansfield et al., 1995; Zhourovleva et al., 1995). They also showed that the human eRF1 specifically interacts with PP2Ac, but not with PR65 or PR55, in both reporter systems, since only PP2Ac-eRF1 double transformants were positive as confirmed by β-galactosidase assays (Figure 6) and did not require histidine for growth (data not shown). From this analysis, we conclude that the PP2A subunit that binds eRF1 is the catalytic subunit itself. On the other hand, eRF3 failed to bind to either of the three PP2A subunits tested in this system (Figure 6).

**Identification of domains required for the interaction of PP2Ac and eRF1**

In addition to the analysis of interactions of full-length proteins in the two-hybrid system, we attempted to map the regions on both eRF1 and PP2Ac required for this association. For this purpose, a series of N- and C-terminally truncated versions of both proteins was constructed (Figure 5) and tested in the same experimental setup as described above. All of the C-terminal deletion mutants of PP2Ac (PP2Ac1-259, PP2Ac1-299 and PP2Ac1-159), but none of the N-terminal deletion mutants (PP2Ac50-309, PP2Ac100-309 and PP2Ac50-209) were able to interact with eRF1 (Figures 5A and 6), suggesting that the region essential for binding was within the N-terminal 50 amino acid residues of the protein. Differences in binding of the C-terminal truncations of PP2Ac to the full-length eRF1 suggest that the C-terminal portion of the protein also contains sequences that influence this
Fig. 3. Alignment of amino acid sequences of eRF1 from different species. Amino acid sequence of human eRF1 (Hum eRF1) is aligned with corresponding sequences of X. laevis (Xen eRF1), S. cerevisiae (Sc eRF1), A. thaliana (Ara eRF1). Amino acid sequences determined for the rabbit homolog are underlined. Dots represent identical residues, while dashes represent spaces to optimize the alignment. The region in the C-terminal half of human eRF1 which was identified as essential for binding to PP2Ac in the two-hybrid system analysis is presented in bold type.

Fig. 4. Analysis of eRF1 mRNA levels in human tissues. Human tissue blot (Clontech) loaded with 2 μg of poly(A)^+ RNA isolated from the indicated human tissues was hybridized with a human eRF1 cDNA probe corresponding to clone BBZ.eRF1-1 labeled to a specific activity of ~1×10^6 c.p.m./μg DNA. Three hybridizing fragments of 4, 2.5, and 2 kb were detected. Hybridization and washing of the blot were performed as described in the manufacturer’s instructions. The blot was exposed to Kodak XAR-5 film for 9 h at –80°C with intensifying screens.

Fig. 5. Schematic representation of deletion mutants of human PP2Ac (A) and human eRF1 (B) tested in the two-hybrid system and their ability to retain the interaction. Regions of the molecules required for binding in the two-hybrid system, located between amino acid residues 1 and 50 in PP2Ac and 338 and 381 in eRF1, respectively, are highlighted.

**Immunoprecipitates of eRF1, but not eRF3, contain PP2A activity**

To test whether complex formation between PP2A and eRF1 occurs in mammalian cells, we examined whether these proteins co-immunoprecipitate. Association between PP2A and PR65 under the same experimental conditions was used as a positive control. Extracts from COS-1 cells transiently transfected with human eRF1, eRF3 or PR65 tagged with the hemagglutinin (HA) epitope at the
N-termini were subjected to immunoprecipitation with the anti-HA tag monoclonal antibody 12CA5.

We measured PP2A activity in transfected COS-1 cell extracts and the immunoprecipitates using a $^{32}$P-labeled peptide (Kemptide Val$^6$, Ala$^7$) as a substrate (Figure 7). Assays were performed in the presence and absence of 10 nM okadaic acid, which is used typically to distinguish between PP1 and PP2A activities. The specific activity of PP2A in the extracts (expressed as mU/mg protein) was in the same range for all transfected cells (Figure 7A). As shown in Figure 7B, 12CA5 immunoprecipitates from cells transfected with HA-tagged eRF1 or PR65 contained significant okadaic acid-sensitive, PP2A-like phosphatase activity as compared with immunoprecipitates from mock-transfected cells. PP2A activity associated with eRF1 accounts for ~1% (0.4–1.6% range) of the total cytoplasmic PP2A activity. Immunoprecipitation of eRF3 did not bring down PP2A activity significantly higher than the background. This result suggests that the interactions of PP2A and eRF1 are mutually exclusive.

**PP2Ac and eRF1 are associated in vivo in mammalian cells**

To determine which PP2A subunits were present in the complex with eRF1, HA–eRF1 immunoprecipitates were subjected to Western blot analysis with rabbit polyclonal anti-peptide antisera specific for different subunits of PP2A (see Materials and methods). These experiments showed that the catalytic subunit of PP2A (PP2Ac) can be detected in immunoprecipitates from HA–eRF1- or HA–PR65-transfected, but not from mock-transfected cells (Figure 8, lower panel). We looked for the presence of other established regulatory subunits of PP2A in eRF1 immunoprecipitates, and were able to detect PR65 (Figure 8, upper panel), but not PR55 or PR72 (data not shown). From this analysis, we conclude that eRF1 apparently complexes with the core dimer of PP2A (consisting of PP2Ac and PR65) to form a novel trimeric complex, which is much less abundant than other previously reported complexes of PP2A. These experiments provide the first evidence for an in vivo association between the catalytic subunit of PP2A and eRF1 protein in mammalian cells, which is to our knowledge the first report of PP2A interacting with a protein involved in the regulation of protein synthesis.

**Expression of HA-tagged eRF1 in COS-1 cells increases the amount of PP2A associated with the polysomes**

We have looked at the distribution of PP2A in fractionated exponentially growing COS-1 cells, as well as in COS-1 cells transiently transfected with HA-tagged eRF1. Ribosomes (80S) from these cells were obtained by high speed centrifugation of cell-free extracts through a 38% sucrose cushion (described in Materials and methods). We performed controls using antibodies specific to ribosomal (S6) and cytosolic (regulatory RII subunit of protein kinase A) proteins to evaluate successful separation (data not shown). PP2A distribution was analyzed in total cell-free extracts, and in the sucrose and ribosomal fractions. The analysis was carried out by Western blotting and activity measurements using $^{32}$P-labeled peptide (Kemptide Val$^6$, Ala$^7$) as a substrate (Figure 9A and B). These experiments showed that in untransfected COS-1 cells, PP2A present in the polysomes was a very small portion of total cytoplasmic PP2A activity (1–2%), which is in agreement with estimates from studies performed using rabbit reticulocyte lysates (Foulkes et al., 1983). However, over-expression of eRF1 significantly increases the amount of PP2A present in the polysomes, suggesting that PP2A can be recruited to the polysomes by increasing the amount of free eRF1 available to bind to PP2Ac (Figure 9A). The data from activity measurements were confirmed subsequently by Western blot analysis (Figure 9B) of
Fig. 7. (A) Specific PP2A activity in extracts of COS-1 cells transiently transfected with HA-tagged eRF1, eRF3 or PR65. (B) PP2A activity in corresponding anti-HA tag immunoprecipitates from 100 µg extracts. Activity measurements were performed in the absence (gray bars) and presence (black bars) of 10 nM okadaic acid, a potent PP2A inhibitor, using 32P-labeled Kempidine Val6, Ala7 as a substrate as described in Materials and methods. Numbers represent the mean values (± SEM) from three independent experiments carried out in duplicate assays.

PP2A and eRF1, which showed a significant increase of PP2Ac and PR65 in COS-1 cells following overexpression of eRF1. The slower migrating band cross-reacting with eRF1 antibody represents the HA-tagged form. In contrast to previous reports on the S.cerevisiae homolog Sup45 (Stansfield et al., 1992), mammalian eRF1 was shown not to be present exclusively in the polysomal fraction, but rather equally distributed between cytoplasmic and polysomal fractions, which may point to its relatively loose attachment to the 40S subunit in mammalian cells.

Subsequent immunoprecipitation experiments from fractionated COS-1 cells described above, followed by activity measurements and immunoblotting, showed that PP2A dimer and associated okadaic acid-sensitive phosphatase activity are present in immunoprecipitated fractions of eRF1, confirming that indeed increased PP2A detected in the polysome fraction was associated with eRF1 (Figure 9C and D).

**Effects of different forms of PP2A on polypeptide chain release factor activity of eRF1**

Since we initially did not observe any effect of eRF1 on basal or protamine-stimulated activity of PP2A, we attempted to look for possible effects of PP2A on polypeptide chain release factor activity of eRF1. Therefore, we measured the stop codon-dependent release of formyl-[35S]-methionine from the formyl-[35S]methionyl-tRNA Met-AUG-80S substrate complex mediated by eRF1 in an in vitro termination assay (Tate and Caskey, 1990). In these experiments, we used recombinant human histidine-tagged eRF1 (His-eRF1) and GST-tagged eRF3 (GST-eRF3) purified to apparent homogeneity, as well as purified preparations of PP2Ac, PP2A2, and PP2A4. His-eRF1 was active as a release factor on its own, but treatment with equimolar concentrations of different forms of PP2A did not have any dramatic effects on the activity of the recombinant protein (Figure 10). As previously reported for the S.cerevisiae and X.laevis homologs (Stansfeld et al., 1995; Zhouravleva et al., 1995), GST-eRF3 was able to stimulate eRF1 release factor activity (data not shown). We did not observe any significant effects of different PP2A preparations on eRF3-stimulated activity of His-eRF1 (data not shown). We also tested the release activity of a MonoQ-purified preparation of PP2A that contains eRF1 (MQ I), as confirmed by Western blotting with Ab eRF1 124−437. eRF1 present in this preparation was also active in termination assays, although to a somewhat lower extent than the recombinant protein, but the activity in the presence of 10 nM okadaic acid (Sigma) was not significantly different from that in untreated samples (Figure 10).

To understand further the interaction of PP2A with eRF1, we determined release activity in immunoprecipitates of HA-eRF1 from transfected COS-1 cells. The data so far available indicate that the activity of eRF1 is extremely low (<5%) compared with the similar amount of recombinant protein. As shown above (Figure 8), these immunoprecipitates contain PP2Ac and PR65. These results suggest at least two possibilities: (i) the eRF1 immunoprecipitates contain additional protein(s) that inhibit its activity (in this case partially purified eRF1 would be devoid of this putative inhibitory protein, hence explaining its activity), or (ii) eRF1 immobilized on protein A-Sepharose beads via 12CA5 monoclonal antibody was unable, due to size restrictions, to get access to the substrate on the ribosome.

Taken together, these results indicate a complex relation-
ship between eRF1 activity and association with PP2A and/or eRF3. Nonetheless, the current results provide a basis for future studies to delineate the role of protein phosphorylation in the regulation of chain termination.

Discussion

Here we report the interaction of the catalytic subunit of PP2A (PP2Ac) with the protein that belongs to a recently established protein family termed eRF1, which functions in the termination of protein synthesis as a polypeptide chain release factor (Frolova et al., 1994). Identification of this protein was facilitated by the analysis of the subunit composition of purified PP2A preparations and co-purified proteins typically found in such preparations. The novelty of this result is that it is the first cellular protein other than the established regulatory subunits of PP2A so far found to interact with the catalytic subunit of PP2A. It also provides a mechanism whereby PP2A could be involved in the regulation of protein synthesis.

Members of the eRF1 protein family have been identified from several higher eukaryotic organisms based on significant sequence similarity to a ribosome-associated protein originally identified from the yeast S. cerevisiae (Breining and Piepersberg, 1986). This protein, termed Sup45, has been implicated in maintaining translational fidelity through genetic analysis of mutants showing

---

**Fig. 9.** Expression of eRF1 increases the amount of PP2A associated with 80S ribosomes. (A) PP2A activity in fractionated COS-1 cells, mock transfected or transfected with HA-tagged eRF1. (B) Western blot of corresponding preparations; the upper panel was probed for PR65 and eRF1, and the lower panel for PP2Ac. (C) PP2A activity in 12CA5 immunoprecipitates of the same fractions brought down from 100 µg of proteins. (D) Western blot of corresponding preparations; the upper panel was probed for PR65, the middle panel for eRF1 and the lower panel for PP2Ac. Numbers represent the mean values (± SEM) from three independent experiments carried out in duplicate assays. The activity measured in the presence of 10 nM okadaic acid was subtracted from all values.

**Fig. 10.** Activity of recombinant eRF1 in an in vitro termination assay. PP2Ac, PP2A2 and PP2A3 are different forms of PP2A; MonoQl is a preparation of PP2A that contains eRF1 (see Materials and methods); OA is okadaic acid. Release factor activity was measured as stop codon-dependent release of formyl-[35S]methionine from the formyl-[35S]methionyl-tRNA<sub>Met</sub>-AUG-80S substate complex. The values of formyl-[35S]methionine released in the absence of stop codon (0.02–0.05 pmol) have been subtracted from all values.
defective nonsense suppression in vivo (reviewed in Stansfield and Tuite, 1994). Yeast Sup45 shows ~70% amino acid identity with its eukaryotic counterparts, and a mutation in the SUP45 gene can be complemented by the X.laevis Sup45 homolog C11 (Tassan et al., 1993), suggesting that its function in translation termination is a conserved evolutionary feature.

It was suggested that eRF1 is not the only release factor required for translation termination in vivo (Tuite and Stansfield, 1994), since it had been shown previously that translational termination in eukaryotes is a GTP-dependent process (Beaudet and Caskey, 1971), yet members of the eRF1 family do not contain any consensus GTP binding sites (Frolova et al., 1994). A likely candidate for this additional component of the eukaryotic translation termination machinery was proposed to be the Sup35 protein (Tuite and Stansfield, 1994). S.cerevisiae mutants in the SUP35 gene show an array of phenotypes similar to SUP45 mutants, suggesting the involvement of Sup35 in the process of translational termination (reviewed in Stansfield and Tuite, 1994). In agreement with this suggestion, Sup35 also contains a consensus GTP binding site (Kushnirov et al., 1988). Indeed, the most recent findings by Zhouravleva et al. (1995) and Stansfield et al. (1995) show that Sup45 (eRF1) and Sup35 (also termed eRF3) actually interact to form a release factor complex, both in yeast and higher eukaryotes. Sup35 (eRF3) is inactive as a release factor on its own but greatly stimulates the activity of eRF1, and one of its functions in the release factor complex could be GTP binding and hydrolysis. Reports that Sup35 is a prion-like protein (Paushkin et al., 1996) which can exist in functionally different conformations suggest a possibility for the regulation of translational termination in eukaryotes.

In order to establish the PP2A subunit responsible for interaction with eRF1, we performed direct interaction analysis using the yeast two-hybrid system (Fields and Song, 1989). One could expect PR65 as the likely candidate for this interaction, since it is known to bind to both the catalytic and variable regulatory subunits, thus performing a scaffold-like function for assembly of PP2A holoenzymes (Ruediger et al., 1994). Surprisingly, eRF1 turned out to interact directly and specifically with the catalytic subunit of PP2A and apparently did not bind either PR65 or PR55 in the yeast two-hybrid system. Our further interest was focused, therefore, on the studies of PP2Ac–eRF1 association in different experimental systems and examining the physiological role of this interaction. Analysis of deletion mutants using the two-hybrid system showed that deletion of 50 N-terminal amino acids on PP2Ac abolishes the binding, and that the eRF1 region responsible for interaction with PP2Ac is in the two-hybrid system most likely located between amino acid residues Thr338 and Asn381. Quantitative analysis of interactions between various deletion partners also pointed to the existence of another region in the C-terminal portion of PP2Ac that also possibly affects binding to eRF1. Sequence analysis of the N-terminal domain of PP2Ac showed that it contains all divergent residues found in α and β isoforms of PP2Ac. Furthermore, this domain displays very low homology to the N-terminal regions of PP1 and other PP2A-like serine/threonine protein phosphatases, such as PPX, so it might provide specificity for eRF1 binding to PP2Ac.

Further experiments showed that indeed a portion of the cytoplasmic PP2A is associated with eRF1 in vivo, since the immunoprecipitation of human eRF1 transiently expressed in COS1 cells brings down PP2Ac and PR65, as determined by Western blot analysis, and PP2A activity, as demonstrated by phosphatase activity measurements. eRF1-associated PP2A activity accounts for ~1% of the total cytoplasmic PP2A activity. This suggests that novel associations of PP2A exist but at much lower levels than the classical subunit configurations previously described.

What are the possible explanations for the functional significance of this interaction? One possibility is that eRF1 is a substrate for PP2A. eRF1 does not contain any established consensus phosphorylation sites for serine/threonine protein kinases, but contains a number of serine and threonine residues that could potentially be phosphorylated. We tried to examine this possibility, but recombinant human eRF1 was poorly phosphorylated by CAMP-dependent protein kinase, protein kinase C and casein kinase II (data not shown). Therefore, the issue of eRF1 phosphorylation remains to be investigated further. We did not observe any effect of recombinant eRF1 on the basal or protamine-stimulated activity of PP2A, therefore making it difficult to postulate that eRF1 is an inhibitory regulatory subunit of PP2A. Since eRF1 was isolated together with a 55 kDa protein of as yet unknown identity (p55), it is possible that both proteins are important for the interaction with PP2A. However, this possibility remains to be investigated after the molecular cloning of the cDNA encoding p55. The partial protein sequence data of p55 show that it is not an eRF3 homolog, as one would expect, although human eRF3 is coincidentally also a 55 kDa protein.

Given that eRF1 was shown to be associated with ribosomal 40S subunit in yeast (Stansfield et al., 1992), and functions, both in yeast and higher eukaryotes, as a polypeptide chain release factor (Frolova et al., 1994; Stansfield et al., 1995; Zhouravleva et al., 1995), one could postulate that PP2A is recruited to the translational machinery through its interaction with eRF1. Indeed, we observed a significant increase in the amount of PP2A associated with the polysomes when eRF1 was transiently overexpressed in COS1 cells. Many components of the eukaryotic translational apparatus are known to be phosphorylated and, in some cases, phosphorylation has been shown to control the rate of translation (Hershey, 1989). Furthermore, elongation and termination factors that directly function in maintaining translational accuracy are phosphorylated (Hershey, 1989). Phosphorylation levels are also implicated in the control of translational fidelity in Schizosaccharomyces pombe, since the allosuppressor gene, sal3, is allelic to cdc25, which is now known to encode a dual-specificity protein phosphatase (Nurse and Thuriaux, 1984; Dunphy and Kumagai, 1991). Other examples of the involvement of protein phosphatases in maintaining the accuracy of translation have been reported; most remarkable in this respect is the yeast translational allosuppressor SAL6, which recently has been identified as a serine/threonine protein phosphatase, termed PPQ (Vincent et al., 1994).

A fundamental question in signal transduction is how
Protein kinases and protein phosphatases are regulated to phosphorylate/dephosphorylate the correct target proteins rapidly and preferentially at the correct time and place. The targeting hypothesis of Hubbard and Cohen (1993) postulates that a 'targeting subunit' directs protein kinases and phosphatases to specific subcellular locations where they act on their targets. A substantial amount of evidence exists to prove that several protein kinases and phosphatases with broad substrate specificity are recruited to their targets by specific interacting proteins. The type II holoenzyme of the cAMP-dependent protein kinase (PKA) can be tethered to specific subcellular locations through the interaction of its regulatory (RII) subunit with A-kinase anchor proteins or AKAPs (Coughlan et al., 1995). Interestingly, PKA anchoring proteins were identified initially as proteins that co-purified with RII after affinity chromatography (Sarkar et al., 1984). Furthermore, a group of proteins termed RACKs (receptors for activated C-kinase) have been shown to bind to the activated form of the protein kinase C isoenzymes and mediate the translocation of the enzyme to the specific subcellular compartment where it exerts its action (Mochly-Rosen, 1995).

For PP2A, this type of regulation was thought to be mediated by its constituent regulatory subunits. However, with regard to the numerous cellular processes in which PP2A is predicted to be involved, it is feasible to speculate that a certain mode of regulation could also come from extrinsic proteins, rather than only from intrinsic holoenzyme components. Besides previously described classical inhibitory regulatory subunits of PP2A, there is a growing list of different cytosolic and nuclear proteins that are reported to bind to the dimeric form of PP2A to mediate specific cellular responses, such as the retinoblastoma protein-related p107 binding protein p59 (M.Voorhoeve, E.M.Hijmans and R.Bernards, personal communication). The results presented here demonstrate for the first time the interaction between PP2A and a factor involved in the termination of protein synthesis. eRF1 appears to play a dual role in (i) polypeptide chain termination, and (ii) recruitment of PP2A to the polysomes and, therefore, provides a basis for future experiments to evaluate the role of PP2A in the regulation of protein synthesis.

Materials and methods

**Purification of PP2A holoenzymes**

PP2A holoenzymes were purified from rabbit skeletal muscle using the protocol described by Tung et al. (1985) as modified by Zolnierowicz et al. (1994) (summarized in Figure 1). For the DEAE-Sepharose chromatography step, (5×40 cm column) proteins were eluted with a 2000 ml gradient of 0.05-0.6 M NaCl. Fractions corresponding to PP2A eluting from DEAE-Sepharose at 0.27-0.32 M, 0.34-0.38 M and 0.39-0.44 M NaCl (designated pool 1, pool 2 and pool 3, respectively) were combined and purified further. PP2A in pools 1 and 3 was purified by sequential chromatography on poly-l-lysine agarose, o-aminohexyl Sepharose and thiophosphorylase a-Sepharose columns. For PP2A in pool 2, the enzyme was purified by poly-l-lysine agarose and thiophosphorylase a-Sepharose. The final purification step for all three PP2A preparations involved ion exchange MonoQ FPLC (0.5×5 cm, Pharmacia) using a 40 ml gradient from 0.2 to 0.5 M NaCl. PP2A activity throughout purification was monitored by assaying protamine-stimulated phosphatase phosphatase activity as described by Waelkens et al. (1987). The purified proteins obtained from the final steps were analyzed by SDS-PAGE and immunodetected by Western blotting.

**Determination of amino acid sequences**

Protein fractions from the MonoQ-FPLC step were analyzed on 10% SDS-polyacrylamide gels and used to obtain protein sequence data. Protein transfer, trypsin digestion, peptide separation and sequencing were carried out as previously described (Hendrix et al., 1993a).

**Molecular cloning**

For molecular cloning of the 54 kDa protein that co-purifies with PP2A, single-stranded cDNA was prepared from a human breast carcinoma cell line T47D as described previously (Healy et al., 1991). Two degenerate oligonucleotides containing a Hind III restriction site (underlined) were synthesized: sense 5'-ACAAGCCTTAYTYGYAGARATI-WSCARGA-3', corresponding to amino acid sequence YFDEISQD and antisense 5'-ACAAGGTTTTCYCTGICGIGRTTA-3', corresponding to amino acids YLTFQJQK. PCR was performed using Ampli-Taq DNA polymerase (Perkin Elmer). PCR products were gel purified as described in Sambrook et al. (1989), subcloned into the Hind III site of pBluescript (Stratagene) and sequenced using Sequenase 2.0 (USB). PCR clone 18 contained an insert of 188 bp encoding a 62 amino acid polypeptide with 57% identity to the yeast SUF45 gene product and was used subsequently for screening a human fetal brain cDNA library (Stratagene). DNA was labeled with [α-32P]dATP by the random priming method of Feinberg and Vogelstein (1983) to a specific activity of ~106 c.p.m/µg. cDNA library screening was carried out as previously described (Hendrix et al., 1993a).

A cDNA encoding human eRF1 (Hoshino et al., 1989) was amplified from reverse-transcribed T47D total cDNA (prepared as described above) using the following PCR oligonucleotides: sense 5'-ATAAGCCTTACCCATGGAACTTCTCAGAACT-3' (Hind III site is underlined) and anti-sense 5'-TATGGATCCTTATCCTTCTGAGGACG-3' (BamHI site is underlined). PCR products were subcloned in pBluescript and sequenced as described above.

**Northern blot analysis**

The mRNA blot from human tissues (Clontech Laboratories, Inc.) was hybridized with the probe corresponding to the cDNA clone BBZeRF1-4b labeled by the random priming method (Feinberg and Vogelstein, 1983). Hybridization and washing of the blot were carried out according to the supplier's instructions.

** Yeast two-hybrid system**

Two-hybrid analysis was carried out using Matchmaker™ Two-Hybrid System (Clontech Laboratories, Inc.). The cDNAs encoding full-length human PP2A cca (Khwed-Goodall et al., 1991), PR65x (Hemmings et al., 1990), PR55x (Mayer et al., 1991), eRF1 and eRF3 were fused in-frame with the GAL4 DNA binding domain and/or transcriptional domain using pGBT9 and pGAD424 expression vectors, respectively. A series of N- and C-terminal deletions were constructed for both PP2Ae and eRF1 using a PCR-based strategy. PCR products encoding three N-terminal (eRF151-437, eRF193-437 and eRF1150-437) and three C-terminal deletions (eRF1-141, eRF1-381 and eRF1-339) of human eRF1 were subcloned into pGAD424 in-frame with the GAL4 transactivation domain. PCR products encoding three N-terminal (PP2Ae300-389 and PP2Ac300-389) and three C-terminal (PP2Ae129-259, PP2Ae129-209 and PP2Ac119-199) truncations of PP2Ac were subcloned into pGBT9 in frame with the GAL4 DNA binding domain. Control plasmids encoding wild-type GAL4 protein (pClI), SV40 large T antigen (pTD1), p53 (pVAX3) and lamin C (pLAM5'), the latter three fused to the appropriate domains of GAL4, were provided by the manufacturer and used for control interactions. Expression constructs were transformed into S.cerevisiae host strains SPY526 (lacZ reporter gene) and HF7c (lacZ and HIS3 reporter genes). Double transformants were selected for growth on selective SD medium lacking leucine and tryptophan. Expression of the lacZ reporter gene was determined by measuring β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and O-nitrophenyl-β-D-galactoside (ONPG) as substrates. Expression of the HIS3 reporter gene was assessed by monitoring growth on triple selective SD medium lacking leucine, tryptophan and histidine. All media and reagents were prepared according to the manufacturer's recommendations.

**Bacterial expression and purification of recombinant eRF1 and eRF3 proteins**

PCR was used to subclone the cDNA encoding full-length human eRF1 into bacterial expression vector pRSET-A (Invitrogen) to add a polyhistidine tag to the N-terminus of the protein. The expression construct was sequenced and subsequently transformed into E.coli
EcoRI-XbaI fragment was subcloned into mammalian E.coli XL-1 cells, recommendations. using glutathione-agarose resin (Sigma) according to the manufacturer's instructions. Purification of PP2A subunits in holoenzymes was performed as described by Hendrix et al. (1993b). Antibodies used to detect PP2A subunits in preparations of PP2Ac, PP2A2 and PP2A1 from rabbit skeletal muscle (10 nM). Assays were performed using two semi-purified preparation of PP2A that contain eRF1 (MonoQ I and MonoQ II), as confirmed by Western blotting analysis with Ab eRF124-437, in the absence and presence of 10 nM okadaic acid (Sigma). The RF (release factor) activity was calculated as pmoles of formyl[35S]methionine released in the presence of stop codon.

Preparation of peptide-specific antisera and Western blot analysis
Antisera were prepared to human eRF1 by immunization with a peptide YQGGDDEFFLDLDY (amino acids 424–437) corresponding to the C-terminus of the protein. Peptide synthesis and coupling to keyhole limpet hemocyanin with glutaraldehyde and subsequent immunization of rabbits were carried out as described by Hendrix et al. (1993b).

Protein transfer and Western blot analysis were performed as described by Hendrix et al. (1993b). Antibodies used to detect PP2A subunits in protein preparations of PP2Ac, PP2A2 and PP2A1 from rabbit skeletal muscle (10 nM). Assays were performed using two semi-purified preparation of PP2A that contain eRF1 (MonoQ I and MonoQ II), as confirmed by Western blotting analysis with Ab eRF124-437, in the absence and presence of 10 nM okadaic acid (Sigma). The RF (release factor) activity was calculated as pmoles of formyl[35S]methionine released in the presence of stop codon.

Preparation of eukaryotic ribosomes
80S ribosomes were prepared from exponentially growing COS-1 cells by centrifugation through a 38% sucrose cushion as described by Spedding (1990). Subsequent immunoprecipitation and phosphatase activity measurements were performed the same way as described for cell extracts.

In vitro termination assay
Synthesis of formyl[35S]methionyl-rRNAMet, preparation of 80S ribosomes from COS-1 cells and in vitro termination assays were performed as described by Turowski et al. (1995). The reaction mixture (50 µl) contained 2 pmol of formyl[35S]methionyl-rRNAOMe-AUG-80S sub-strate, 5 nM of stop codon-containing octanucleotide (UGAAAAGA), 1 mM GTP, 20 mM Tris–HCl pH 7.4, 60 mM KCl, 10 mM MgCl2 and 10 mM of purified recombinant His-eRF1. To test whether PP2A affects release factor activity of eRF1, samples were treated with purified preparations of PP2A, PP2A2 and PP2A1 from rabbit skeletal muscle (10 nM). Assays were also performed using two semi-purified preparation of PP2A that contain eRF1 (MonoQ I and MonoQ II), as confirmed by Western blotting analysis with Ab eRF124-437, in the absence and presence of 10 nM okadaic acid (Sigma). The RF (release factor) activity was calculated as pmoles of formyl[35S]methionine released in the presence of stop codon.

Acknowledgements
We thank M.Voorhoeve and Dr R.Bernards (The Netherlands Cancer Institute, Amsterdam) for providing pMV vector and HA-PR565a expression construct and sharing their unpublished results with us, Dr P.Mathias for the gift of pEV3S expression vector, G.Aeshbacher and F.Fischer for oligonucleotide and peptide synthesis, and R.Mathies and Dr J.Hofsteenge for protein microsequencing. The authors are grateful to Professor J.F.W.Merlevede for continuous support and encouragement, Professor W.T.Ball, Professor M.Veekmans and V.Feytons for excellent technical assistance. We are also indebted to Professor W.Tate, Dr K.McCaughan and Dr H.J.B. Jeffries for expert advice on eukaryotic termination assays and ribosome preparation, and to M.Andjelkovic, R.Reve and Dr H.B.J.Jeffries for excellent and helpful comments on the manuscript. This work was supported by a fellowship from Krebsliga beider Basel (to N.A.), the European Commission DGXII Biomedical and Health Research Program and the National Fonds voor Wetenschappelijk Onderzoek (to C.V.H. and J.G.).

References
show strongly reduced ability to dephosphorylate substrates of p34cdc2.


Stansfeld, I. *et al.* (1995) The products of the SUP45 (erf1) and SUP35 genes interact to mediate translation termination in **Saccharomyces cerevisiae. EMBO J.,** 14, 4365–4373.


Received on December 5, 1995; revised on September 13, 1996