Mapping the human translation elongation factor eEF1H complex using the yeast two-hybrid system

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In eukaryotes, the eukaryotic translation elongation factor eEF1A responsible for transporting amino-acylated tRNA to the ribosome forms a higher-order complex, eEF1H, with its guanine-nucleotide-exchange factor eEF1B. In metazoans, eEF1B consists of three subunits: eEF1Bα, eEF1Bβ and eEF1Bγ. The first two subunits possess the nucleotide-exchange activity, whereas the role of the last remains poorly defined. In mammals, two active tissue-specific isoforms of eEF1A have been identified. The reason for this pattern of differential expression is unknown. Several models on the basis of *in vitro* experiments have been proposed for the macromolecular organization of the eEF1H complex. However, these models differ in various aspects. This might be due to the difficulties of handling, particularly the eEF1Bβ and eEF1Bγ subunits *in vitro*. Here, the human eEF1H complex is for the first time mapped using the yeast two-hybrid system, which is a powerful *in vivo* technique for analysing protein–protein interactions. The following complexes were observed: eEF1A1:eEF1Bα, eEF1A1:eEF1Bβ, eEF1Bβ:eEF1Bβ, eEF1Bα:eEF1Bγ, eEF1Bβ:eEF1Bγ and eEF1Bα:eEF1Bγ:eEF1Bβ, where the last was observed using a three-hybrid approach. Surprisingly, eEF1A2 showed no or only little affinity for the guanine-nucleotide-exchange factors. Truncated versions of the subunits of eEF1B were used to orientate these subunits within the resulting model. The model unit is a pentamer composed of two molecules of eEF1A, each interacting with either eEF1Bα or eEF1Bβ held together by eEF1Bγ. These units can dimerize via eEF1Bβ. Our model is compared with other models, and structural as well as functional aspects of the model are discussed.

Key words: isoform, protein–protein interaction, three-hybrid system.

INTRODUCTION

The elongation step of protein biosynthesis in eukaryotes requires two different elongation factors, eEF1A and eEF1B [1]. The former transports amino-acylated tRNA to the programmed ribosome, and requires the concomitant binding of GTP. Upon correct codon–anticodon interaction, GTP hydrolysis is triggered. The resulting eEF1A:GDP complex has to be recycled by eEF1B, which promotes the exchange of GDP with GTP to prepare eEF1A for a new elongation cycle. The exchange step has been shown to be crucial *in vivo* [2,3].

In humans, eEF1A is a monomeric protein of 50 kDa, whereas eEF1B is composed of three subunits: eEF1Bα (25 kDa), eEF1Bβ (31 kDa) and eEF1Bγ (48 kDa). The four elongation factor subunits assemble into a 'heavy' complex, eEF1H [4,5].

eEF1Bα possesses guanine-nucleotide-exchange activity [6]. The functional site of eEF1Bα from the brine shrimp *Artemia* was found to be localized at the C-terminal domain of the factor [7], and this was later confirmed for the human protein as well [8]. At present, three three-dimensional structures of eEF1Bα, or fragments thereof, are known [9–11].

eEF1Bβ is only found in higher eukaryotes. Like eEF1Bα, eEF1Bβ also has nucleotide-exchange activity [12]. In humans, these two proteins are 47% identical, and particularly the C-terminal region is well conserved, with an identity of 82%. Therefore it is not surprising that the nucleotide-exchange activity of eEF1Bβ is also located within the C-terminal domain. A leucine-zipper pattern has been identified in the N-terminal domain of eEF1Bβ comprising amino acids 80–115, and this motif appears to be conserved in *Artemia*, *Xenopus* and humans [13].

eEF1Bγ does not have any nucleotide-exchange activity, but seems to stimulate the activity of eEF1Bα [2]. In contrast, no stimulatory effect on the exchange activity of eEF1Bβ has been detected [14]. eEF1Bγ has hydrophobic properties enabling it to become attached to membranes, and in *Artemia salina* it has been found to co-precipitate with tubulin [6]. eEF1Bγ has been localized to the endoplasmic reticulum [15], reinforcing the suggestion that the role of eEF1Bγ is to anchor the eEF1H complex to the membrane.

eEF1H binds valyl-tRNA synthetase (ValRS) tightly [14,16,17], suggesting an activity for channelling of tRNA [18]. This channelling not only increases the efficiency of the overall reaction, but also allows the compartmentalization of all of the components taking part in protein biosynthesis at membranes and the cytoskeletal framework of the cell. This theory is supported further by the finding that a large fraction of the mRNA is tightly associated with the cytoskeleton [19].

Most organisms have more than one active eEF1A gene in their genome. These genes are often differentially expressed during development. In mammals, two active eEF1A genes have been identified [20–23]. The same isoforms from different organisms are up to 100% conserved, whereas eEF1A1 and eEF1A2 from the same organism are approx. 93% identical with respect to amino acid sequence. The expression of eEF1A2 is found exclusively in terminally differentiated cells (of the brain, muscle and heart), where the expression of eEF1A1 is reduced [21,23]. The two eEF1A isoforms perform equally well in translation *in vitro*, whereas they interact slightly differently with guanine nucleotides [23]. However, the two elongation factors may have different additional roles. *Wasted* mice suffer from a genetic disorder, which is specifically caused by a lack of eEF1A2

Abbreviations used: AD, activation domain; 3-AT, 3-aminotriazole; BD, binding domain; MCSI/II, multiple cloning sites I and II respectively; ValRS, valyl-tRNA synthetase.

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resulting from a 15.8 kb deletion that removes the promoter and the first exon of the *eEF1A2* gene [24].

All of the subunits of the eEF1H complex have been reported to be targets of kinases [2,17,25–28]. The functional significance of these phosphorylations is poorly understood, but they are assumed to play a regulatory role. This was demonstrated recently in the case of the cell cycle protein kinase CDK1/cyclin B, which acts on the eEF1Bγ and eEF1Bβ subunits [29].

Apart from playing prominent roles in protein biosynthesis, the factors comprising the eEF1H complex have also been associated with other important cellular functions, such as cell cycle progression [30,31], cytoskeletal organization [6,32,33], oncogenesis [30,34,35] and viral infection [36–38]. Knowledge about the topography of the eEF1H complex may therefore impact on a large number of different biological fields. The supramolecular organization of the eEF1H complex, along with the numerous phosphorylation sites present in the three eEF1B subunits, suggest a function of eEF1B as a regulator of the functions of eEF1A; not only in the elongation step of protein biosynthesis, but also in many of the proposed alternative functions of this factor.

At present, four different models of eEF1H complexes from various higher eukaryotes exist [4,14,16,17]. These models share a number of similarities. However, none of the models are equivalent, and none of them have taken eEF1A2 into account. All of the existing models are on the basis of *in vitro* experiments, such as reconstitution of purified subunits [14], polyacrylamide gel electrophoresis [4], limited proteolysis [4], analysis of the isolated, native complex [16] and *in vitro* phosphorylation [17].

Manipulations of the subunits constituting the eEF1H complex are difficult, since they tend to denature or aggregate into multimeric complexes [6,14]. Furthermore, the unnatural *in vitro* conditions are prone to induce artifacts, which may explain the differences. However, these could also arise from the use of different sources of factors for formation of the complex.

In the present study, an *in vivo* approach (the yeast two-hybrid system [39]) has been applied for the first time to map the human eEF1H complex. The cDNAs encoding the subunits that constitute the eEF1H complex, including eEF1A2, were cloned into yeast two-hybrid expression vectors, with the goal of mapping their specific protein–protein interaction pattern. Furthermore, truncated versions of eEF1Bα, eEF1Bβ and eEF1Bγ were used for a refined mapping of the identified interactions. Finally, a three-hybrid strategy [40] was applied to eliminate ambiguities.

This approach has the advantage compared with previous studies of taking place inside a eukaryotic cell, i.e. under conditions that are much more physiologically relevant than any *in vitro* study. Furthermore, the sensitivity of this technique allows the detection of weak interactions (K_d up to 70 μM) that can be expected in a dynamic system, such as the eEF1H complex [41].

On the basis of our findings, we propose a macromolecular topographical model of the human eEF1H complex, and discuss structural and functional features of the new model. Surprisingly, no (or only very little) interaction could be detected between the tissue-specific isoforms of eEF1A, eEF1A2 and the exchange factors of the eEF1H complex.

EXPERIMENTAL

Yeast strains

The yeast strains AH109 (*MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4*Δ, *gal80*Δ, *LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3*, *MEL1*, *GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2*, *URA3::MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ*; Clontech Matchmaker Two-hybrid System 3) and PJ69-4A (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*; [42]) carrying reporter genes *Ade2*, *His3* and *lacZ* were applied in the present study.

Plasmid construction

The following two-hybrid vectors (Clontech) were applied in our two-hybrid study: pGADT7 [GAL4 DNA-binding domain (BD) fusion], pGBKT7 [GAL4 activation domain (AD) fusion] and pBridge™ (expresses two proteins: a GAL4 DNA-BD fusion and an untagged protein). The constructs made in these vectors are summarized in Table 1.

Truncated versions of eEF1Bα and eEF1Bβ were made according to sequence comparison. eEF1Bγ was truncated according to descriptions of fragments of eEF1Bγ from *A. salina* resulting from tryptic digests [7] and sequence comparison with human eEF1Bγ in order to conserve the distribution of functions described previously [4].

cDNAs encoding human eEF1A1 [43], eEF1A2 [21], eEF1Bα (A.T.C.C. 721384R) and eEF1Bβ (A.T.C.C. 720603R) were amplified by PCR with primers containing the restriction sites indicated in Table 1. Truncated versions of human eEF1Bα and eEF1Bβ were PCR-amplified from the resulting full-length clones. Furthermore, the genes encoding *Escherichia coli* EF1A [44] and EF1B [45] were cloned into pGBKT7 and pGADT7 respectively after PCR amplification using primers introducing the appropriate restriction sites (Table 1). These constructs served as a reference point and to confirm the validity of the approach.

Pwo polymerase (Roche) was used for all PCR amplifications. The reading frame and sequence of all constructs made by PCR cloning were confirmed by sequencing using the Thermo Sequenase™ II sequencing kit (Amersham Biosciences).

The cDNA encoding human eEF1Bγ was subcloned from pET16b-eEF1Bγ (kindly given by Dr G. M. C. Janssen, Leiden University, Leiden, The Netherlands) into pGADT7, and thereafter subcloned into pGBKT7.

Several constructions were made in pBridge: pBridge-eEF1Bα-$\varnothing$, pBridge-eEF1Bα-eEF1Bγ, pBridge-eEF1Bβ-$\varnothing$ and pBridge-eEF1Bβ-eEF1Bγ. These constructs contain the cDNA encoding eEF1Bα or eEF1Bβ in multiple cloning site I (MCSI), which allows the expression of a protein of interest as a GAL4-BD fusion. In two of the constructs, the second multiple cloning site (MCSII) is empty ($\varnothing$), whereas in the other two constructs, the presence of the cDNA encoding eEF1Bγ allows the expression of this protein (in its native form) as a potential bridging protein. The eEF1Bα cDNA was subcloned from pGBKT7-eEF1Bα as a *Xho*I/*Pst*I fragment, and eEF1Bβ cDNA was subcloned from pGBKT7-eEF1Bβ as a *Xho*I/*Sal*I fragment, taking advantage of the unique *Xho*I site in GAL4 BD (amino acids 1–147) shared by pGBKT7 and pBridge. The eEF1Bγ cDNA was PCR-amplified, and one of the primers introduced a silent mutation at Ile^{479} in the naturally occurring *Bgl*II site of the gene to be able to clone the full-length human eEF1Bγ cDNA into the *Bgl*II cloning site II of pBridge.

All constructions applied in this study are summarized in Table 1.

Yeast two-hybrid assay

All media, buffers and methods for the yeast two-hybrid assay were adopted from the Matchmaker Two-hybrid System 3 protocol and the Yeast Protocols Handbook (both from Clontech). Yeast cells were co-transformed with the different

Table 1 Vector constructs

Shown in (**a**) are vector constructs applied in the conventional two-hybrid assay, whereas (**b**) encompasses constructs in the vector pBridge used for the three-hybrid assay. Restriction sites used for the cloning of cDNA are indicated along with the amino acids present in the protein resulting upon expression.

(**a**)

GAL4 binding-domain plasmids			GAL4 activation-domain plasmids		
Construct	Restriction sites 5′/3′	Amino acids	Construct	Restriction sites 5′/3′	Amino acids
pGBKT7-eEF1A1	*Eco*RI/*Pst*I	1–462	pGADT7-eEF1A1	*Eco*RI/*Xho*I	1–462
pGBKT7-eEF1A2	*Eco*RI/*Sal*I	1–463	pGADT7-eEF1A2	*Eco*RI/*Xho*I	1–463
pGBKT7-eEF1Bα	*Eco*RI/*Pst*I	1–225	pGADT7-eEF1Bα	*Eco*RI/*Xho*I	1–225
pGBKT7-eEF1Bβ	*Nde*I/*Eco*RI	1–281	pGADT7-eEF1Bβ	*Nde*I/*Eco*RI	1–281
pGBKT7-eEF1Bγ	*Nde*I/*Pst*I	1–437	pGADT7-eEF1Bγ	*Nde*I/ClaI	1–437
pGBKT7-N_t-eEF1Bα	*Eco*RI/*Pst*I	1–96	pGADT7-N_t-eEF1Bγ	*Nde*I/*Xho*I	1–235
pGBKT7-N_t-eEF1Bβ	*Nde*I/*Eco*RI	1–150	pGADT7-C_t-eEF1Bγ	*Nde*I/*Xho*I	236–437
pGBKT7-EF1A	*Eco*RI/*Pst*I	1–393	pGADT7-EF1B	*Sma*I/*Pst*I	1–282

(**b**)

	MCS I		MCS II	
pBridge constructs	Restriction sites 5′/3′	Amino acids	Restriction sites 5′/3′	Amino acids
pBridge-eEF1Bβ-∅	*Xho*I/*Sal*I	1–281	Not used	0
pBridge-eEF1Bβ-eEF1Bγ	*Xho*I/*Sal*I	1–281	*Bgl*II/*Bgl*II	1–437
pBridge-eEF1Bα-∅	*Xho*I/*Pst*I	1–225	Not used	0
pBridge-eEF1Bα-eEF1Bγ	*Xho*I/*Pst*I	1–225	*Bgl*II/*Bgl*II	1–437

pairs of AD and BD vectors to be tested, and evaluated for prototrophy for adenine or histidine by respectively growing cell suspensions in circular patches (approx. 5 mm in diameter) on synthetic dropout medium lacking either tryptophan, leucine and adenine (SD/−Trp/−Leu/−Ade) or tryptophan, leucine and histidine (SD/−Trp/−Leu/−His). The latter plates contained different concentrations of the His3 protein inhibitor, 3-aminotriazole (3-AT), to evaluate the strength of the two-hybrid interaction. Furthermore, co-transformants were assayed for β-galactosidase activity.

The positive controls provided with the Matchmaker Two-hybrid System 3 were successfully carried out (results not shown). Likewise, the appropriate negative controls (transformation against empty vectors) demonstrated that none of the interactions tested were prone to self-activation (results not shown).

pBridge™ assay

In this assay, two-hybrid fusion proteins are expressed as in the conventional two-hybrid assay, but one of the vectors, pBridge™ (Clontech), allows the expression of a third protein in addition to the GAL4 fusion proteins. The two GAL4 fusion proteins are unable to trigger the reporter gene of the selected yeast strain when they are co-expressed alone if there is no direct binding between them. However, the third protein may be able to bridge them, leading to reporter-gene activation [40]. As for the conventional two-hybrid fusion proteins, the potential bridging protein is also expressed with a nuclear localization signal, allowing the protein to enter the nucleus, where the interaction assay takes place.

Yeast cells were co-transformed with the different pairs of pBridge™ and AD vectors to be tested, and reporter-gene activation was evaluated as described for the conventional two-hybrid assay.

None of the interactions tested were prone to self-activation, as demonstrated by transformation against empty vectors (results not shown).

RESULTS

Analysis of potential interactions among eEF1A1, eEF1A2, eEF1Bα and eEF1Bβ

Full-length constructs of eEF1A1, eEF1A2, eEF1Bα and eEF1Bβ were co-transformed into yeast strains AH109 and PJ69-4A. Reporter-gene activation was evaluated, as shown in Table 2. Surprisingly, eEF1A1 interacts much more strongly

Table 2 Associations between eEF1A1, eEF1A2, eEF1Bα and eEF1Bβ

pGBKT7 and pGADT7 constructs were co-transformed into yeast strains AH109 and PJ69-4A, as indicated, and growth on SD/−Trp/−Leu/−Ade and SD/−Trp/−Leu/−His was measured. In case of the *His3* reporter, the strength of the interaction was monitored in terms of growth on plates containing different levels of 3-AT, i.e. ⩾ 10 mM (10), 6 mM (6), 3 mM (3), 1 mM (1) and 0 mM (0). The intensity of growth on SD/−Trp/−Leu/−Ade is indicated by the number of + signs. '−' means no growth on medium lacking histidine (in the absence of 3-AT) or on SD/−Trp/−Leu/−Ade medium. β-gal, β-galactosidase.

Co-transformed vectors		Strains ...	AH109 Reporter-gene activation		PJ69-4A Reporter-gene activation		
pGBKT7	pGADT7		*Ade2*	*His3*	*Ade2*	*His3*	*lacZ* (β-gal units)
eEF1A1	eEF1A1		−	−	−	−	1.0
eEF1A1	eEF1Bα		−	−	−	−	1.0
eEF1A1	eEF1Bβ		−	−	−	−	1.0
eEF1A2	eEF1A1		−	−	−	−	1.0
eEF1A2	eEF1Bα		−	−	−	−	1.0
eEF1A2	eEF1Bβ		−	−	−	−	1.0
eEF1Bα	eEF1A1		−	10	−	3	2.7
eEF1Bα	eEF1A2		−	0	−	0	1.6
eEF1Bα	eEF1Bα		−	−	−	−	1.0
eEF1Bα	eEF1Bβ		−	−	−	−	1.0
eEF1Bβ	eEF1A1		−	10	−	6	8.9
eEF1Bβ	eEF1A2		−	0	−	0	2.0
eEF1Bβ	eEF1Bα		−	−	−	−	1.6
eEF1Bβ	eEF1Bβ		−	0	+	3	2.2

Table 3 Interaction between eEF1Bγ and eEF1A1, eEF1A2, eEF1Bα and eEF1Bβ

pGBKT7 and pGADT7 constructs were co-transformed into yeast strain PJ69-4A as shown in the Table, and growth on SD/−Trp/−Leu/−Ade and SD/−Trp/−Leu/−His was monitored. In the case of the *His3* reporter, the strength of the interaction is indicated in terms of growth on plates containing different levels of 3-AT, i.e. ⩾ 10 mM (10), 6 mM (6), 3 mM (3), 1 mM (1) and 0 mM (0). The intensity of growth on SD/−Trp/−Leu/−Ade is indicated by the number of + signs. '−' means no growth on medium lacking histidine (in the absence of 3-AT) or on SD/−Trp/−Leu/−Ade medium, whereas 'nd' means 'not determined'. β-gal, β-galactosidase.

Co-transformed vector constructs		Reporter-gene activation		
pGBKT7	pGADT7	*Ade2*	*His3*	*lacZ* (β-gal units)
eEF1A1	eEF1Bγ	−	−	1.0
eEF1A2	eEF1Bγ	−	−	1.0
eEF1Bα	eEF1Bγ	+++	6	9.5
eEF1Bβ	eEF1Bγ	+++	10	44.0
eEF1Bγ	eEF1Bγ	−	−	1.0
eEF1Bγ	eEF1A1	−	−	1.0
eEF1Bγ	eEF1A2	−	−	1.0

Table 4 Three-hybrid experiment

Various pBridge™ and pGADT7 constructs were co-transformed into yeast strain PJ69-4A, as indicated, and growth on SD/−Trp/−Leu/−Ade and SD/−Trp/−Leu/−His was monitored. In the case of the *His3* reporter, the strength of the interaction is indicated in terms of growth on plates containing different levels of 3-AT, i.e. ⩾ 10 mM (10), 6 mM (6), 3 mM (3), 1 mM (1) and 0 mM (0). The intensity of growth on SD/−Trp/−Leu/−Ade is indicated by the number of + signs. '−' means no growth on medium lacking histidine (in the absence of 3-AT) or on SD/−Trp/−Leu/−Ade medium; '∅' designates a vector without an insert, and 'nd' means 'not determined'. β-gal, β-galactosidase.

Co-transformed vector constructs		Reporter-gene activation		
pBridge™ (MCSI/MCSII)	pGADT7	*Ade2*	*His3*	*lacZ* (β-gal units)
eEF1Bα/∅	eEF1Bα	−	−	1.0
eEF1Bα/∅	eEF1Bβ	−	−	1.0
eEF1Bα/∅	∅	−	−	1.0
eEF1Bβ/∅	eEF1Bβ	−	0	2.3
eEF1Bβ/∅	eEF1Bα	−	−	1.0
eEF1Bα/eEF1Bγ	∅	−	−	1.0
eEF1Bα/eEF1Bγ	eEF1Bα	−	−	1.0
eEF1Bα/eEF1Bγ	eEF1Bβ	−	0	2.2
eEF1Bβ/eEF1Bγ	∅	−	−	1.0
eEF1Bβ/eEF1Bγ	eEF1Bα	+	6	7.2
eEF1Bβ/eEF1Bγ	eEF1Bβ	−	0	1.5

with eEF1Bα and eEF1Bβ than does eEF1A2, which gives no, or only a very faint, signal in the two-hybrid system. It appears that the signals involving eEF1A are asymmetric, i.e. a signal obtained with a particular constellation of pGADT7 and pGBKT7 constructs may disappear if the inserts of the vectors are swapped. Thus the interactions of eEF1A are only detected when eEF1A is expressed as an AD fusion.

All possible dimerizations were tested, but only eEF1Bβ turned out to be able to interact with itself, albeit only weakly in strain AH109. It should be mentioned that the presence of human eEF1Bβ in yeast, which does not have a counterpart to this elongation factor, seems to have a negative effect on cell growth and survival.

Interactions among eEF1Bγ and the other subunits of the eEF1H complex

The cDNA encoding human eEF1Bγ was cloned into the yeast two-hybrid vectors pGADT7 and pGBKT7, and these were co-transformed into yeast strain PJ69-4A either together or with pGADT7/pGBKT7 constructs containing the cDNAs of the other three eEF1H subunits. Reporter-gene activation was scored as shown in Table 3. We observe an interaction of both eEF1Bα and eEF1Bβ with eEF1Bγ.

The pBridge™ assay

After having tested the four subunits of eEF1H against each other, a first preliminary model of the composition of the complex was made. However, it was not clear whether or not eEF1Bγ has two binding sites to accommodate eEF1Bα and eEF1Bβ at the same time, or whether there exists one binding site that is shared by eEF1Bα and eEF1Bβ, i.e. whether eEF1Bα and eEF1Bβ can bind simultaneously to eEF1Bγ or whether the binding of one exchange factor to eEF1Bγ excludes the binding of the other. In order to answer this question, a three-hybrid experiment was set up, where untagged eEF1Bγ was expressed to serve as a bridging protein between eEF1Bα and eEF1Bβ. In this experiment, eEF1Bα and eEF1Bβ are expressed as GAL4 fusion proteins (AD fusion from pGADT7 and BD fusion from MCSI of pBridge™), whereas eEF1Bγ is expressed without a fusion tag (from MCSII of pBridge™). Controls without the cDNA encoding eEF1Bγ was made in order to determine whether or not a positive signal was strictly dependent on the presence of eEF1Bγ. Yeast strain PJ69-4A was co-transformed with the appropriate constructs, and growth on SD/−Trp/−Leu/−Ade and SD/−Trp/−Leu/−His media as well as β-galactosidase activities was monitored. These results are shown in Table 4.

Our results show that the presence of eEF1Bγ allows an interaction to take place between eEF1Bα and eEF1Bβ. The interaction relies on the co-expression of eEF1Bγ. In contrast, eEF1Bγ is not able to mediate an interaction between two molecules of eEF1Bα or two molecules of eEF1Bβ. In fact, eEF1Bγ seems to have a negative effect on the formation of eEF1Bβ dimers, as detected using the conventional two-hybrid system (Tables 2 and 4).

Estimation of the strengths of the interactions

The strength of the interactions taking place within the eEF1H complex can be evaluated in a qualitative manner on the basis of the levels of reporter-gene activation [46]. The *Ade2* reporter provides a growth readout, and the activation is simply evaluated by inspection of the size and density of the patches obtained. Also the *His3* reporter gives a signal related to the growth level. However, in this case, titration with 3-AT, which is a competitive inhibitor of the His3 protein, enables a numerical readout, since the concentration of 3-AT needed to inhibit growth is proportional to the level of reporter-gene activation. Finally, the activity of β-galactosidase resulting upon *lacZ* activation can be measured directly in a colorimetric assay.

For comparison, a positive control plasmid encoding the full-length wild-type GAL4 protein resulted in the scores '+++', 10 and 134 β-galactosidase units for the *Ade2*, *His3* and *lacZ* reporters respectively, whereas the negative controls resulted in no growth on SD/−Trp/−Leu/−Ade and SD/−Trp/−Leu/−His plates and gave a readout of 1.0 β-galactosidase unit for the *lacZ* reporter (based on an average of 20 independent tests).

It appears that the interactions between eEF1Bγ and the exchange factors eEF1Bα and eEF1Bβ are considerably stronger

Table 5 Interactions between truncated and full-length versions of eEF1H subunits

pGBKT7 and pGADT7 constructs were co-transformed into yeast strain AH109, as indicated, and growth on SD/—Trp/—Leu/—Ade and SD/—Trp/—Leu/—His was monitored. In the case of the *His3* reporter, the strength of the interaction is indicated in terms of growth on plates containing different levels of 3-AT, i.e. ⩾ 10 mM (10), 6 mM (6), 3 mM (3), 1 mM (1) and 0 mM (0). The intensity of growth on SD/—Trp/—Leu/—Ade is indicated by the number of + signs. '—' means no growth on medium lacking histidine (in the absence of 3-AT) or on SD/—Trp/—Leu/—Ade medium, whereas 'nd' means 'not determined'. N_t and C_t denote 'N-terminally truncated' or 'C-terminally truncated' respectively. β-gal, β-galactosidase.

Co-transformed vector constructs		Reporter gene activation		
pGBKT7	pGADT7	*Ade2*	*His3*	*lac Z* (β-gal units)
N_t-eEF1Bα	eEF1A1	—	—	1.0
N_t-eEF1Bα	eEF1Bα	—	—	1.0
N_t-eEF1Bα	eEF1Bγ	+++	10	15
N_t-eEF1Bβ	eEF1A1	—	—	1.0
N_t-eEF1Bβ	eEF1Bβ	—	—	1.0
N_t-eEF1Bβ	eEF1Bγ	++	10	35.6
eEF1A1	N_t-eEF1Bγ	—	—	1.0
N_t-eEF1Bα	N_t-eEF1Bγ	++	10	22.0
N_t-eEF1Bβ	N_t-eEF1Bγ	+++	10	51.0
eEF1Bα	N_t-eEF1Bγ	+++	10	7.0
eEF1Bβ	N_t-eEF1Bγ	+++	10	9.3
eEF1Bγ	N_t-eEF1Bγ	—	—	1.0
N_t-eEF1Bα	C_t-eEF1Bγ	—	—	1.0
N_t-eEF1Bβ	C_t-eEF1Bγ	—	—	1.0
eEF1Bα	C_t-eEF1Bγ	—	—	1.0
eEF1Bβ	C_t-eEF1Bγ	—	—	1.0
eEF1Bγ	C_t-eEF1Bγ	—	—	1.0

than the interactions among eEF1A1 and the exchange factors, as shown in Tables 2 and 3. Notice that the strength of the reporter response depends on the constellation of BD and AD vectors. Thus we compare the situations in which eEF1Bα and eEF1Bβ are always present as BD fusions, i.e. in pGBKT7. In comparison, the interaction between the prokaryotic EF1A (equivalent to eEF1A) and EF1B (equivalent to eEF1Bα/eEF1Bβ) represents an intermediate situation. This interaction is characterized by a K_d of approx. 10 μM in the presence of guanine nucleotides [47], as is the case *in vivo*. This interaction resulted in the scores '++' and 6 (in strain PJ69-4A) for the *Ade2* and *His3* reporters respectively. The eEF1Bβ dimer appears to be weaker than this, but interacts in the same range as the complex formed between eEF1A1 and eEF1Bα.

Truncated forms of eEF1Bα, eEF1Bβ and eEF1Bγ

Our model was refined further using truncated forms of eEF1Bα, eEF1Bβ and eEF1Bγ in order to orientate the domains of the different proteins comprising eEF1B. The functional diversity of the domains of these proteins is well documented using *A. salina* [4,7]. Co-transformations of pGBKT7 and pGADT7 constructs expressing truncated and full-length versions of the elongation factors were carried out, and the results from platings on SD/−Trp/−Leu/−Ade and SD/−Trp/−Leu/−His media along with β-galactosidase activities are shown in Table 5.

The N-terminal domain of either eEF1Bα or eEF1Bβ is sufficient to sustain the interaction of these two factors with eEF1Bγ, since the strength of the interactions seems to be unchanged or reinforced when compared with the results obtained with the full-length proteins, and seems stronger for the pair eEF1Bβ:eEF1Bγ than for eEF1Bα:eEF1Bγ (compare with the results shown in Table 3). The interaction appears to take place via the N-terminal domain of eEF1Bγ, whereas the C-terminal region is completely lacking this binding capacity. In contrast, no dimerization of the N-terminal domain of eEF1Bβ can be observed. Thus at least part of the C-terminal domain appears to be necessary for dimerization to occur.

DISCUSSION

Proposal of a human eEF1H model

The yeast two-hybrid system as well as a three-hybrid strategy has been used to delineate the composition of the human eEF1H complex and to determine the orientation of the individual subunits within the complex. The following interactions were detected: eEF1A:eEF1Bα, eEF1A:eEF1Bβ, eEF1Bβ:eEF1Bβ, eEF1Bα:eEF1Bγ, eEF1Bβ:eEF1Bγ and eEF1Bα:eEF1Bγ:eEF1Bβ. However, only eEF1A1 was able to interact with the guanine-nucleotide-exchange factors eEF1Bα and eEF1Bβ.

As shown in Table 5, the eEF1A:eEF1Bα interaction could not be mediated by the N-terminal domain of eEF1Bα alone, and must therefore require the C-terminal domain (or parts thereof) as well. This finding is in line with the structure of yeast eEF1A and a functional C-terminal fragment of yeast eEF1Bα [10]. On the basis of this structure, we interpret that domains 1 and 2 of eEF1A are involved in this interaction. The interaction between eEF1A and eEF1Bα is commonly observed (e.g. see [4,14,17]).

Also, the interaction between eEF1A and eEF1Bβ was found to involve the C-terminal domain of eEF1Bβ (or parts thereof), which is strongly homologous with the C-terminal domain of eEF1Bα. This interaction has also been observed in the eEF1H complexes of *Artemia* [4] and the rabbit [14,17].

The interactions involving eEF1A were found to display directionality. This is a common phenomenon in the two-hybrid system, probably caused by steric hindrance [46,48]. Either the binding characteristics of eEF1A or the GAL4-BD may be obscured by the fusion partner.

The existence of a eEF1Bβ:eEF1Bβ dimer has previously been proposed on the basis of the potential of the leucine zipper in the N-terminal region of the factor to facilitate dimerization [14]. In the present study, for the first time, we demonstrate directly that this interaction takes place *in vivo*. However, our experiments reflect that the N-terminal domain of eEF1Bβ alone is not sufficient to sustain this dimerization.

The eEF1Bα:eEF1Bγ:eEF1Bβ complex has previously been reported to exist in rabbit [14] and *Artemia* [4]. However, in contrast with earlier studies on rabbit eEF1H [14,17], we have been able to detect direct binding between eEF1Bγ and eEF1Bβ. In addition, we also detected the eEF1Bα:eEF1Bγ and eEF1Bβ:eEF1Bγ complexes separately, indicating that the binding of eEF1Bβ to eEF1Bγ is independent of the prior binding of eEF1Bα, in contrast with previous suggestions [4]. Furthermore, we demonstrate using a three-hybrid strategy that the ternary complex is formed in human eEF1H, and show that eEF1Bα and eEF1Bβ bind concomitantly and independently via their N-terminal domains to distinct parts of the N-terminal domain of eEF1Bγ. The N-terminal domain of eEF1Bγ contains a sequence similar to that of glutathione S-transferase, which has been proposed to be involved in the regulation of the assembly of multi-subunit complexes [49]. The N-terminal regions of eEF1Bα and eEF1Bγ have been shown previously to interact [7].

The existence of the interactions described above led us to propose the topographical model shown in Figure 1. The model has several similarities with the models on the basis of other studies. However, this new model differs from the existing

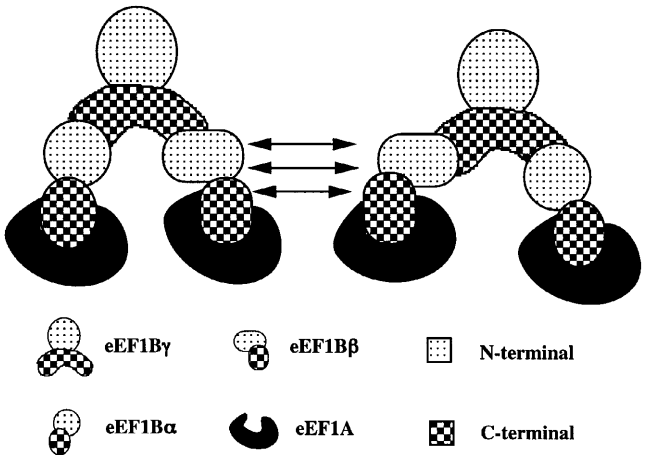

Figure 1 Model of the human eEF1H complex

A minimal complex composed of two molecules of eEF1A and one molecule of each of the eEF1B subunits resulted from our studies. The orientations of the eEF1B subunits with respect to N- and C-terminal domains are indicated using different fill-in patterns. The eEF1H complex holds the potential of dimerizing via the eEF1Bβ subunits, as indicated by the arrows. Neither domain of eEF1Bβ is sufficient for this dimerization to take place.

Table 6 Comparison of the model derived in the present study with existing models

'X' indicates that the interaction of interest has been observed.

Interaction	Present model	Janssen et al. [4]	Bec et al. [14]	Minella et al. [16]	Sheu and Traugh [17]
1A:Bα	X	X	Not included	Not included	X
1A:Bβ	X	X	Not included	Not included	X
Bβ:Bβ	X		X	X (trimer)	X (aggregate)
Bα:Bγ:Bβ	X	X	X	X	
Bα:Bγ_2:Bβ					X
Bα:Bγ	X	X	X	X	X
Bβ:Bγ	X				X
Bγ:Bγ				X (hexamer)	X
Bα:Bα				X (trimer)	X

models in a number of ways. The models are compared in Table 6. The model of the eEF1H complex from *A. salina* is identical with the present model, except for the potential dimerization of eEF1Bβ [4]. In contrast, the model obtained after studies of rabbit eEF1H includes the eEF1Bβ dimerization, whereas no interaction is observed between eEF1Bβ and eEF1A [14]. In the latest model of the eEF1H complex from rabbit on the basis of phosphorylation experiments [17], dimers of the type eEF1Bα:eEF1Bα and eEF1Bγ:eEF1Bγ are proposed. We also tested the possible formation of these dimers using the yeast two-hybrid system, but were unable to identify these.

The differences described above may reflect the use of other sources of elongation factors and/or the use of *in vitro* experiments.

Functional aspects of the model

According to the level of reporter-gene activation, the strengths of the interactions between eEF1Bγ and the two exchange subunits, eEF1Bα and eEF1Bβ, suggest that this part of the complex is fairly stable. In contrast, we observe that the eEF1A molecules interacting with the exchange factors are bound in a more labile manner, allowing the dissociation of eEF1A:GTP, as well as eEF1A:GDP when needed.

The appearance of several binding partners for eEF1Bβ (eEF1A, eEF1Bβ, eEF1Bγ and ValRS [50]) raises the question of whether or not all the potential interactions take place at the same time. We have shown that eEF1Bγ and eEF1Bβ interact via their N-terminal domains, and crystallographic studies reveal that the C-terminal region of eEF1Bα (highly identical with the C-terminal region of eEF1Bβ) binds to eEF1A [10]. This is in agreement with the results obtained after analysis of the eEF1H complex from *A. salina* [4]. In principle, both interactions could occur simultaneously. We have shown that the N-terminal part of eEF1Bβ alone is not capable of sustaining eEF1Bβ dimerization. Thus this dimerization could possibly exclude the binding of either eEF1Bγ or eEF1A. As the eEF1Bβ:eEF1Bγ interaction is by far the more stable of the two, we suggest that it is eEF1A that dissociates, as proposed previously for rabbit eEF1H [14].

The binding of eEF1Bβ to ValRS has been located to the N-terminal region of eEF1Bβ, but has not been clearly mapped [14]. It is questionable whether the very short stretch of amino acids of eEF1Bβ available after eEF1Bγ binding and eEF1Bβ dimerization can account for the binding of ValRS. Thus we suggest that the binding of ValRS to the N-terminal part of eEF1Bβ takes place after the dimerization of eEF1Bβ, thereby enabling more contact surface of the eEF1Bβ protein to become available for binding the N-terminal stretch of the synthetase.

Despite the high conservation in the C-terminal domains of eEF1Bα and eEF1Bβ, it is difficult to imagine that they exert exactly the same effect on eEF1A. However, no differences in their nucleotide-exchange activities have been detected so far, but the effect of eEF1Bγ on the two factors seems to differ [14]. Our findings showing the potential of eEF1Bβ to dimerize and the simultaneous binding of the two exchange factors to different sites on eEF1Bγ indicate that eEF1Bα and eEF1Bβ have different regulatory roles. This is supported by a number of observation made by others. The eEF1Bβ exchange factor is absent in lower eukaryotes, suggesting that this factor may be related to some of the additional roles of eEF1A reported in higher eukaryotes. Also, the differing phosphorylation patterns of the two exchange factors [17,29] lends evidence to the notion that the roles of these two factors are divergent. Another possibility is that the same (or similar) roles are regulated in different manners. Finally, eEF1Bβ seems to be the specific binding or phosphorylation target of various viral proteins, probably with the aim of optimizing the protein synthesis machinery of infected cells [37,38].

Differential binding of the two isoforms of eEF1A to eEF1B

In contrast with eEF1A1, eEF1A2 presented no, or only a very weak, response in the two-hybrid system. Other studies in our laboratory have shown that eEF1A2 in fusion with the GAL4 DNA-binding domain is fully competent in interacting with other eEF1A-binding proteins identified in a yeast two-hybrid screening (F. Mansilla and C. R. Knudsen, unpublished work). Thus the differential binding of the two isoforms of eEF1A to the exchange factors is not due to problems inherent in the two-hybrid set-up. Our results indicate that the eEF1H complex involving eEF1A2 is more unstable than the corresponding complex involving eEF1A1. However, the present study does not allow an evaluation of whether or not this instability makes the eEF1H complex involving eEF1A2 too unstable to function properly in protein synthesis *in vivo*. eEF1A1 binds GTP more strongly than GDP, whereas the opposite is true for eEF1A2

[23]. This implies that eEF1A2 should depend more strongly on the presence of a guanine-nucleotide-exchange factor.

In both rice ([51]; Genbank® accession numbers D23674 and D12821) and *Xenopus laevis* [52], functional isoforms of eEF1Bα have been detected. Also in humans, the existence of multiple chromosomal isoforms of eEF1Bα has been demonstrated [53]. One gene, *EF1β5a*, transcribes a cDNA in a tissue-specific manner, showing high cDNA levels in brain and skeletal muscle only. This expression pattern is in accordance with that of eEF1A2. We suggest that these exchange factor isoforms operate specifically on eEF1A2 or eventually on other as-yet-unknown isoforms of eEF1A.

The amino acid sequences of the two human isoforms of eEF1A are 93% identical. Furthermore, the majority of differences are semi-conservative. We have mapped the distribution of all non-identical residues on the basis of the known structure of yeast eEF1A [10], and clearly all of these cluster on one side of the molecule, whereas the interface that binds the C-terminal fragment of eEF1Bα is at the opposite side. Considering this structural information, the explanation of our two-hybrid system results is challenging in terms of structure, since both human eEF1A isoforms present a very conserved binding interface to eEF1Bα. However, the N-terminal part of eEF1Bα, not present in the crystallographic structure, may also be involved in the interaction with eEF1A. Furthermore, subtle structural divergences caused by the amino acid differences may be transmitted to the opposite site of the molecule.

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