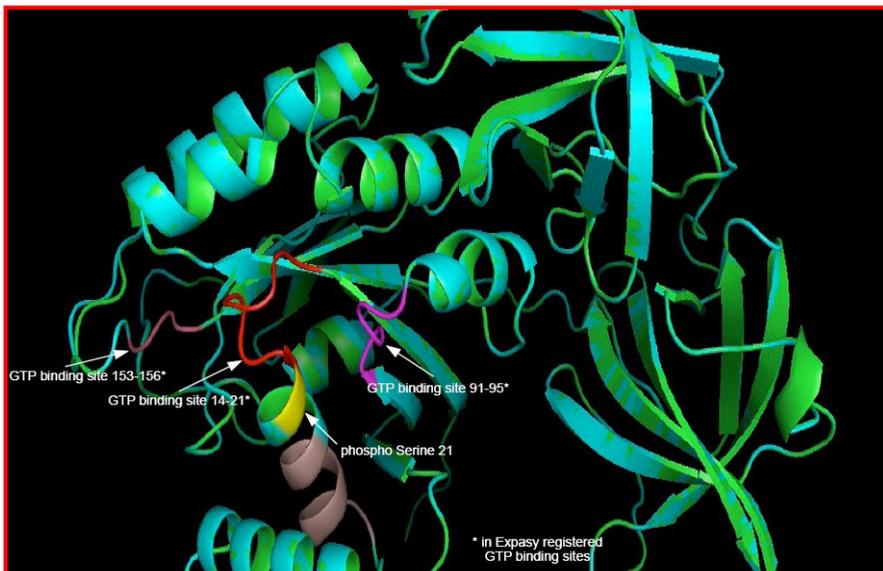




**UNIVERSITA' DI NAPOLI FEDERICO II**

**DOTTORATO DI RICERCA IN BIOCHIMICA  
E BIOLOGIA MOLECOLARE E CELLULARE  
XXII CICLO**

*Regulatory phosphorylations on the eukaryotic elongation  
factor 1A mediated by RAF kinases*



**Dottoranda: Carmen Sanges  
Relatore: Paolo Arcari**



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***REGULATORY PHOSPHORYLATIONS ON THE  
EUKARYOTIC ELONGATION FACTOR 1A MEDIATED BY  
RAF KINASES***

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**Anno Accademico 2008/2009**



## SUMMARY

The elongation factor 1A has a crucial role in protein biosynthesis. EF1A belongs to the GTP-binding family of proteins and is able to deliver the aa-tRNA to the A-site of the ribosome because of its GTPase activity. Higher eukaryotes express two tissue specific isoforms of EF1A. In the last years, these isoforms were found to be involved in several different processes like senescence, apoptosis and transformation. In particular EF1A2 is overexpressed in several tumor cells and participates in drug resistance processes. Previous experiments in H1355 cells demonstrated that IFN $\alpha$  treatment results in an antiapoptotical answer during which EF1A2 is increasingly expressed due to its RAF kinase mediated phosphorylation. As this interaction establishes a new link between the mitogenic cascade and protein biosynthesis, *in vitro* kinase assays were performed in presence of B- and C-RAF kinase and recombinant EF1A1/2 to investigate this important interaction. Mass spectrometry analysis identified treonine 88 (exclusively mediated by B-RAF) and serine 21 on EF1A1 and serine 21 on EF1A2 as B-/ and C-RAF mediated phosphorylation sites. Interestingly, serine 21 belongs to the consensus sequence of the GTP/GDP binding domain of EF1A. Its phosphorylation prevents the binding of the nucleotide suggesting that this potentially RAF mediated modification has a regulatory role affecting the function of the elongation factor. Moreover, the C-RAF mediated phosphorylation on S21 is enhanced when both isoforms were incubated together prior to the kinase/phosphorylation reaction. This suggests that the heterodimerization of the two isoforms results in a

conformational change which increases the accessibility of serine 21 and thus supports the phosphorylation on this residue. Overexpression of EF1A1 in proliferating COS 7 cells and mass spectrometry analysis followed by pull-down of GST-EF1A1 confirmed the phosphorylation of T88 *in vivo*. This indicates that B-RAF is directly implicated in the protein biosynthesis process. Further, generated mutants of EF1A1 (T88A/D and S21A/D) and EF1A2 (S21A/D) were overexpressed in COS 7 cells. In comparison to the wt, the S21A/D mutants were less stable and more rapidly degraded in a proteasome dependent manner. This demonstrates that S21 is an essential site for the activity of EF1A. Taken together, the RAF mediated regulation of this site could be a crucial mechanism involved in the functional switching of EF1A between its role in protein biosynthesis and its participation in several other cellular processes, including drug resistance and transformation.

## RIASSUNTO

Il fattore di allungamento 1A possiede un ruolo primario nella biosintesi delle proteine essendo artefice del corretto posizionamento dell' aa-tRNA sul ribosoma. Questa funzione ed il suo corretto svolgimento sono garantite dall'attività GTPasica dello stesso fattore, il quale infatti appartiene alla famiglia delle GTP binding protein. EF1A negli organismi eucariotici superiori è presente in due isoforme (EF1A1 ed EF1A2) tessuto specifiche e negli ultimi anni è stato riscontrato il loro coinvolgimento in diversi processi cellulari quali senescenza, apoptosi e trasformazione. In particolare l'isoforma 2 risulta overespressa durante diversi tipi di tumore e partecipa a fenomeni di resistenza ai chemioterapici. Infatti, risultati precedenti hanno messo in luce che in un sistema cellulare di carcinoma epidermoide polmonare umano (H1355), il trattamento con  $INF\alpha$  induce una risposta antiapoptotica mediata dalle chinasi RAF ed esplicitata tramite overespressione e fosforilazione maggiormente a carico di EF1A2. In questo lavoro abbiamo cercato di chiarire la natura dell'interazione verificata tra il fattore di allungamento e le RAF chinasi ed il ruolo funzionale di questa interazione *in vivo*. Saggi di fosforilazione *in vitro* in presenza di RAF chinasi (B/C-RAF) e analisi di spettrometria di massa hanno rivelato la presenza di gruppi fosfato su entrambe le isoforme, in particolare i siti coinvolti nella modificazione risultano essere la treonina 88 (mediata esclusivamente da B-RAF) e la serina 21 per EF1A1 e la serina 21 per EF1A2. Da sottolineare che la serina 21 appartiene alla prima sequenza di consenso per il legame al GTP/GDP. Quando entrambe le isoforme sono

preincubate e successivamente saggiate in un saggio di chinasi *in vitro* la fosforilazione ad opera di C-RAF sembra essere favorita suggerendo un eterodimerizzazione di EF1A che consente una maggiore disponibilità dei siti in S21 per entrambe le isoforme. Overespressione di EF1A1 in cellule COS 7 in attiva proliferazione ha confermato la fosforilazione in T88 suggerendo il coinvolgimento diretto della chinasi B-RAF durante il processo di sintesi proteica. Inoltre, overespressione in COS 7 di entrambe le isoforme wt e mutate per i siti di fosforilazione ha rivelato una minore stabilità dei mutanti S21A/D rispetto al wt e ai mutanti T88A/D. I risultati ottenuti suggeriscono che la fosforilazione in serina 21 faccia parte di un sistema regolatorio delle funzioni di EF1A mediato dalle RAF chinasi che potrebbe spiegare lo switch del fattore tra il suo ruolo nella sintesi proteica e quello in altri processi cellulari (legame all'F-actina, organizzazione del citoscheletro, etc.) coinvolti nella tumorigenesi e nella resistenza ai chemioterapici.

# TABLE OF CONTENTS

	<b>Pag.</b>
<b>1 INTRODUCTION</b>	<b>1</b>
<b>1.1 The eukaryotic elongation factor 1A</b>	<b>1</b>
1.1.1 Role in protein biosynthesis: the elongation factor 1 complex	1
1.1.2 Structures and isoforms	5
1.1.3 Regulation of the elongation cycle in protein biosynthesis	9
1.1.4 The eukaryotic elongation factor 1A in different biological process	12
<b>1.2 The mitogenic cascade and RAF kinases</b>	<b>14</b>
<b>1.3 Scientific hypothesis and aim of the work</b>	<b>19</b>
<b>2 MATERIALS AND METHODS</b>	<b>21</b>
<b>2.1 Materials</b>	<b>21</b>
2.1.1 Working with bacteria	21
2.1.2 Working with mammalian cells	24
2.1.3 Electrophoresis and western blot	25
<b>2.2 Methods</b>	<b>28</b>
2.2.1 Purification of His-tagged proteins from <i>E. coli</i>	28
2.2.2 <i>In vitro</i> Kinase assay	29
2.2.3 Mass Spectrometry Measurements	30
2.2.4 Site Directed Mutagenesis	31
2.2.5 Transformation of competent bacteria	32
2.2.6 Isolation of plasmid DNA	33
2.2.7 Transfection and lyses of mammalian cells	33
2.2.8 Pull down of GST- or His-tagged proteins	34
<b>3 RESULTS</b>	<b>35</b>
<b>3.1 Purification of recombinant EF1A1 and EF1A2 from <i>E. Coli</i></b>	<b>35</b>
<b>3.2 B-RAF but not C-RAF phosphorylates EF1A1 and EF1A2 <i>in vitro</i></b>	<b>36</b>

<b>3.3</b>	<b>Reduction of B-RAF activity on EF1A1 mutated in the predicted phosphorylation sites</b>	<b>38</b>
<b>3.4</b>	<b>Phosphorylation of a potential EF1A heterodimer by C-RAF <i>in vitro</i></b>	<b>40</b>
<b>3.5</b>	<b>Identification of phosphorylation sites on EF1A1 and EF1A2 mediated by RAF kinases <i>in vitro</i></b>	<b>41</b>
<b>3.6</b>	<b>Purification of EF1A1 wt and EF1A2 wt expressed in COS 7 cells</b>	<b>42</b>
<b>3.7</b>	<b>Identification of phosphorylation sites and interaction partners of EF1A1 expressed in COS 7 cells</b>	<b>44</b>
<b>3.8</b>	<b>Purification of EF1A1 mutants expressed in COS 7 cells</b>	<b>46</b>
<b>3.9</b>	<b>Stability of the S21 mutants of EF1A1 and EF1A2 <i>in vivo</i></b>	<b>47</b>
<b>3.10</b>	<b>Ubiquitination and stability of EF1A1 wt, EF1A2 wt and their mutants <i>in vivo</i> experiments</b>	<b>48</b>
<b>3.11</b>	<b>Heterodimerization of EF1A1 and EF1A2 <i>in vivo</i></b>	<b>53</b>
<b>4</b>	<b>DISCUSSION</b>	<b>55</b>
<b>4.1</b>	<b>The heterodimerization of EF1A1/2 enhances a RAF isoform specific phosphorylation of EF1A</b>	<b>55</b>
<b>4.2</b>	<b>The importance of the EF1A serine 21 site</b>	<b>57</b>
<b>4.3</b>	<b>Does the RAF mediated phosphorylation of EF1A regulate its ability to bind or bundle actin?</b>	<b>60</b>
<b>5</b>	<b>REFERENCES</b>	<b>65</b>

## FIGURES AND TABLES LIST

<b>Fig. 1.1</b>	Initiation cycle in eukaryots	2
<b>Fig. 1.2</b>	Elongation cycle in eukaryots	4
<b>Fig. 1.3</b>	Solved structures of EF-Tu	5
<b>Fig. 1.4</b>	Multiple sequence alignment of elongation factor 1 from different organisms	8
<b>Fig. 1.5</b>	Phosphorylation sites on eukaryotic elongation factors	11
<b>Fig. 1.6</b>	Functional domains and phosphorylation sites of eEF1A1/2	13
<b>Fig. 1.7</b>	Cancer relevant mutation in B-RAF	14
<b>Fig. 1.8</b>	Schematic representation of the mitogenic cascade	17
<b>Fig. 3.1</b>	EF1A1 His-Tag purification	36
<b>Fig. 3.2</b>	In vitro kinase assay of EF1A1	37
<b>Fig. 3.3</b>	In vitro kinase assay of EF1A2	37
<b>Fig. 3.4</b>	In vitro kinase assay of EF1A1 mutants	39
<b>Fig. 3.5</b>	In vitro kinase assay of EF1A1 wt and double mutant	40
<b>Fig. 3.6</b>	C-RAF DD kinase assay of EF1A1 and EF1A2	41
<b>Fig. 3.7</b>	Purification of EF1A1 wt GST-Tag	43
<b>Fig. 3.8</b>	Interaction partners EF1A1 expressed in COS 7	45
<b>Fig. 3.9</b>	Purification of EF1A1 wt and mutants from COS 7	46
<b>Fig. 3.10</b>	Immuno blotting of EF1A1 and EF1A2 protein expression levels in COS 7	47
<b>Fig. 3.11</b>	Immuno blotting after pull-down of the endogenous ubiquitination of	

	EF1A1, EF1A2 and their mutants	49
<b>Fig. 3.12</b>	Immuno blotting after pull-down of the ubiquitination of EF1A1, EF1A2 and their mutants	50
<b>Fig. 3.13</b>	Immuno blotting after pull-down of the ubiquitination of EF1A1, EF1A2 and their mutants	52
<b>Fig. 3.14</b>	Heterodimerization of EF1A1 EF1A2 in vivo	54
<b>Fig. 4.1</b>	Merge of EF1A1 EF1A2 structures	57
<b>Fig. 4.2</b>	Guanine-binding pocket in yeast and human variants	58
<b>Fig. 4.3</b>	Schematic representation of GDP binding to SsEF1A	59
<b>Fig. 4.4</b>	Co-crystal structure of EF1A-EF1B C terminus-GDP	62
<b>Tab. 1</b>	Identified phosphorylation sites on EF1A1 EF1A2	42
<b>Tab. 2</b>	Phosphorylation on EF1A1 expressed in COS 7	44
<b>Tab. 3</b>	EF1A1 interaction partners identified after GST pull-down	47

## **1 INTRODUCTION**

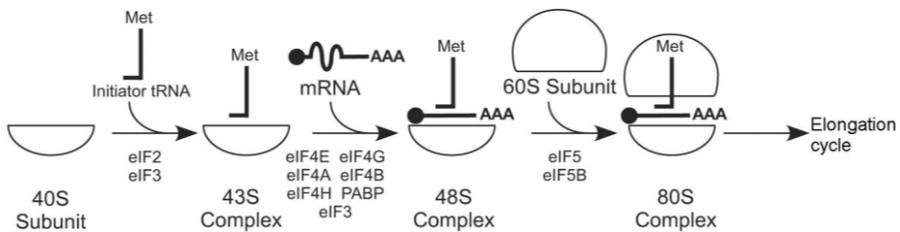
### **1.1 The eukaryotic elongation factor 1A (eEF1A)**

eEF1A is the second most abundant protein in the cell. It belongs to the GTP-binding elongation factor family and promotes the GTP-dependent binding of aminoacyl-tRNA (aa-tRNA) to the A-site of the ribosome during the elongation cycle in protein biosynthesis. Moreover, the GTPase activity of eEF1A is also used to enhance the accuracy of codon recognition. The functions of the elongation factor 1A in the elongation cycle have been extensively investigated in eubacteria, e. g. *E.Coli* (EF Tu) as well as in archaea e.g. *Sulfolobus Sulfataricus* (Krab I.M. *et al.*, 1998; Arcari P. *et al.*, 1994) and eukaryotes, e. g. *Saccharomyces cerevisiae*. These studies showed the high conservation of the elongation factor between the three kingdoms as a well as among a wide range of different species underlining the importance of this protein for the life.

#### **1.1.1 Role in protein biosynthesis: the elongation factor 1 complex**

The protein biosynthesis is a process present in all organisms, prokaryotes and eukaryotes, sharing a similar mechanisms. It starts from the ribosome and involves four different stages: initiation, elongation, termination and recycling (Noble, *et al.*, 2008). All of these steps are tightly controlled by specific translation factors. Many of these proteins are GTPases, which are activated by binding to the ribosome, on a site called the GTPase-activating center (GAC) (Ramakrishnan *et al.*, 2002). In eukaryotes, at the initiation

point, the 40S ribosomal subunit is bound by a ternary complex consisting of the initiation factor 2 (eIF2), GTP and methionyl initiator tRNA (Met-tRNA<sub>i</sub>) to form the 43S complex. The recruitment of mRNA is due to the eIF4 that, together with the poly(A)-binding protein (PABP), recognise the 5'-terminal cap or the 3'-terminal poly(A) tract of mRNA. The mRNA will be transferred to the 43S complex resulting in the 48S complex. Once the first AUG in good sequence context has been found, the initiation factors are replaced by the 60S subunit to form the 80S initiation complex, via GTP hydrolysis on eIF2 mediated by eIF5 (Rhoads *et al.*,2006). Now the complex is ready to receive the first elongator tRNA and to start with the elongation stage of the biosynthesis. In higher eukaryotes the elongation factors work always as a complex, the elongation factor 1 complex, consisting of eEF1A and the three subunit of eEF1B (Sonenberg, *et al.* 2000)

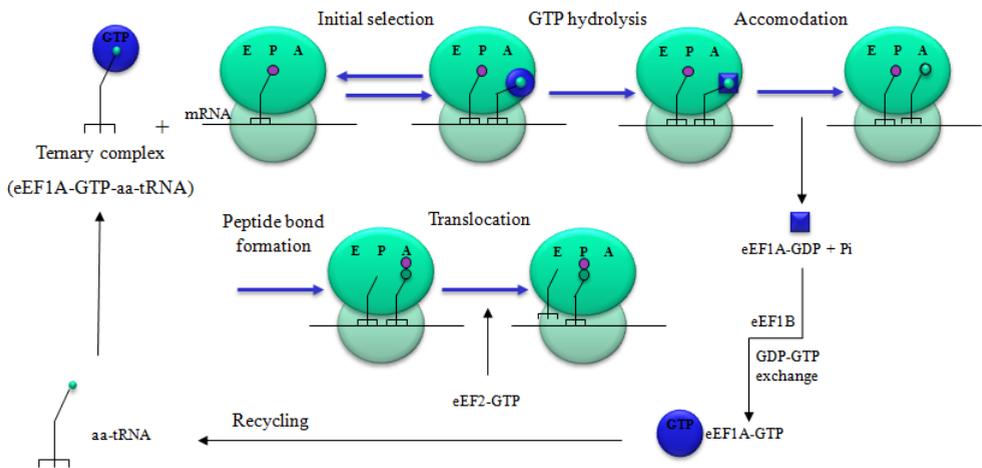


**Fig. 1.1 Initiation cycle in eukaryots :** to complete the initiation cycle, the hydrolysis of the GTP bound to eIF2 is required. released eIF2-GDP is than recycled to eIF2-GTP by the guanine nucleotide exchange eIF2B. Fig.s adapted from *Rhoads R.E. et al.*

The elongation phase of protein synthesis is a cyclic process consisting of basic steps repeated until the entire coding sequence of the mRNA is translated and a termination codon come out in the decoding site. During

elongation, the active form of the elongation factor 1A binds the GTP and transports the new aminoacyl-tRNA (aa-tRNA), as a ternary complex (Fig. 1.2), to the empty A site of the 80S initiation complex (Stark *et al.*, 1997). In particular, eEF1A-GTP protects the aa-tRNA against hydrolysis and assists the ribosome in making a correct interaction between the current codon on the mRNA and the anticodon of the transported aa-tRNA (Pape *et al.*, 1999). Such a decoding event triggers the ribosome to induce GTP hydrolysis on eEF1A (Nilsson, *et al.*, 2005) and leads its major conformational change, which causes the release of aa-tRNA and the accommodation of the 3' end in the PT (peptidyl transferase) centre on the 60S subunit, followed by peptide-bond formation (Nissen, *et al.*, 2000). In parallel, the inactive GDP-bound eEF1A is released from the ribosome. This inactive form of eEF1A, unable to bind another aa-tRNA, is recycled on the active form by the elongation factor 1B (eEF1B). eEF1B consists of three subunits  $\alpha$ ,  $\beta$  and  $\gamma$  and works as a guanine nucleotide-exchange factor (GEF) for eEF1A (Andersen, *et al.*, 2001; Andersen, *et al.* 2003). In the following step, elongation factor 2 (eEF2) catalyzes the translocation of A and P site tRNAs to the P and E sites respectively, as well as movement of the mRNA by exactly one codon to allow a new round of elongation (Rodnina, *et al.*, 1997). A-site-bound aa-tRNA reacts with P-site-bound pept-tRNA (peptidyltRNA) to form a peptide bond, resulting in deacylated tRNA in the P site and pept-tRNA prolonged by one amino acid in the A site.

## Introduction

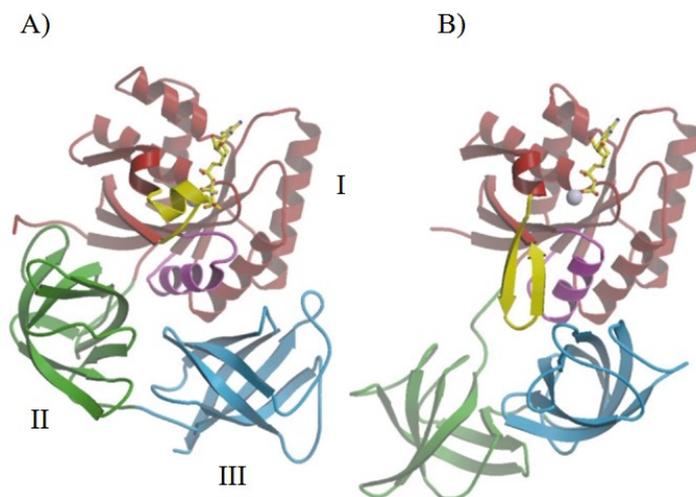


**Fig. 1.2 Elongation cycle in eukaryotes:** crucial step in the elongation stage is the selection of the correct aa-tRNA, based on codon-anticodon interaction. The ribosome supervises this point during an initial selection of the ternary complex and during proofreading of the aa-tRNA. The initial selection utilizes the ability of cognate tRNA to stimulate the GTPase activity of eEF1A much faster than non-cognate and near-cognate tRNA.

When the ribosome come across one of the stop codons, UAA,UAG or UGA, release factor 1 (eRF1) is recruited to the ribosome to promote the release of the newly synthesized polypeptide from the ribosome. After termination, the ribosome dissociates into its constituent subunits and the mRNA and deacylated tRNA are released, allowing the ribosome to be recycled, by release factor 3 (eRF3) in cooperation with eEF2 (Noble, *et al.*, 2008).

### 1.1.2 Structures and isoforms

Thanks to the recent development in macromolecular crystallography and cryo-electron microscopy, huge progress has been made in structure determination of components of the translation machinery, including atomic structures of many translation factors and both subunits of the bacterial ribosome. In particular several structural results have been obtained about the prokaryotic homolog of eEF1A, EF-Tu. Crystal structures of full-length EF-Tu from *Thermus Thermophilus*, *Thermus Aquaticus* and *Escherichia Coli* were solved in complex with the non-hydrolyzable GTP analogue and with bound GDP (Berchtold, *et al.*, 1993; Kjeldgaard, *et al.*, 1993; Polekhina *et al.*, 1996; Abel, *et al.*, 1996).



**Fig. 1.3 Solved structures of EF-Tu:** **A)** Crystal structure of full-length EF-Tu from *T. Aquaticus* in complex with the non-hydrolyzable GTP analogue, **B)** EF-Tu from *E. Coli* in complex with GDP. The three domains of EF-Tu are in red, green and blue, respectively; nucleotides are represented in stick model, whereas Mg<sup>2+</sup> ion as grey ball. In yellow the switch regions.

## *Introduction*

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These structures showed clearly that EF-Tu consists of three domains. The GTP binding domain, domain I or the G domain, has the same basic structure compared to the GTPase domains of other G proteins. Domains II and III are both consisting of  $\beta$  barrels and, in all known structures, are in the same relative orientation, suggesting that they function as a single rigid unit. Moreover, EF-Tu shows a large conformational change upon hydrolysis of GTP to GDP, that causes a substantial rotation of the G domain with respect to domains II and III and a switch of a specific region in the same domain. Co-crystal structure of yeast eEF1A bound with the guanylic nucleotide offer a detailed overview on the guanine binding domain in eukaryotes. This confirms, together with a solved structure of an archeal GDP bound EF1A (REF), a well conserved folding of the protein among different species. Although eEF1A contains additional inserts within the G domain. Interestingly, the consensus sequences of the GTP/GDP binding site are identical for all of them (Fig. 1.4). Structures have also been solved for EF-Tu bound to EF-Ts, facilitating to understand how EF-Ts catalyzes the exchange of GDP to GTP. EF-Ts binds between the G domain and domains I and II and weakens the affinity of EF-Tu for the GDP by altering the GDP-binding pocket. This allows GDP to dissociate and the rebinding of GTP. The structures of prokaryotic EF-Tu and the homologous eukaryotic elongation factor, eEF1A, are very similar, although eEF1A contains additional inserts within the G domain. Therefore it was surprising to find that the GEF for eEF1A, eEF1B $\alpha$ , works by a different mechanism from that of EF-Ts. The crystal structure of eEF1A bound to the catalytic domain of eEF1B $\alpha$  showed that eEF1B $\alpha$  bound to eEF1A in an entirely different manner. E. g. Mg<sup>2+</sup> does not appear to be required for the exchange

mechanism, in contrast to EF-Tu, since the structure of archaeal EF1A indicated that the GDP-bound form of eEF1A no longer coordinates  $Mg^{2+}$  in the nucleotide-binding site, suggesting that eEF1B $\alpha$  induces local conformational changes that cause GDP dissociation. It is also thought that eEF1B $\alpha$  prevents the binding of eEF1A to an aa-tRNA, thus preventing it from forming a ternary complex until GTP is rebound.

In higher vertebrates, eEF1A is found in two variant forms, encoded by distinct genes (Lund *et al.*, 1996), and with different expression patterns. The near-ubiquitous form, eEF1A1, is expressed in all tissues throughout development but is absent in adult muscle and heart (Lee *et al.*, 1992; Chambers *et al.*, 1998). The latter tissues express instead eEF1A2 as do certain other cell types including, notably, large motor neurons, islet cells in the pancreas and enteroendocrine cells in the gut (Newbery *et al.*, 2007). Despite sharing 92% sequence identity (Fig. 1.4), paralogous human eEF1A1 and eEF1A2 have different functional profiles.



They exhibit similar translation activities, but have different relative affinities for GTP and GDP (Kahns *et al.*, 1998). eEF1A1 binds GTP more strongly than GDP, whereas the opposite is the case for eEF1A2. The GDP dissociation rate constant is seven-fold higher for eEF1A1 than for eEF1A2, and the GDP/GTP preference ratio is 0.82 for eEF1A1, but 1.50 for eEF1A2. Surprisingly, since this would predict its greater reliance on GTP-exchange factors, eEF1A2 appears to show little or no affinity for the components of the guanine-nucleotide exchange factor (GEF) complex eEF1B in yeast-two-hybrid experiments (Mansilla *et al.*, 2002). Moreover, from the pair-wise sequence alignment between the two human variants (Fig. 1.4), it is apparent that many of the changes involve substitution of Ser or Thr (total of 11); although no Tyr amino acid residues are lost or gained.

### **1.1.3 Regulation of the elongation cycle in protein biosynthesis**

In the last several years the importance of translational control in cell growth, proliferation, development as well as survival, learning and memory has been underlined by the elucidation of the signalling intermediates involved in protein synthesis. The most important pathways in eukaryotes that control the translation apparatus are the PI3K/Akt/mTOR and the Ras-MAPK signalling cascades (Sonenberg *et al.*, 2007). In particular they phosphorylate the translation factors directly, like mTOR with 4E-BPs and S6 kinase and indirectly eIF4E and eIF4G. The Ras-MAPK pathway executes the phosphorylation of eIF4E and eIF4B. Usually this modification results in increased translation rates and cell growth/proliferation. So far the better understood mechanisms of translational control involved the initiation

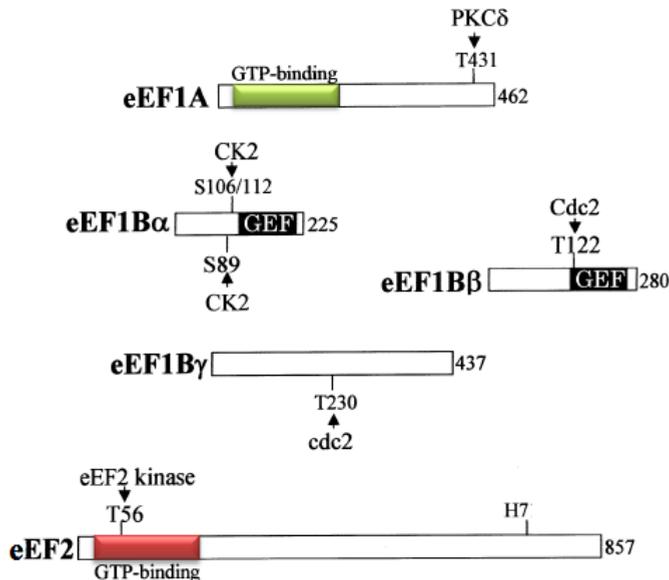
## *Introduction*

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factors (2, 2B, 4B, 4E e 4G), the elongation factor 2 and the ribosomal protein (rp) S6. Less is known about the regulation of eEF1A and eEF1B even though several groups have studied the phosphorylation and regulation of these two factors (Browne *et al.*, 2002). In the following are summarized current knowledges. The casein kinase 2 (CK2) phosphorylates the  $\alpha$  subunit of eEF1B *in vitro* (Sheue *et al.*, 1999) leading to an hypothetical less ability of the complete eEF1B to interacts with eEF1A. Insulin or phorbol esters are able to enhance the phosphorylation of eEF1A, eEF1B $\alpha$  and eEF1B $\beta$  *in vivo* (Venema *et al.*, 1991). From experimental results, which include phosphopeptide mapping data, is possible to suggest the involvement of the protein kinase MS6K (multipotential S6 kinase) for all of them and further the activity of not-identified insulin-stimulated kinases for eEF1A. Anyway, phosphorylation of eEF1A/B *in vitro* by MS6K result in a modest stimulation of its activity (Chang *et al.*,1997). eEF1A and eEF1B are also substrates for phosphorylation by the classical protein kinase C (PKC) isoforms *in vitro* and this may explain the ability of phorbol esters (which activate several PKCs) to increase the phosphorylation of these proteins *in vivo* (Venema *et al.*, 1991). More precisely, PKC  $\delta$  phosphorylates eEF1A at Threonine 431 (based on murine sequence, Kielbassa *et al.*, 1995). Phorbol esters also increase the phosphorylation of the valyl-tRNA synthetase that associates with eEF1A/B. The available evidence suggests that phosphorylation of eEF1A/B and of valyl-tRNA synthetase by PKC increases their activities in translation elongation and amino acylation, respectively. The increased activity of eEF1A/B appears to result from enhanced GEF activity. Lastly, in *Xenopus* oocytes, eEF1B $\gamma$  is phosphorylated during meiotic maturation (Belle *et al.*, 1989). It appears to

## Introduction

be a direct substrate for the protein kinase activity of the maturation-promoting complex MPF (Janssen *et al.*,1991) and the major site of phosphorylation was identified as Thr230, which is conserved residues in mammals. The kinase present in MPF, *cdc2*, also phosphorylates eEF1B $\beta$ , in this case at Thr122 (Mulner-Lorillon *et al.*,1994). Although protein synthesis is increased during maturation, there is so far no evidence that these phosphorylation events actually alter the activity of eEF1A/eEF1B.

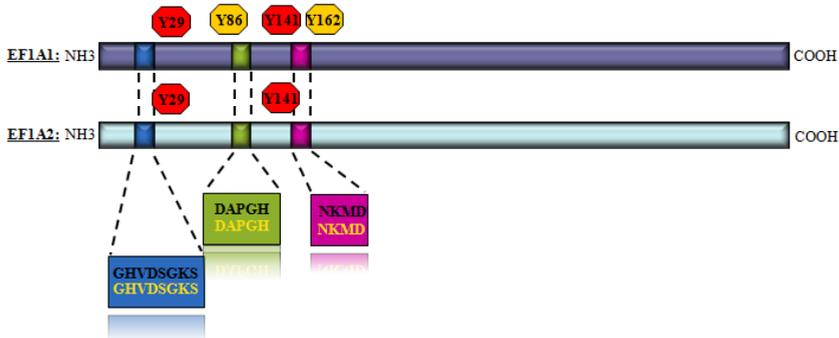


**Fig. 1. 5 Phosphorylation sites on eukaryotic elongation factors.**

The Fig. describe the residues involved as substrate for identified kinases on the elongation factors.

### **1.1.4 The eukaryotic elongation factor 1A in different biological process**

eEF1A1 has been shown to be involved in additional non-canonical functions, including actin-binding and bundling, apoptosis, nuclear transport, proteasomal-mediated degradation of damaged proteins, heat shock and transformation. eEF1A2 has been less extensively studied at the biochemical level, so it is not yet clear how many of these non-canonical functions are shared by this variant. For example, eEF1A2 has been shown to have a role in actin remodeling in cells (Jeghanathan et al., 2008), but has not been shown directly to bind to actin. In humans, eEF1A2 has been shown to have oncogenic properties when inappropriately overexpressed, and has been implicated in ovarian, breast, pancreatic, liver and lung cancer (ref putative oncogene), although the mechanism for overexpression remains elusive, and no mutations have been identified in ovarian tumors (Tomlinson *et al.*, 2007). Loss of expression of eEF1A2, on the other hand, has been shown in mice to result in motor neuron degeneration reminiscent of motor neuron disease, or amyotrophic lateral sclerosis (Chambers et al., 1998). How eEF1A2 isoform is involved in malignant transformation is not as yet understood. Since tyrosine kinases are prominent players in cancer development, direct comparison of the ability of eEF1A1 and eEF1A2 to be involved in phospho Tyr-specific signalling processes could help to interpret cancer-related properties of eEF1A2. Large-scale proteomics studies (Molina, *et al.*, 2007) revealed that conserved Tyr residues (Tyr29, Tyr85, Tyr86, Tyr141, Tyr162, Tyr254) in both human eEF1A variants are phosphorylated (Fig. 1.6).

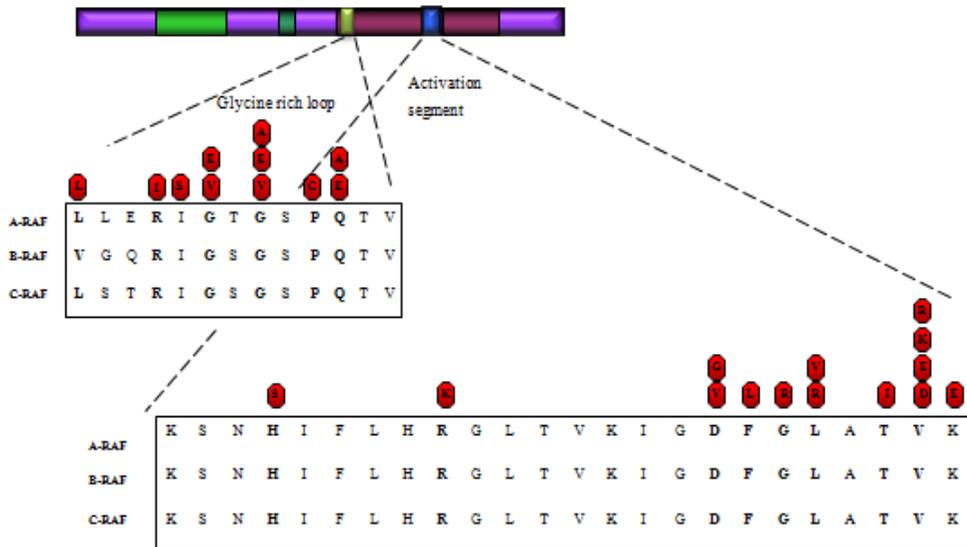


**Fig. 1.6 Functional domains and phosphorylation sites of eEF1A1/2:** eEF1A binds GDP/GTP through three different binding sites (aa 14-21, blue box; aa 91-95, green box; aa 153-156, violet box). As indicated, the consensus sequences of these sites are conserved between the two isoforms (eEF1A1, black; eEF1A2, yellow). Albeit both isoforms share a sequence homology of 98%, only eEF1A1 is phosphorylated on Y86 and Y162 in addition to the conserved phosphorylation sites Y29 and Y141 of eEF1A1 and eEF1A2.

## 1.2 The mitogenic cascade and RAF kinases

The discovery of the transforming activity of the viral oncogene v-RAF (an oncogenic product of the murine sarcoma virus 3611; Rapp et al., 1983) was the basic finding to understand the control of proliferation, cell growth, survival and differentiation via extracellular stimuli (e. g. growth factors). The family of RAF kinases are serine/threonine kinases that exhibit important roles in the regulation of the highly conserved RAS RAS-RAF RAF-MEK MEK-ERK cascade. In mammalian cells three isoforms of RAF kinases exist, called A-RAF, B-RAF and C-RAF. As C-RAF was the first kinase described to induce tumors in prior healthy organisms (Rapp et al., 1983), nowadays B-RAF gained much attention because of its role in cancer progression. This isoform carries a cancer relevant mutation (valin at

position 600 is mutated to glutamate; Fig. 1.7) which is found in several cancer types, e. g. melanoma or colorectal carcinoma (Davies et al., 2002).



**Fig. 1.7: Cancer relevant mutations in B-RAF**

Mutations of B-RAF that are relevant in human cancer are present in the glycin – rich loop and the activation segment (corresponding sequences are aligned to A-/ C-RAF as indicated in boxes).

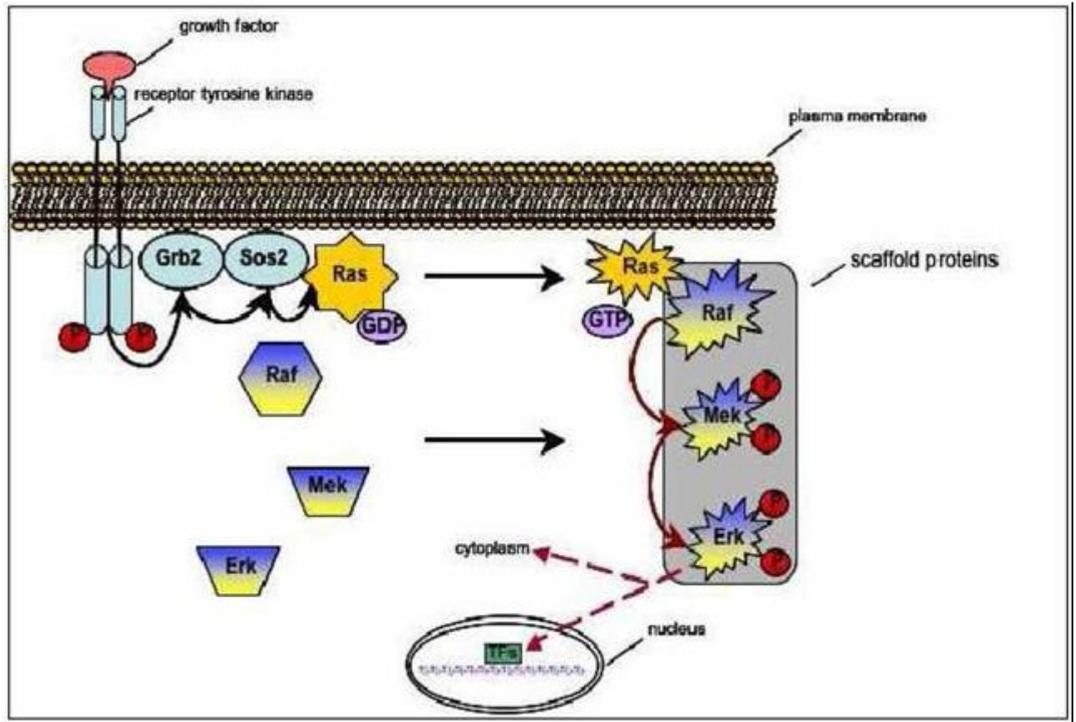
Whereas B- as well as C-RAF play a role in cancer, the relevance of A-RAF in this field is unclear. However, tissue specific expression levels of all three isoforms were found by Storm and co-workers (Storm et al., 1990). Remarkably, B-RAF is mainly expressed on higher levels in the nervous system, whereas the highest C-RAF levels are found in striated muscle, cerebellum and fetal brain. A-RAF is found in high amounts in urogenital organs (Luckett et al., 2000). Independently from their tissue specific expression, all isoforms activate the downstream cascade, but differ in their

individual function. Knocking out RAF proteins in mice, Pritchard and other colleagues showed (Pritchard et al., 1996; Wojnowski et al. 1997; Mikula et al. 2001; Huser et al. 2001), that each RAF isoform has a distinct function in development. A-RAF  $-/-$  embryos are for example viable, but show neurological and intestinal disorders, whereas C- as well as B-RAF embryos die early in development (Pritchard et al., 1996). The latter embryos show vascular defects and die because of vascular haemorrhage. This is accompanied by overall growth retardation and increased apoptosis in endothelial tissues (Wojnowski et al., 1997). C-RAF  $-/-$  embryos were small in size and death was caused by a high apoptosis rate in the liver. Furthermore, these embryos died during midgestation and showed poor development of the placenta and especially of the liver and the haematopoietic compartment (Mikula et al., 2001; Huser et al., 2001). Taken together, these findings indicate, that RAF signalling is crucial for cellular processes like proliferation, development and differentiation, whereas its deregulation can also lead to cell transformation (Leicht et al. 2007). Investigating the functions of the mitogenic cascade, these Ras connected serine/threonine kinases were found to activate further downstream kinases (MEK, ERK), transmitting the signal into the nucleus. As shown in Fig. 1.8, the binding of growth factors activates via an exchange of a guanine nucleotide Ras that binds to the Ras-binding-domain (RBD) of RAF (Wittinghofer et al., 1996). Besides this RBD dependent interaction, RAS also binds to the cysteine rich domain (CRD) of RAF (Hancock et al., 2003). This, from the GTP status of RAS independent process, recruits RAF to the plasma membrane. Although this interaction does not boost the kinase activity, it initializes the activation of RAF.

## *Introduction*

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Activated RAF phosphorylates its downstream target MEK (MEK 1: S217/S221) further activating ERK on threonine and tyrosine residues (ERK 1: T202/Y204). Finally, activated ERK is able to mediate this extracellular response and targets multiple cytosolic, but also nuclear effector molecules. ERK can shuttle inside the nucleus, where it phosphorylates and regulates several transcription factors, particularly myc, Elk1, p53, Ets1/2 and the AP-1 complex that comprises various heterodimers of c-fos and c-jun family members (Treisman, 1996; Adhikary and Eilers, 2007; Gille et al., 1992; Milne et al., 1994; Yang et al., 1996; Buchwalter et al. 2004; Balmano and Cook, 1999; Shaulian and Karin, 2002). The biological consequences of this mitogenic signalling cascade depend on signal intensity as well as signal duration. As shown in fibroblasts, induction of proliferation with platelet-derived-growth-factor (PDGF) depends on continuing ERK activity, whereas strong RAF signalling results in a cell cycle arrest due to the transcription of cell cycle inhibitors such as p21 (Woods et al., 1997; Kerkhoff et al., 1998).



**Fig. 1.8: Schematic representation of the mitogenic cascade**

Growth factor binding to the receptor tyrosine kinase results in the dimerization of the receptor, activating the Grb2/Sos2 complex further activating Ras via GDP/GTP exchange. RAS mediated membrane recruitment of RAF activates this kinase. Active RAF phosphorylates its downstream kinase MEK that further phosphorylates ERK. Downstream substrates of ERK are either cytoplasmatic or nuclear. The stabilization and coordination of the cascade is supported by scaffolding proteins. These scaffolding proteins directly regulate the efficiency of the activation process as well as the maintenance of the signal.

### **1.3 Scientific hypothesis and aim of the work**

*Interferes a potential direct interaction between RAF kinase(s) and the elongation factor 1A with the regulation of protein biosynthesis?*

Protein biosynthesis is a fundamental process in the evolution and maintenance of life. Considering the huge biodiversity on our planet, it is intriguing that molecules participating in this process are found to be conserved from some of the earliest single cell organisms, e. g. *Archeobacteria* to the most recent species - humans. Regarding the development of a differentiated multicellular organism, cellular proliferation is an essential step that requires proteinbiosynthesis to fullfill the need for elevated levels of macromolecules. A recent finding connects both of these necessary events via two molecules: the proliferation as well as other cell process affecting incidences (e. g. transformation) regulating kinase RAF and the elongation factor 1A that plays a central role in the translational elongation as well as several other processes (e. g. apoptosis) (Lamberti *et al.*, 2007). Until today, it is not entirely clear, which RAF isoforms (A-, B-, or C - RAF) can directly interact with EF1A and how this potential RAF mediated phosphorylation of EF1A affects the elongation factor itself. Keeping in mind, that both proteins are overexpressed (mutated) in several kinds of cancer, a potential direct interaction that affects the protein biosynthesis rate and subsequently accelerates cellular proliferation is plausible. First aim of this work was to identify RAF isofom(s) that directly phosphorylate EF1A. The identification of residues on EF1A, that are targeted by RAF, further allows to characterise the subsequent outcome of

## *Introduction*

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this direct interaction on molecular level. Studying the underlying mechanism of a potential RAF mediated regulation of EF1A also links the activity of one of the most cancer relevant proteins to a fundamental process in the cell. This connection could shed light on a so far unknown interaction between two of the most important events during the life of (multi-) cellular organisms as well as cancer - cellular proliferation and protein biosynthesis.



## 2 MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Working with bacteria

##### Instruments:

Bacterial incubator (Heraeus B 6200), Bacterial shaker (New Brunswick Scientific innova 4330), Centrifuge RC 5B plus (Sorvall), Thermocycler (PeqLab), Thermomixer (Eppendorf), NanoDrop (Thermo).

##### Chemicals:

Ampicillin (SIGMA), Bacto-Agar (DIFCO), Bacto-tryptone (Roth), Sodium Chloride (AppliChem), Yeast extract (DIFCO), IPTG, Isopropyl  $\beta$ -D-thiogalactoside (SIGMA), Ni-IDA (Macherey&Nagel), PMSF (Fluka). ATP, ADP (SIGMA), [ $\gamma$ ]<sup>32</sup>ATP (Hartmann Analytics), Magnesiumchloride (AppliChem).

##### Enzymes and Oligonucleotides:

Polymerase (NEB), Pfu polymerase (Stratagene), T4 Ligase (Fermentas), Oligonucleotides (Metabion), dNTPs (Fermentas).

##### Constructs:

pET30a-EF1A1-(His)<sub>5</sub> (MSZ institute)

Pet22-EF1A2-(His)<sub>6</sub> (Lamberti *et al.*)

pcDNA3.1-(His)<sub>6</sub>-EF1A1 (this work)

pcDNA3.1-(His)<sub>6</sub>-EF1A2 (Knudsen *et al.*)

pEBG-GST-EF1A1(this work)

pEBG-GST-EF1A1 T88A/D (this work)

pEBG-GST-EF1A1 S21 A/D (this work)

pcDNA3.1-(His)<sub>6</sub>-EF1A1 T88A/D (this work)

pcDNA3.1-(His)<sub>6</sub>-EF1A1 S21A/D (this work)

pcDNA3.1-(His)<sub>6</sub>-EF1A2 S21A/D (this work)

Primers:

EF1A1 pcDNA3.1-(His)<sub>6</sub> for

5`-tag ctt ggt acc gag atg gga tcc gaa aag act-3`

EF1A1 pcDNA3.1-(His)<sub>6</sub> rev

5`-agt ctt ttc gga tcc cat ctc ggt acc aag cta-3`

EF1A1 pEBG for

5`-aaa aaa gga tcc atg gga aag gaa aag act cat atc aac att gtc-3`

EF1A1 pEBG rev

5`-aaa aaa tet aga tea ttt agc ctt ctg agc ttt ctg ggc aga ctt -3`

EF1A1 S21A for

5`-gta gat tcg ggc aag gcg acc act act ggc cat-3`

EF1A1 S21A rev

5`-atg gcc agt agt ggt cgc ctt gcc cga atc tac-3`

EF1A1 S21D for

5`-gta gat tcg ggc aag gat acc act act ggc cat-3`

EF1A1 S21D rev

5`-atg gcc agt agt ggt atc ctt gcc cga atc tac-3`

EF1A1 T88A for

5`-agc aag tac tat gtg gcg atc att gat gcc cca-3`

EF1A1 T88A rev

5`-tgg ggc atc aat gat cgc cac ata gta ctt gct-3`

EF1A1 T88D for

5`-agc aag tac tat gtg gat atc att gat gcc cca-3`

EF1A1 T88D rev

5`-tgg ggc atc aat gat atc cac ata gta ctt gct-3`

EF1A2 S21A for

5`-gtg gac tcc gga aag gcg acc acc acg ggc cac-3`

EF1A2 S21A rev

5`-gtg gcc cgt ggt ggt cgc ctt tcc gga gtc cac-3`

EF1A2 S21D for

5`-gtg gac tcc gga aag gat acc acc acg ggc cac-3`

EF1A2 S21D rev

5`-gtg gcc cgt ggt ggt atc ctt tcc gga gtc cac-3`

### Kits:

BCA-protein assay kit (Pierce), MiniPrepKit (Metabion/PeqLab), QIAGEN Plasmid Kit (Maxi) (QIAGEN), QIAquick Gel Extraction kit (QIAGEN)

### Buffers:

#### **LB (Luria-Bertani) medium**

10g/L Bacto-tryptone

10g/L NaCl

5g/L yeast extract

Adjust pH to 7.5 with NaOH

For plates, add 15 g Bacto-agar per liter and ampicillin

### **LEW buffer**

50mM NaH<sub>2</sub>PO<sub>4</sub>

300mM NaCl

pH 8,0

### Bacterial strains:

For overexpression of recombinant proteins in *E. coli*, different expression optimized strains were used: BL21C43 and Rosetta. Cloning as well as SD mutagenesis included the transformation of the cloning optimized *E. coli* DH5a strain.

## **2.1.2 Working with mammalian cells**

### Instruments:

Cell culture hood (Heraeus Instrument), Cell culture incubator (Heraeus Instrument), Light microscope (Leica), Megafuge 1.0 R (Heraeus), Mini centrifuge refrigerated (Eppendorf), Water bath (Kötterman)

### Chemicals and cell culture compounds:

10xPBS (Fluka), DMEM (Gibco), L-Glutamin (Gibco), JetPEI (Polyplus-transfection), MG132 (Calbiochem), Penicillin/Streptomycin (Gibco), Trypsin EDTA (Invitrogen), Ni-NTA agarose (QIAGEN), Glutathion-sepharose (Amersham), NP40 (SIGMA), Orthovanadat (SIGMA), Tris-(hydroxymethyl)-aminomethane (Tris) (AppliChem),  $\beta$ -glycerophosphate (SIGMA), Dithiothreitol (DTT) (Roth), Triton-X100 (SIGMA), Imidazole (SIGMA).

Buffers:

**Ni-NTA buffer**

100mM NaH<sub>2</sub>PO<sub>4</sub>

10mM Tris-Cl

pH 8,0

**Glutathion sepharose buffer:**

20mM Hepes

150mM NaCl

50mM Tris

0,1% NP-40

pH 7,5

For washing of glutathion sepharose beads, NaCl was increased to 250mM

Cell line:

The cell line used in this study was cultured in Dulbeccos Modified Eagle Medium (DMEM) with 10% FCS , 2 mM L-glutamine, and antibiotics (penicillin-streptomycin). All works using cell culture methods were performed under a sterile bench. Cell line used was the following: COS 7 cells (African green monkey kidney).

### **2.1.3 Electrophoresis and Western blotting**

Instruments:

Developing machine (AGFA), Electrophoresis power supply (Bio-Rad)  
Electrophoresis unit Bio-Rad Mini-Protean II, pH meter (WTW pH525),  
Spectrophotometer Ultraspec 3000 (Pharmacia Biotech)

Chemicals:

Acrylamide (30%)/Bisacrylamide (0.8%), 37,5:1 (Roth), SDS (Fluka), TEMED (SIGMA), Ammonium peroxydisulfate (APS) (SIGMA), Glycine (Roth), Protein ladder (Fermentas), Bromphenolblue (SIGMA), Ponceau S (SIGMA), , Tween20 (SIGMA), Whatman 3MM Paper (Schleicher & Schüll), PROTRAN nitrocellulose membrane (Schleicher & Schüll), Milk powder (AppliChem), X-ray film Super RX (Fuji), Agarose ultra pure (Roth), Ethidiumbromide (SIGMA), 10xTBE (tris-borate-EDTA) (AppliChem), 1 kb DNA ladder (Fermentas)

Antibodies:

mouse  $\alpha$ -EF1A (Upstate), mouse  $\alpha$ -HA (Santa Cruz), rabbit  $\alpha$ -GST (Santa Cruz), mouse  $\alpha$ -Ubiquitin (Santa Cruz), mouse  $\alpha$ -actin (Santa Cruz) mouse  $\alpha$ -penta-His (QIAGEN).

Kits:

ECL detection kit (Amersham), ECL SuperSignal (West Pico kit Pierce)

Buffers:

**10xSDS-PAGE running buffer:**

0,025M Tris

0,25M Glycine

1,0% SDS

pH of 10x is 8,8 (1x buffer has pH 8,3)

**SDS-PAGE stacking-gel buffer:**

250mM Tris-HCl

0,2% SDS

pH 6,8

**SDS-PAGE separating-gel buffer:**

750mM Tris-HCl

0,2% SDS

pH 8,8

**5xLämmli sample buffer:**

31mM Tris-HCl, pH6.8

1% SDS

5 % Glycerin

2.5 %  $\beta$ -Mercaptoethanol

0.05 % Bromphenolblue

**1xBlotting buffer:**

25mM Tris

192mM Glycine

15% Methanol

**10xTBST:**

500mM Tris-HCl, pH 7,4

1,5M NaCl

0,05% Tween20

## **2.2 METHODS**

### **2.2.1 Purification of His-tagged proteins from *E. coli***

Recombinant proteins were overexpressed in 500 ml of appropriate *E. coli* strains (*Rosetta*, *BL21C43*) using IPTG . To prevent the formation of inclusion bodies during the expression of recombinant EF1A1 and EF1A2, the overexpression was induced at 15° C overnight and 120 rpm in a shaker. On the next day, the cells were centrifuged at 4000 rpm and 4°C for 20 minutes. The supernatant was discarded and the resulting debris pellet was resuspended in 10 ml LEW buffer containing freshly added protease inhibitors (PMSF, leupeptin, aprotinin, benzamidin) and Dnase (10U/μl). After 30 min incubation at 4°C the cells were sonicated 45 sec with bursts of 1 sec. The lysed cells were centrifuged at 15000 rpm at 4°C for 30 min. The protein containing supernatant was added to the equilibrated (with 2 ml of the corresponding LEW buffer) Protino Ni-IDA column. After draining, the column was washed two times with 2 ml washing buffer (containing 10 mmol, 20 mmol immidazol, respectively). The bound recombinant protein was finally eluted 4 times with 1,5 ml elution buffer (containing 50 mmol, 100 mmol, 180 mmol, 200 mmol immidazol). The samples were mixed with glycerol and frozen at -20° C for further investigations. Successful purifications were analyzed by Comassie blue staining and/or immunoblotting.

### **2.2.2 *In vitro* Kinase assay**

In general, kinases bind and hydrolyse ATP into ADP + P. The transfer of the free phosphate group to their substrate protein results in a conformational change of these proteins. This phosphorylation reaction is restricted to exoponated amino acids on the substrate protein. In the case of tyrosine kinases a tyrosine whereas in the case of a serine/threonine kinase a serine and/or a threonine on the target protein is phosphorylated. The interaction depends on the ratio between kinase and substrate and potentially other participating proteins (e. g. scaffolding proteins) or post-translational modifications (e. g. ubiquitination etc.). An *in vitro* kinase assay renders the above described biochemical kinase/substrate interaction. To test the activity of kinases as well as of substrates, recombinant proteins, purified from *SF9* cells or *E. coli*, were used. Depending on the kinase and the substrate, recombinant proteins were mixed in a 1:3 to 1:5 ratio ensuring a substrate excess. Further components were added as indicated below.

#### **Component Volume in $\mu$ l Final Concentration**

Kinase Variable Approx. 300 ng  
Substrate Variable Approx. 900 – 1500 ng  
10 x Kinase Buffer 3 1 x  
ATP\* 3-100  $\mu$ mol  
DTT 1 1 mM  
MgCl<sub>2</sub> 1-10 mM  
ddH<sub>2</sub>O ad final volume 30  $\mu$ l

\*In the case of radioactive labelling, 5 - 10  $\mu\text{Ci}$  [ $\gamma^{32}\text{P}$ ] ATP, corresponding to 0,5 – 1,0  $\mu\text{l}$ , are added. The mixture was incubated at 37°C and 600 rpm for 1 h on a thermomixer. The reaction was stopped by addition of 4 x Laemmli buffer to a final 1 x concentration and analyzed by immunoblotting with phosphospecific antibodies or, in case of radioactive labelling, preposition of the nitrocellulose membrane to an X-ray film at -80° C overnight.

### **2.2.3 Mass Spectrometry Measurements**

Samples were separated by SDS-PAGE using NuPAGE Novex 4-12% Bis-Tris gels (MOPS buffer system). Gels were subjected to silver staining, and the respective bands were excised and washed according to Shevchenko *et al.*. Briefly, gel pieces were washed 3 times alternately with 50  $\mu\text{l}$  of 50 mM  $\text{NH}_4\text{HCO}_3$  and 25 mM  $\text{NH}_4\text{HCO}_3$  in 50% acetonitrile. Subsequently, the gel slices were dried in a vacuum centrifuge. 5  $\mu\text{l}$  of trypsin solution (12.5 ng/ $\mu\text{l}$  in 50 mM ammonium bicarbonate) were added to each gel piece and incubated at 37 °C overnight for in-gel digestion. The obtained peptides were eluted with 20  $\mu\text{l}$  of 5% formic acid and subjected to nano-liquid chromatography-MS/MS analysis. Thereby, an Agilent 1100 nano-HPLC system (Agilent Technologies GmbH, Boeblingen, Germany) was used. The samples were preconcentrated on a 100- $\mu\text{m}$  inner diameter, 2-cm C18 column (nanoseparations, Nieuwkoop, The Netherlands) using 0.05% trifluoroacetic acid with a flow rate of 8  $\mu\text{l}/\text{min}$ . The peptides were then separated on a 75- $\mu\text{m}$  inner diameter, 15 cm, ZorbaxSB300-C18-column (flow rate 300  $\mu\text{l}/\text{min}$ ; Agilent Technologies GmbH, Boeblingen, Germany)

using a 2-h binary gradient from 5 to 50% solvent B (solvent A: 0.2% formic acid; solvent B: 0.2% formic acid, 84% acetonitrile). The nano-HPLC was directly coupled to a QTOF-mass spectrometer (QStar XL, Applied Biosystems GmbH, Darmstadt, Germany) acquiring repeatedly one full-MS and two tandem-MS spectra of the most intensive ions in the respective full MS scan. The tandem-MS spectra were searched against the NCBI nr data base using the Spectrum Mill software (Rev A.03.03.080 SR1; Agilent Technologies GmbH, Boeblingen, Germany) using the following adjustments: taxonomy (*Homo sapiens*), trypsin as protease, one missed cleavage site, oxidation of methionine, phosphorylation of serine, threonine and tyrosine, pyroglutamic acid for N-terminal Gln as variable modifications, 0.2-Da tolerance for MS and MS/MS signals, and only doubly and triply charged ions.

#### **2.2.4 Site Directed Mutagenesis**

Specific mutations at a single nucleotide in a DNA sequence are introduced via site directed mutagenesis. For this PCR reaction each oligonucleotide (forward and reverse) consists of in total 11 base triplets. 10 of those base triplets are complementary to the parental strand, whereas the sixth base triplet contains the point mutation which should be introduced into the vector. The amplification of the complete insert containing vector is carried out by a *Pfu* polymerase (stratagene, metabion, respectively) with a 3'-5' exonuclease activity (proof reading activity) providing a low error rate. Following amplification, the SD mutagenesis product is treated with the *Dpn I* endonuclease (1 hour at 37°C plus inactivation for 30 minutes at

80°C) specifically digesting the methylated parental DNA strand. Thus, the final product theoretically consists mainly of daughter strands that contains the desired mutation. SD mutagenesis was performed according to the following PCR program:

**Standard PCR program for amplification of DNA**

**Step Temperature Time Cycle(s)**

Denaturing 95°C 3 min 1

Denaturing 95°C 0,5 min

Annealing TM-3-5°C 0,5 min

Elongation 72°C variable min\*

Completion 72°C 10 min 1

Storage 4°C hold ∞

\*The duration of the elongation cycle depends on the total length of the vector and was calculated based on the processivity of the average Pfu polymerase (2 min per 1000 bp)

**2.2.5 Transformation of competent bacteria**

Thaw frozen bacteria on ice and add at least 0,5 µg/µl DNA to 100 µl competent bacteria. Gently mix the solution and place it on ice for 30 min. Incubate the cells at 42°C for 50 sec and place them on ice for 30 min. Add 1 ml room temperature LB media and incubate the bacteria at 37°C and 1000 rpm on a mixer. Centrifuge the cells at 11.000 rpm, invert the tube to discard the supernatant and resuspend the cells in 100 µl LB-media. Plate

the cell suspension on a LB agarplate (containing antibiotics for selection) and incubate at 37° C overnight. The selected colonies are transformed with DNA according to the encoded resistance on the plasmid.

### **2.2.6 Isolation of plasmid DNA**

For the isolation of plasmid DNA 2 ml of a 5 ml overnight culture is used. The plasmid is prepared according to the manufacturer's instructions of the Miniprep Kit from met@bion or PeqLab based on the alkaline lysis of bacterial cells (Birnboim and Doly, 1979). For the transfection of mammalian cells, DNA is prepared from 200 ml of overnight cultured bacteria according to the manufacturer's protocol of the Maxi Prep Kit (QIAGEN). In general, contaminating proteins, lipids and chromosomal DNA are precipitated, whereas the aqueous plasmid DNA is bound on a silica matrix in the presence of high salt.

### **2.2.7 Transfection and lyses of mammalian cells**

For overexpression of proteins in mammalian cells, cells are transfected with JetPEI solution according to the manufacturer's protocol. In general, transfected cells are harvested 48 h after transfection by cell scraper in 1xPBS. Harvested cells are washed two times with 1ml 1xPBS (centrifuge each time at 2000 rpm for 2 min). After the final centrifugation step, the cell pellet is either lysed immediately or frozen at -80° C.

For lysis, cells are resuspended in lyses buffer (1ml/2x10<sup>6</sup> cells) containing freshly added protease inhibitors and rotated at 4°C for 30 min. Afterwards

cell debris is pelleted by centrifugation at 13.200 rpm and 4°C for 30 min. The protein containing supernatant is transferred into a new reaction tube for following experiments or frozen at -20°C.

### **2.2.8 Pull down of GST - or His – tagged Proteins**

Depending on the tag of the recombinant protein, a glutathion - / Ni - matrix based isolation of in mammalian cells overexpressed tagged proteins has been performed. For this purpose, the cells are lysed as described above. According to the manual, protein amounts were quantified with protein detection kit. Equal protein amounts were bound on pre-equilibrated beads and the desired protein is bound to the matrix via incubation of 2 hours at room temperature (RT) or overnight (ON) at 4°C . The bound protein is washed 2 times (centrifugation for 2 min at 2000 rpm and 4° C) in 1 ml of an appropriate buffer by inverting the reaction 6 – 8 times. For the final washing step (centrifugaton for 2 min at 2000 rpm and 4° C), the buffer contains 20 mM freshly added imimidazole or, in case of glutathion sepharose up to 250 mM NaCl, reducing to the matrix unspecifically bound proteins. 100 µl of the appropriate elution buffer as well as 50 µl 4 x Laemmli buffer are added to the beads and, in the last case, the mixture is heated for 5 min at 100° C.

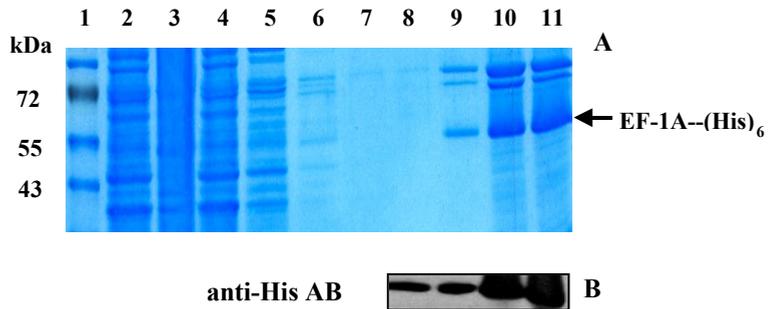
### **3. RESULTS**

Previous experiments showed *in vivo* an interaction between the elongation factor 1A and C-Raf kinase during a survival response, mediated by the RAS/Erk pathway, and induced by the EGF-R during treatment of the human epidermoid cancer cell line H1355 with IFN $\alpha$ . In particular this interaction resulted in a phosphorylation of EF1A that seems to be involved in the upregulation of its protein levels.

#### **3.1 Purification of recombinant EF1A1 and EF1A2 from *E.Coli***

In order to identify the sites on EF1A that are involved in RAF kinase mediated phosphorylation, both EF1A isoforms were expressed and purified from *E. Coli* according to the procedure described in the methods section. After the cell lysis, the protein purification was performed using a Ni-IDA matrix and the fractions were analyzed by SDS-PAGE. As reported in Fig. 3.1 A, the elution profile revealed that fraction 2 was, in all preparations, the most suitable for the following phosphorylation experiments. The presence of EF1A was also confirmed by immunoblotting with antibody anti-EF1A (Fig. 3.1 B). Data related to the purification of EF1A2 are not shown.

## Results



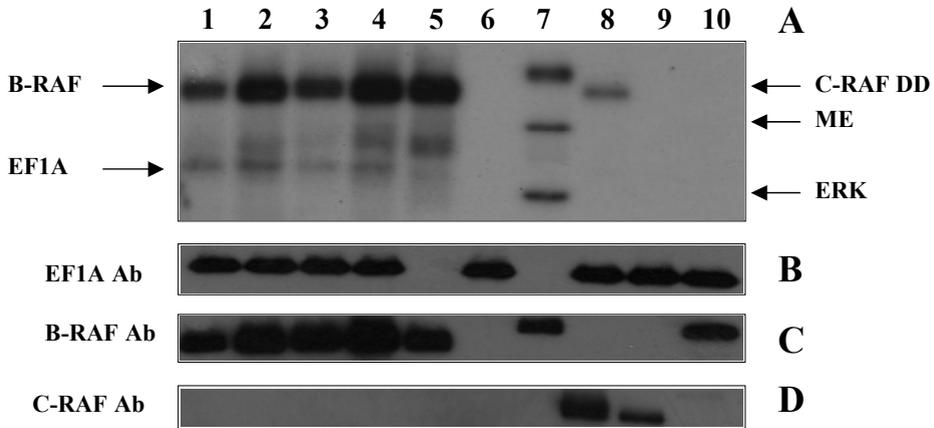
**Fig. 3.1 EF1A1 His-Tag purification.** A) Coomassie staining and B) Western blotting analysis with Ab anti-His of the elution fractions. Lanes: 1, size marker; 2, lysate; 3, pellet; 4, supernatant; 5, 6, 7, washes; 8, 9, 10, 11, elution fractions

### 3.2 B-RAF but not C-RAF phosphorylates EF1A1 and EF1A2 *in vitro*

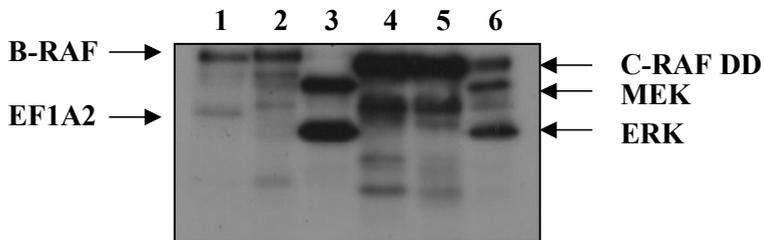
Purified recombinant EF1A1 and EF1A2 from *E. Coli* were used to perform *in vitro* kinase assays in presence of [ $\gamma$ -<sup>32</sup>P]ATP and recombinant B-RAF (B-RAF wt or constitutively active B-RAF mutant V600E\*) as well as C-RAF (constitutively active C-RAF DD) purified from Sf9 cells. C-RAF K75D and B-RAF K75D\*\* were used as negative controls. After the phosphorylation reaction, samples were separated by SDS-PAGE and transferred on a nitrocellulose membrane that was exposed to a X-ray film at -80°C over night. The autoradiography (Fig. 3.2) showed that B-RAF wt and the constitutively active B-RAF mutant V600E were able to phosphorylate both, EF1A1 and EF1A2 (Fig. 3.3) *in vitro*. C-RAF instead, did not show any phosphorylation activity on both EF1A isoforms.

\*Constitutively active B-RAF mutant V600E is a more active form of the kinase in which the exchange of valine against glutamate create a negative charge that mimics the phosphate group

\*\* C-RAF K75D and B-RAF K75D are kinases with a negatively modified amino acid in the ATP binding pocket



**Fig. 3.2** *In vitro* kinase assay of EF1A1. A) Autoradiography. B), C) and D) Immunoblotting against antibody anti EF1A, B-RAF and C-RAF, respectively. EF1A1 wt was incubated in presence of B-RAF wt, B-RAF V600E and C-RAF DD, respectively. Lanes: 1, B-RAF wt (diluted 1:5) + EF-1A1 wt; 2, B-RAF wt + EF-1A wt; 3, B-RAF V600E (diluted 1:5) + EF-1A1 wt; 4, B-RAF V600E + EF-1A1 wt; 5, B-RAF wt; 6, EF-1A1 wt; 7, B-RAF wt + MEK + ERK; 8, C-RAF DD + EF-1A1 wt; 9, C-RAF K75W + EF-1A1 wt; 10, B-RAF K75W + EF1A1 wt.



**Fig. 3.3** *In vitro* kinase assay of EF1A2. EF1A2 wt was incubated in presence of B-RAF wt and C-Raf DD, respectively. Lanes: 1, B-RAF wt + EF-1A2 wt; 2, B-RAF wt; 3, B-RAF wt + MEK + ERK; 4, C-RAF DD + EF-1A2 wt; 5, C-RAF DD; 6, C-RAF DD + MEK + ERK.

### 3.3 Reduction of B-RAF activity on EF1A1 mutated in the predicted phosphorylation sites

Combining *in silico* molecular modelling and molecular dynamic simulation, putative EF1A serines and threonines were predicted as phosphorylation sites (Lamberti *et al.*, 2007).

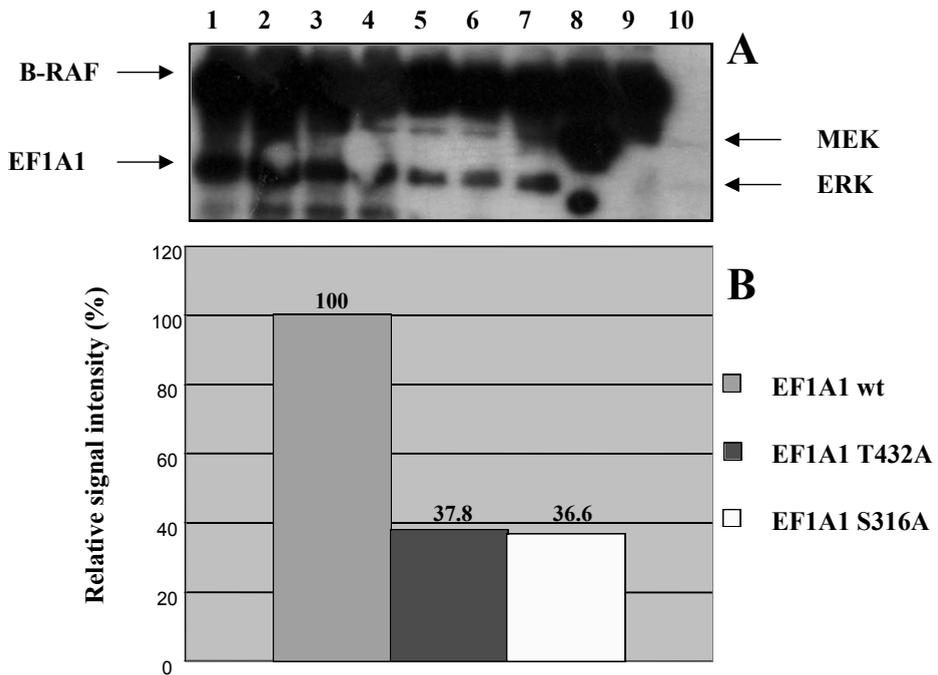
Based on this analysis and considering the sites with the highest probability, mutants of EF1A1 (S18A, S157A, T242A, S316A, S383A and T432A) were generated in order to verify their involvement in the RAF kinases mediated phosphorylation. To this purpose, an *in vitro* kinase assay, as above described, was performed. After exposure of the membrane to the X-Ray film, it was found that, with respect to EF1A wt, the B-RAF mediated phosphorylation of EF1A1 appeared to be reduced only for the EF1A1 single mutant T432A and S316A (Fig. 3.4 A). Densitometric evaluation of band intensity revealed a reduction of 62.2 and 63.4 % for the T432A mutant and the S316A mutant, respectively (Fig. 3.4 B).

To assess if the contemporary presence of both mutations would have further reduced the phosphorylation signal previously observed, the double mutant S316A/T432A was generated, purified and assayed for phosphorylation as already described. As reported in Fig. 3.5, compared to EF1A wt, the extent of the phosphorylation signal observed was reduced also for the double mutant.

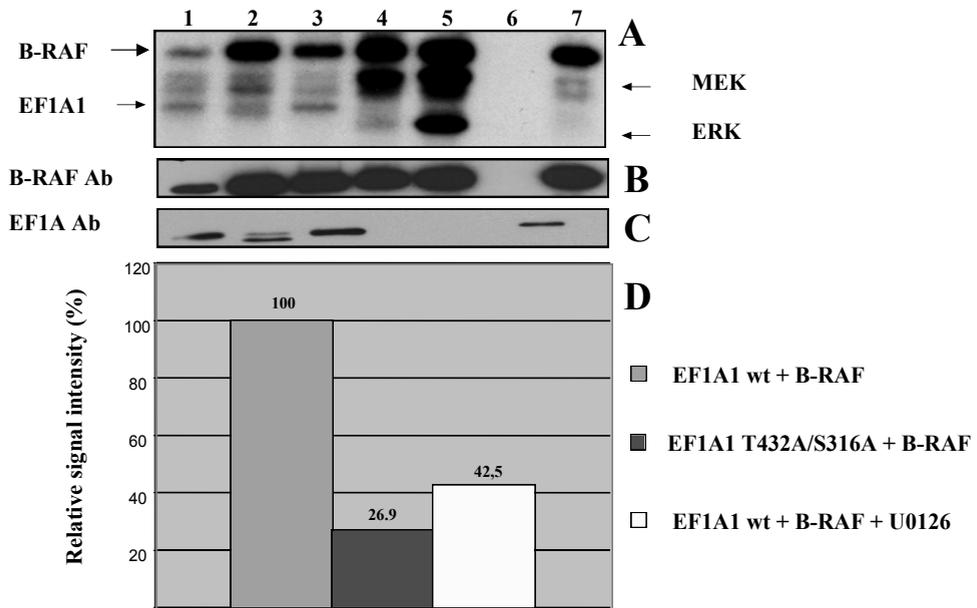
To exclude the involvement of a Mek homologue, possibly present as a contaminant in the B-RAF preparation from Sf9 cells, B-RAF wt and EF1A1 wt were incubated in presence of Mek inhibitor U0126 (Fig. 3.5).

## Results

Densitometric evaluation of band intensity revealed a reduction of 73.1 and 57.5 % for the double mutant and EF1A1 wt in the presence of U0126, respectively (Fig. 3.5 B).



**Fig. 3.4 *In vitro* kinase assay of EF1A1 mutants.** A) Autoradiography. The kinase assay was performed incubating B-RAF wt in the presence of EF1A1 wt (lane 1) and EF1A1 mutants T242A (lane 2), S18A (lane 3), S157A (lane 4), T432A (lane 5), S316A (lane 6) and S383A (lane 7), respectively. B) Densitometric evaluation.

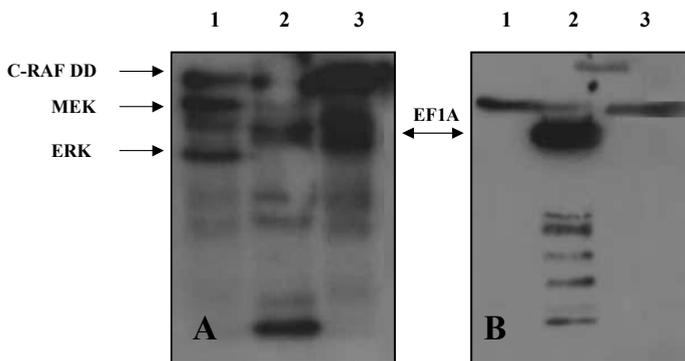


**Fig. 3.5 *In vitro* kinase assay on EF1A1 wt and double mutant.** A) Autoradiography. B) and C) immunoblotting against B-RAF and EF1A antibodies, respectively. The kinase assay was performed incubating B-RAF wt in presence of EF1A1 wt (lane 1), EF1A1 S316A/T242A (lane 2), respectively, The assay was also performed in the presence of MEK inhibitor U0126 (lane 3). D) Densitometric evaluation.

### 3.4 Phosphorylation of a potential EF1A heterodimer by C-RAF *in vitro*

In precedent *in vitro* experiments it was found that C-RAF was not able to phosphorylate EF1A1 as well as EF1A2, contrary to what previously observed in H1355 cells (Lamberti *et al.*, 2007). These results raise the question whether C-RAF needs, for its *in vivo* phosphorylation activity observed on EF1A, a potential heterodimerization of the two EF1A isoforms. For that reason, to determine whether C-RAF was able to

phosphorylate a potential EF1A heterodimer *in vitro*, EF1A1 and EF1A2 were mixed, preincubated in the kinase buffer for 10 min and then assayed for phosphorylation by the addition of C-RAF DD kinase. The results obtained showed the presence of a phosphorylation signal corresponding to the size of EF1A (Fig. 3.6 A). This correspondence was confirmed by probing the same membrane with anti-His Ab (Fig. 3.6 B).



**Fig. 3.6 C-RAF DD kinase assay of EF1A1 and EF1A2.** A) Autoradiography; B) Anti-His antibody detection. Lanes: 1, positive control; 2, C-RAF DD + EF1A1 + EF1A2; 3, C-RAF DD.

### 3.5 Identification of phosphorylation sites on EF1A1 and EF1A2 mediated by RAF kinases *in vitro*

To identify the amino acid residues of EF1A involved in the RAF-mediated phosphorylation, equal amounts of EF1A1 and EF1A2, alone or together, were incubated with either B-RAF or C-RAF in the presence of cold ATP and analyzed by mass spectrometry. Analysis of the phosphopeptides

## Results

indicated that in presence of B-RAF, EF1A1 was phosphorylated at residues Serine 21 and Tyrosine 86 or Threonine 88 and EF1A2 at residue Serine 21. In the presence of C-RAF, EF1A2 was phosphorylated at residue of Serine 21. No data was collected for the C-RAF mediated phosphorylation of EF1A1. When the assay was performed on both isoforms, EF1A1 and EF1A2 were phosphorylated on Serine 21 either in the presence of B-RAF or C-RAF (Table 1).

**Table 1. Identified phosphorylation sites on EF1A1 and EF1A2**

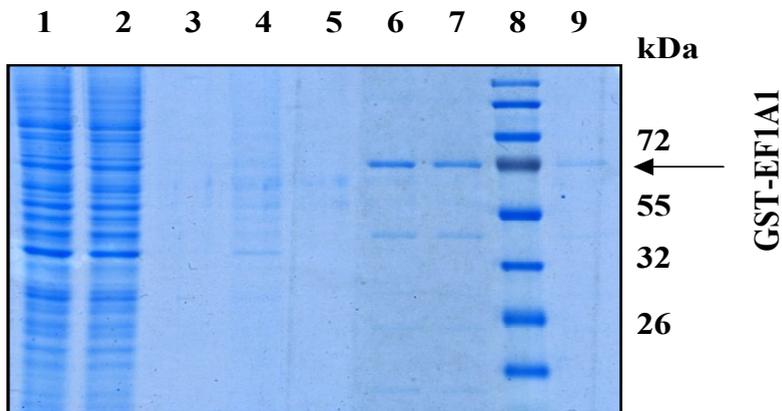
<b>Kinases</b>	<b>Substrates</b>	<b>Phosphorylated sites</b>
B-RAF	EF1A1	S21, Y86/T88
B-RAF	EF1A2	S21
B-RAF	EF1A1 + EF1A2	S21; S21
C-RAF	EF1A1	n.d.
C-RAF	EF1A2	S21
C-RAF	EF1A1 + EF1A2	S21; S21

### **3.6 Purification of EF1A1 wt and EF1A2 wt expressed in COS 7 cells**

EF1A1 and EF1A2 were cloned in mammalian expression vectors (pEBG and pCDNA 3.1, respectively) and expressed in COS 7 cells, in order to determine if post-translational modifications, normally not occurring in the *E. coli* heterologous expression system, could influence the *in vitro* phosphorylation of EF1A mediated by RAF kinases. Because of the lack of

## Results

a specific antibody raised against the two isoforms, EF1A1 was fused to GST whereas EF1A2 was provided with a His-Tag. 48h after transfection, the cells were harvested and GST-EF1A1 was purified from the lysate using glutathione sepharose. The SDS-PAGE of purified proteins (Fig. 3.7) showed the presence of a stained band of about 72 kDa, corresponding to the size of EF1A1 (46 kDa) plus GST (26 kDa). A similar result was obtained for the purification procedure of EF1A2-His (about 52 kDa, data not shown).



**Fig. 3.7 Purification of EF1A1 wt GST-Tag.** Coomassie staining of the purification procedure: lysate and supernatant (lanes 1 and 2), washes (lanes 3, 4, 5), elutions (lanes 6, 7, 9), size marker (lane 8). GST-EF1A1 corresponds to the band around 72 kDa.

### 3.7 Identification of phosphorylation sites and interaction partners of EF1A1 expressed in COS 7 cells

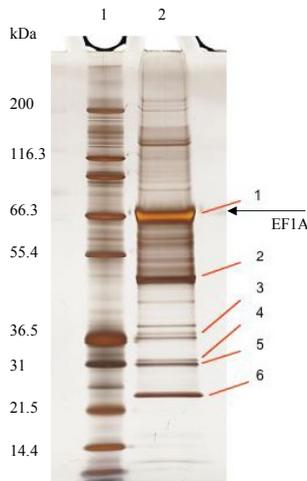
EF1A1 wt fused to GST was expressed in COS 7 cells and purified according to the procedure described above. The purified GST-EF1A1 was extracted from the silver stained SDS-PAGE gel and analysed by mass spectrometry. The phosphopeptides obtained after tryptic digestion of the protein, led to the identification of two phosphorylation sites on EF1A1 (Table 2). These sites were coincident with those found in the previous mass spectrometry analysis performed on recombinant EF1A1 purified from *E. Coli* after the *in vitro* kinase assay in the presence of B-RAF (Tab. 3).

**Tab. 2. Phosphorylation sites identified on EF1A1 expressed in COS 7 cells**

Phosphopeptide sequence	Modified aa residues
K.YYVpTIIDAPGHRDFIK.N	T 88
K.p(YY)VTIIDAPGHR.D	Y 86

Moreover, silver staining of the SDS-PAGE gel revealed the presence, in comparison to the GST control (not shown), of several additional bands that were also analysed by mass spectrometry (Fig. 3.8). The proteins identified are listed in Table 3. These include all components of the protein synthesis elongation cycle, heat shock proteins (HSP), laminin, polyadenylate binding protein 1 and Peroxiredoxin-1. These findings are in line with the already known literature data (Bohnsack *et al.*, 2002; Khacho *et al.*, 2008; Marchesi, 1993; Chang *et al.*, 2006).

## Results



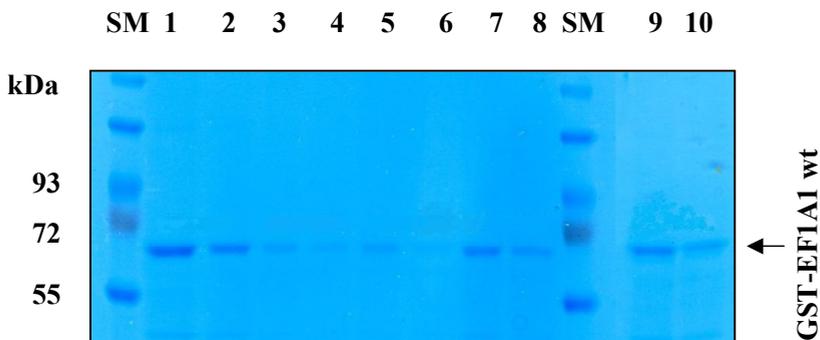
**Fig. 3.8 Interaction partners of EF1A1 expressed in COS 7 cells.** Silver staining of the SDS-PAGE gel. 1) Size Markers; 2) Proteins identified in the elution of GST pull down experiment. The number is referred to the proteins identified that are reported in Table 3.

**Table 3. EF1A1 interaction partners identified after GST pull-down**

Band	Accession N.	Protein	Sequence coverage (%)
1	IPI00396485	EEF1A1 Elongation factor 1-alpha 1	64
1	IPI00007765	HSPA9 Stress-70 protein, mitochondrial	56
		HSPA8 Isoform 1 of Heat shock cognate	
1	IPI00003865	71 kDa protein	47
1	IPI00003362	HSPA5 HSPA5 protein	41
1	IPI00021405	LMNA Isoform A of Lamin-A/C	14
		PABPC1 Isoform 1 of Polyadenylate-	
1	IPI00008524	binding protein 1	13
		HSP90AB1 Heat shock protein HSP 90-	
1	IPI00414676	beta	7
2	IPI00747497	EEF1G 50 kDa protein	28
2	IPI00396485	EEF1A1 Elongation factor 1-alpha 1	18
3	IPI00023048	EEF1D Elongation factor 1-delta	24
4	IPI00178440	EEF1B2 Elongation factor 1-beta	8
5	IPI00178440	EEF1B2 Elongation factor 1-beta	18
6	IPI00219757	GSTP1 Glutathione S-transferase P	21
6	IPI00000874	PRDX1 Peroxiredoxin-1	13

### 3.8 Purification of EF1A1 mutants expressed in COS 7 cells

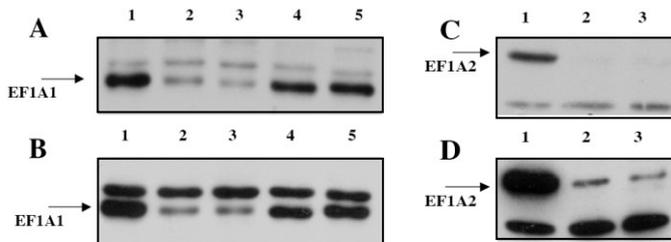
To study the effect of the B-RAF and C-RAF mediated phosphorylation on EF1A, recombinant EF1A1 and EF1A2, already cloned in a mammalian vector (pEBG and pCDNA 3.1, respectively), were mutated in the positions Serine 21 and Threonine 88 on EF1A1 and Serine 21 on EF1A2. To mimic the negative charge of the phosphate group, Serine/Threonine was replaced by Glutamate (dominant positive), whereas the exchange of Serine/Threonine against Alanine prevents the phosphorylation on these sites (dominant negative). These proteins were expressed in COS 7 cells and purified by GST pull-down. As reported in Fig. 3.9, the proteins are expressed in COS 7 cells at different levels. In particular, the S21A and S21D mutants seem to be less expressed compared to EF1A1 wt as well as T88A and T88D EF1A1 mutants. These results suggest that these proteins are *in vivo* less stable and that the RAF kinase mediated phosphorylation of S21 might play a regulatory role affecting EF1A1 expression levels.



**Fig. 3.9 Purification of EF1A1 wt and mutants from COS 7 cells.** Coomassie staining of the SDS-PAGE. Lanes: SM, size markers; 1 and 2, elution fractions of EF1A1 wt; 3 and 4, elution fractions of S21A; 5 and 6, elution fractions of S21D; 7 and 8, elution fractions of T88A; 9 and 10, elution fractions of T88D.

### 3.9 Stability of the S21 mutants of EF1A1 and EF1A2 *in vivo*

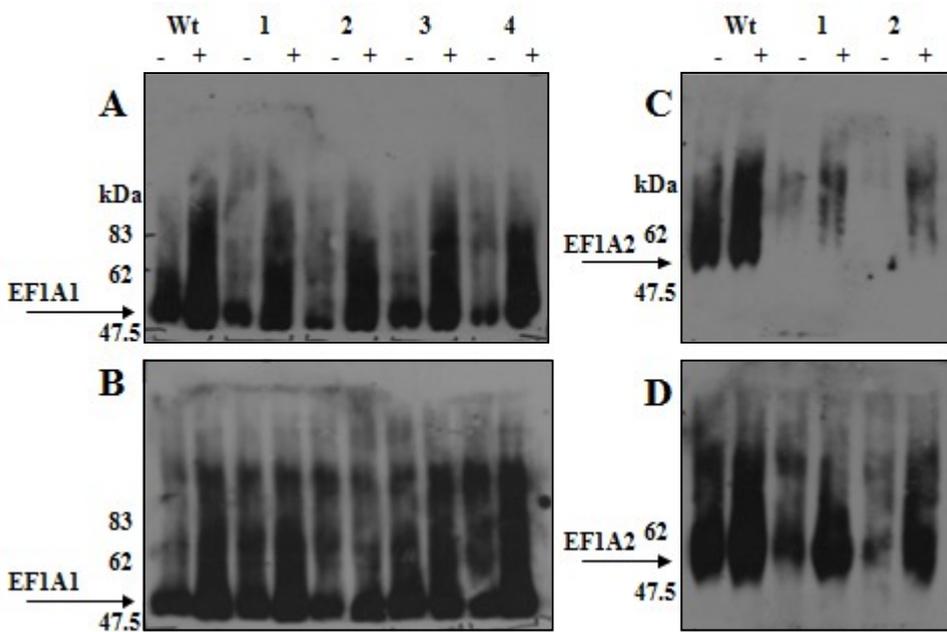
To compare the stability of the S21 mutants of EF1A1 with those of EF1A2 *in vivo*, the expression levels of both EF1A isoforms were evaluated. To this purpose, EF1A1 was cloned in the mammalian vector pCDNA 3.1 and expressed as His-EF1A1 in COS 7 cells. 36 h after transfection, cells were harvested and lysed. Following the His pull-down, the protein samples were analysed by immunoblotting with anti-His antibody. As reported in Fig. 3.10, compared to EF1A1 wt and EF1A2 wt, the expression of S21 mutants of EF1A1 and EF1A2 appear negatively regulated *in vivo*. In particular, the S21 mutants of EF1A2 seem to be more sensitive to this regulation.



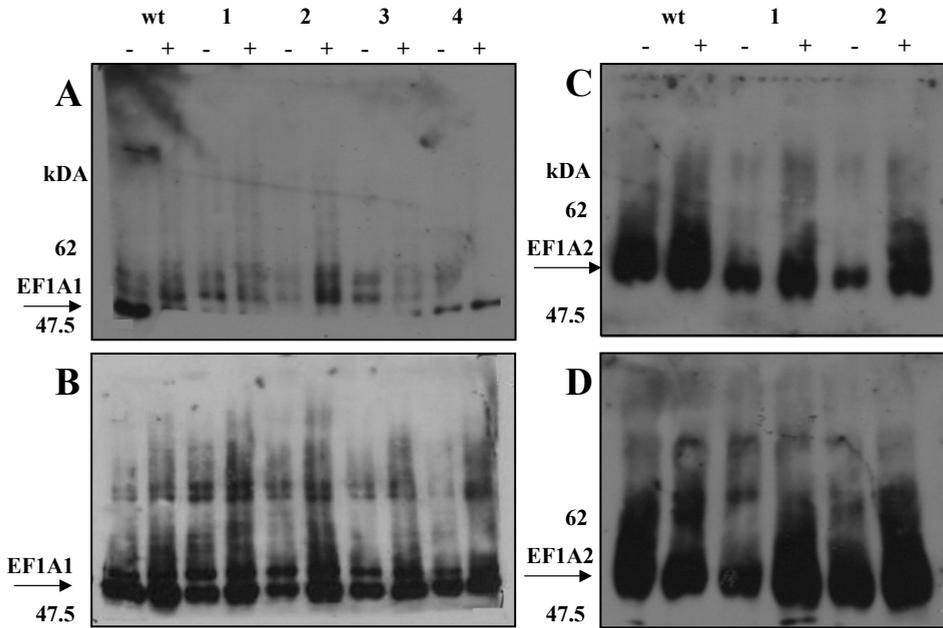
**Fig 3.10 Immunoblotting of EF1A1 and EF1A2 protein expression levels in COS 7 cells.** COS 7 cells, after transfection, were analysed for EF1A1 expression with antibody anti-His. A) Cell lysate, B) pull-down. Lanes: 1, EF1A1 wt; 2, S21A; 3, S21D; 4, T88A; 5, T88D. COS 7 cells, after transfection, were analysed for EF1A2 expression with antibody anti-His. C) Cell lysate, D) pull-down. Lanes: 1, EF1A2 wt; 2, S21A; 3, S21D.

### **3.10 Ubiquitination and stability of EF1A1 wt, EF1A2 wt and their mutants *in vivo***

The above results revealed different expression levels of EF1A1 and EF1A2 mutants suggesting an ubiquitin-dependent degradation of these mutated enzymes. Therefore, to better evaluate the stability of EF1A1 wt, EF1A2 wt and their mutants as well as their endogenous ubiquitination, COS 7 cells were transfected with the EF1A1 and EF1A2 constructs for 36 h and subsequently incubated in the presence or absence of MG 132 for 12 h. Immunoblotting analysis with anti-His antibody of the cell extracts and corresponding His-pull-down samples showed that compared to EF1A1 (Fig. 3.11 A and 3.11 B) also for the EF1A2 mutants, the polyubiquitination level was higher than that of EF1A2 wt (Fig. 3.11 C and Fig. 3.11 D). To compare the level of the endogenous ubiquitination with that observed when also ubiquitin was co-expressed, COS 7 cells were cotransfected with EF1A1 and EF1A2 constructs and ubiquitin wt for 36 h and then incubated in the presence or absence of the proteasome inhibitor for 12 h. As reported in Fig. 3.12, also in these cases, the polyubiquitination levels of EF1A1 wt and EF1A2 wt appeared lower than the corresponding mutants.



**Fig 3.11 Immunoblotting after pull-down of the endogenous ubiquitination of EF1A1 and EF1A2 and their mutants.** After cotransfection and treatment of cells in the absence (-) or in the presence (+) of MG132, cell extracts were analysed before and after pull-down for EF1A1 and EF1A2, respectively by immunoblotting with antibody anti-His. EF1A1: A) lysates, B) pull-down. Lanes: wt, EF1A1 wt; 1, EF1A1 S21A; 2, EF1A1 S21D; 3, EF1A1 T88A; 4, EF1A1 T88D. EF1A2: C) lysates, B) pull-down. Lanes: wt, EF1A2 wt; 1, EF1A2 S21A; 2, EF1A2.



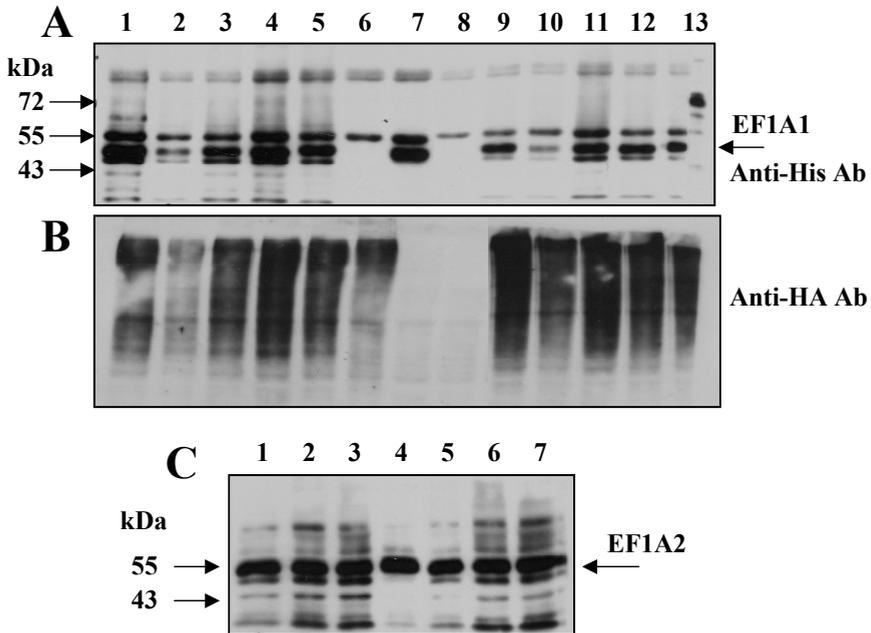
**Fig 3.12 Immunoblotting after pull-down of the ubiquitination of EF1A1 and EF1A2 and their mutants.** After cotransfection with ubiquitin wt and treatment of cells in the absence (-) or in the presence (+) of MG132, cell extracts were analysed before and after pull-down for EF1A1 and EF1A2, respectively by immunoblotting with antibody anti-His. EF1A1: A) lysates, B) pull-down. Lanes: wt, EF1A1 wt; 1, EF1A1 S21A; 2, EF1A1 S21D; 3, EF1A1 T88A; 4, EF1A1 T88D. EF1A2: C) lysates, B) pull-down. Lanes: wt, EF1A2 wt; 1, EF1A2 S21A; 2, EF1A2.

## *Results*

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To confirm that reduced expression of EF1A1 and EF1A2 mutants was ascribed to a reduced stability of the proteins i.e. to an increase of their ubiquitination and thus to proteasome-dependent degradation, the same EF1A samples were cotransfected with ubiquitin K48\* or ubiquitin wt for 36 h followed by subsequent incubation of transfected cells for 12 h with the proteasome inhibitor MG 132. Cell extracts were then analysed by pull down with glutathion sepharose or a Ni-NTA matrix for EF1A1 and EF1A2 expression, respectively, and the membranes were subsequently probed with antibody anti-His to reveal the level of EF1A and anti-HA to detect HA-ubiquitin K48 (Fig. 3.13). As reported in Fig 3.13 A, the inhibition of the proteasome by MG 132 led to an accumulation of polyubiquitinated proteins that, compared to EF1A1 wt, was higher for the EF1A1 mutants (Fig. 3.13 B). Regarding EF1A2 and its mutants, the protein levels, immunodetected after pull-down showed that also for the EF1A2 mutants, the polyubiquitination level was higher than that of EF1A2 wt (Fig. 3.13 C). Thus, either if the transfection was done with HA-ubiquitin K48 or HA-ubiquitin wt, the polyubiquitination levels appeared quite similar. These results indicate that in the presence of the proteasome inhibitor, although at different extents, all EF1A1 and EF1A2 mutants appeared to be more polyubiquitinated than the corresponding wild type factors. This suggests that the instability of the expressed mutants is due to a more rapid degradation mediated by the proteasome.

\*Ubiquitin K48. A mutant that can only form K48-branched ubiquitin chains.



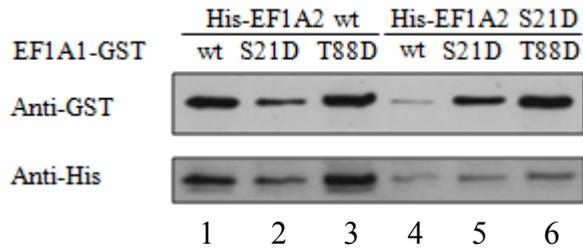
**Fig. 3.13 Immunoblotting after pull-down of the ubiquitination of EF1A1 and EF1A2 and their mutants:** after cotransfection with either ubiquitin K48 or ubiquitin wt and MG132 treatment, cell extracts were analysed after pull-down for ubiquitination of EF1A1 and EF1A2, respectively by immunoblotting with antibody anti-His and anti-HA. A) and B) EF1A1. Lanes: 1 EF1A1 wt + UB HA K48; 2, EF1A1 S21A + UB HA K48; 3, EF1A1 S21D + UB HA K48; 4, EF1A1 T88A + UB HA K48; 5, EF1A1 T88D + UB HA K48; 6, UB HA K48; 7, EF1A1 wt; 8, UB HA wt; 9, EF1A1 wt + UB HA wt; 10, EF1A1 S21A + UB HA wt; 11, EF1A1 S21D + UB HA wt; 12, EF1A1 T88A + UB HA wt; 13, EF1A1 T88D + UB HA wt. C) EF1A2. Lanes: 1, EF1A2 wt + UB HA k48; 2, EF1A2 S21A + UB HA k48; 3, EF1A2 S21D + UB HA k48; 4, EF1A2 wt; 5, EF1A2 wt + UB HA wt; 6, EF1A2 S21A + UB HA wt; 7, EF1A2 S21D + UB HA wt.

### 3.11 Heterodimerization of EF1A1 and EF1A2 *in vivo*: preliminary experiments

Previous *in vitro* experiments (section 3.4) showed a potential heterodimerization of both elongation factor isoforms. To further investigate on this potential heterodimerization *in vivo*, recombinant GST-EF1A1 wt and its mutants were cotransfected in different combination with His-EF1A2 wt and His-EF1A2 S21D in COS 7 cells. After 24 h, the cells were harvested and lysed. GST-EF1A1 and associated proteins were isolated via GST pull-down and the resulting eluates were blotted and probed against antibody anti the corresponding tags of the recombinant proteins (GST and His). As shown in figure 3.14, all samples revealed an interaction between the two isoforms thus confirming *in vivo* the heterodimerization observed *in vitro*. In general, the overexpression of EF1A2 wt positively affects the stability of the heterodimerization with EF1A1 wt and mutants (Fig. 3.14, lanes 1-3) compared to the overexpression of EF1A2 S21D (lanes 4-6). This observed increase in complex stability is enhanced in the case of EF1A1 T88D overexpressing cells (lane 3 compared to lane 1). In contrast, the overexpression of EF1A2 S21D seems to reduce the complexes stability (lanes 3-6). In particular, overexpression of EF1A2 S21D seems to have two different outcomes: negatively affecting the complex formation and the stability of EF1A1 wt.

## Results

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**Fig. 3.14 Heterodimerization of EF1A1 and EF1A2 *in vivo*.** EF1A1-GST and the indicated mutants were co-expressed in COS 7 cells with EF1A2-His wt and EF1A2-His S21D. EF1A1-GST and interacting proteins were isolated via GST pull down and analysed by immunoblotting with anti-GST and anti-His antibody.

## **4. DISCUSSION**

In this study, it has been examined the involvement of the translation elongation factor 1A isoforms in post-translational modifications induced by their interaction with RAF kinases. In addition, it has been evaluated if the phosphorylation of EF1A could also modulate its proteasome-dependent degradation and its GDP/GTP binding activity. This work plan was started on the basis of preliminary results here summarized: following treatment of a human hepidermoid cancer cell line (H1355) with interferon alpha (INF $\alpha$ ), it was observed **1.** anti-apoptotic role of EF1A2 isoform; **2.** phosphorylation of EF1A (indirectly?) mediated by C-RAF kinase, following activation of a survival pathway induced by EGF receptor.

Therefore, the first aims included the determination of the specific EF1A proteins' amino acid residues (EF1A1 or EF1A2) phosphorylated by RAF isoforms using proteomic strategies. To this purpose, recombinant EF1A1 and EF1A2 were expressed and purified from *E. coli* (Fig. 1.1) and radioactively assayed in presence of B-RAF or C-RAF. In these conditions, B-RAF but not C-RAF was able to phosphorylate the EF1A1/2 isoforms (Fig. 1.2) whereas the result regarding the C-RAF mediated phosphorylation did not show an evident signal.

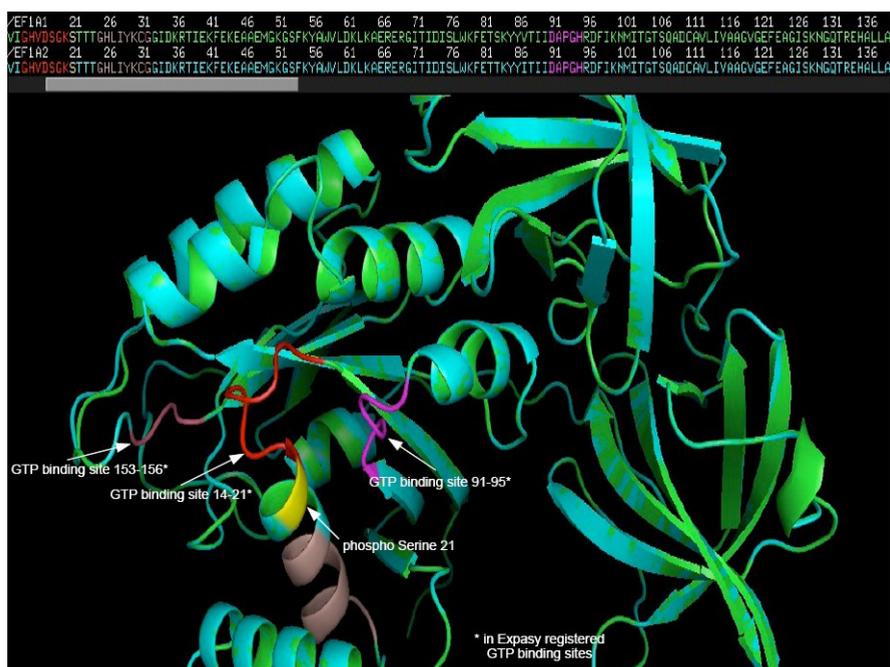
### **4.1 The heterodimerization of EF1A1/2 enhances a RAF isoform specific phosphorylation of EF1A**

While both isoforms (solely) were phosphorylated by B-RAF *in vitro*, a phosphorylation of a potential EF1A heterodimer by C-RAF *in vitro* was

observed. This finding indicates that the RAF specific phosphorylation of the elongation factor might depend on the formation of EF1A1•EF1A2 complex. Thus it is possible that this heterodimerization enhances the activity of C-RAF on EF1A. This notion is underlined by the finding that no C-RAF mediated phosphorylation signal on EF1A2 alone was detected in the radioactive *in vitro* kinase assay, even though mass spectrometry analysis identified S21 as a phosphorylated residue in the same kinase assay. In the B-RAF/EF1A1 kinase assays, besides S21 also T88 was found to be a phosphorylated residue using mass spectrometry analysis. Remarkably, T88 was not identified when both isoforms were mixed together in the *in vitro* kinase assay. Mass spectrometry analysis also revealed the B-RAF mediated phosphorylation of T88 on overexpressed EF1A1 isolated from COS7 cells. Moreover, in the same sample, all members of the elongation factor 1 complex were found as well as the endogenous EF1A1 probably associated with overexpressed GST-EF1A1. A phospho-mimicking mutation of T88 (T88D) on EF1A1 enhances its activity to form a complex with EF1A2 in COS7 cells as observed in a pull-down experiment (Fig. 3.14). Taken together, these results suggest a role for the B-RAF specific phosphorylation of T88 on EF1A1 affecting the formation of the elongation factor 1 complex as well as a homo/heterodimerization of EF1A participating in the complex. This is in accordance to structural modeling, where T88 is part of the binding region of EF1A involved in its association with EF1B $\alpha$  (Andersen, *et al.*, 2000; Soares *et al.*, 2009).

## 4.2 The importance of the EF1A serine 21 site

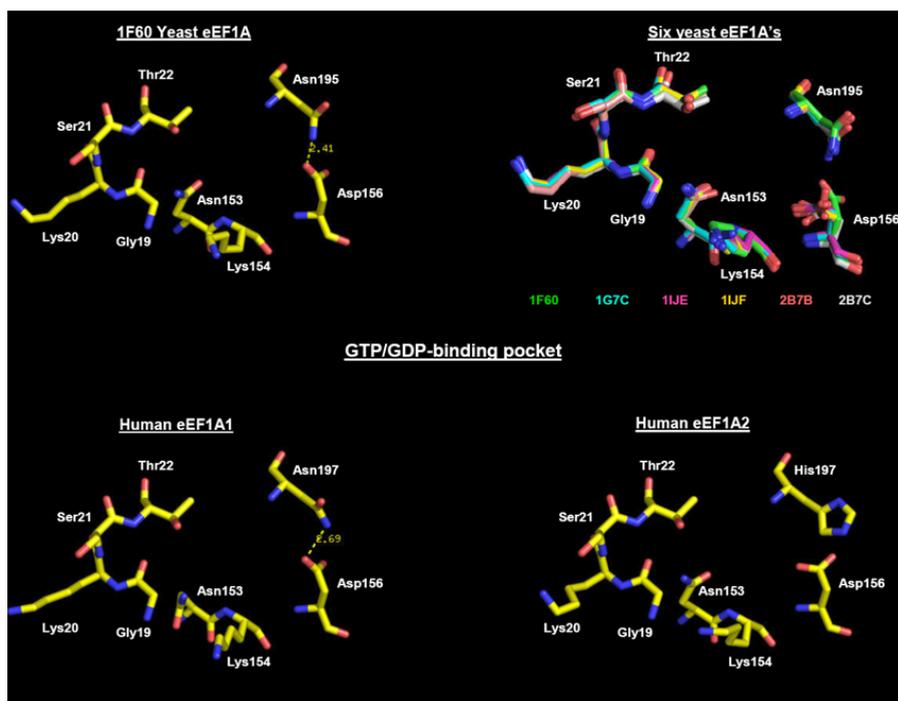
Both EF1A isoforms are phosphorylated by B-RAF and C-RAF on S21. As shown in the 3-D model (Fig. 4.1), this residue belongs to one of the three GTP/GDP binding site consensus sequences (Dever *et al.*, 1987) and is almost located in the center of the GTP/GDP binding pocket.



**Fig. 4.1 Merge of EF1A1 and EF1A2 model structures.** The model shows the position of the consensus sequences on both EF1A isoforms 3-D structures with particular regard to the position of the serine 21.

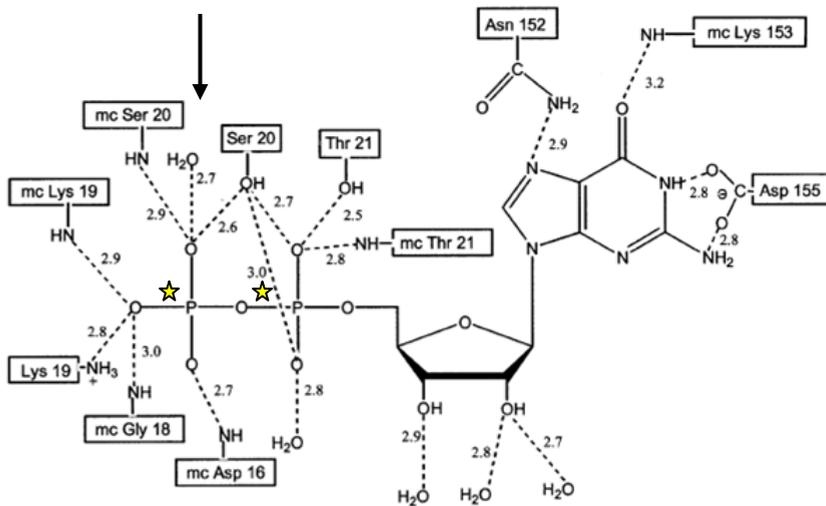
Structural models of both EF1A1 and EF1A2 revealed the critical importance of Gly19, Lys20, Ser21, Thr22, Asn153, Lys154 and Asp156 (all in domain I) within the binding sites for GTP/GDP ligands (Soares *et*

*al.*, 2009). These observations were in agreement with previously performed mutagenesis studies in yeast (Carr-Schmid, *et al.*, 1999, Ozturk *et al.*, 2006). In fact, the Asn153Thr and Asp156Asn mutations of yeast EF1A resulted in dramatic reduction in translational fidelity. The guanine-binding pocket is highly conserved in both human variants. Thus, their differences in GDP/GTP preference ratios must be explained in another way.



**Fig. 4.2 Guanine-binding pocket in yeast and human variants.** Close-up equivalent views of residues involved in binding of GTP/GDP: the yeast template (top left); all six yeast EF1A structures superposed (top right) and colored differently (see their corresponding PDB IDs below); human EF1A1 (bottom left); and human EF1A2 (bottom right). Labeled residues include: Gly19, Lys20, Ser21, Thr22, Asn153, Lys154, Asp156 [GTP/GDP-binding residues] and Asn195 (yeast), Asn/His197 (human EF1A1/EF1A2) that show presence of H-bond (indicated by a yellow dashed line and distance in Å) with Asp156, absent in human EF1A2. (Fig. adapted from Soares *et al.*, 2009; supplementary file S5)

Overexpression of the S21A/D mutants in COS7 cells revealed reduced protein levels due to the proteasomal degradation of the two EF1A mutants. According to models of EF1A from *S. Sulfataricus* (Fig. 4.3) the S20 side chain is linked directly to the oxygens of both phosphates of the GDP nucleotide (Vitagliano, *et al.*, 2001) Therefore, the binding of the guanine nucleotide should be potentially prevented when this site becomes phosphorylated by RAF kinases (personal communication, M. Masullo).



**Fig. 4.3 Schematic representation of GDP binding to *Ss*EF1A.** Serine 20 (black arrow) binds the  $\gamma$ - and  $\beta$ - phosphates (yellow star) of GDP via a hydroxyl group.

Moreover, the same region of EF1A is involved in the binding of EF1B $\alpha$  and precisely, the EF1B $\alpha$  C-terminal fragment lies mainly on domains I and II, with only a single contact with domain III (Andersen *et al.*, 2000).

Therefore, closing the access of this site *via* phosphorylation for both GTP and EF1B could result in a conformational change that directs EF1A to interact with other partners leading to different functions. However, both overexpressed mutated isoforms (S21A, S21D) are degraded to almost the same extent. Thus, mutations of this site cause a severe modification that leads to the proteasomal degradation. This indicates that the S21 residue is a crucial site for the half-life of EF1A because its blockage prevents the protein from switching between its role in protein biosynthesis and its involvement in other functions. This leads to the hypothesis that RAF mediated phosphorylation of EF1A on S21 negatively regulates the GTP binding and/or nucleotide exchange of the enzyme and that this phosphorylation can be a regulatory switch for other functional roles of EF1A. Thus, RAF kinases potentially regulate the function of EF1A.

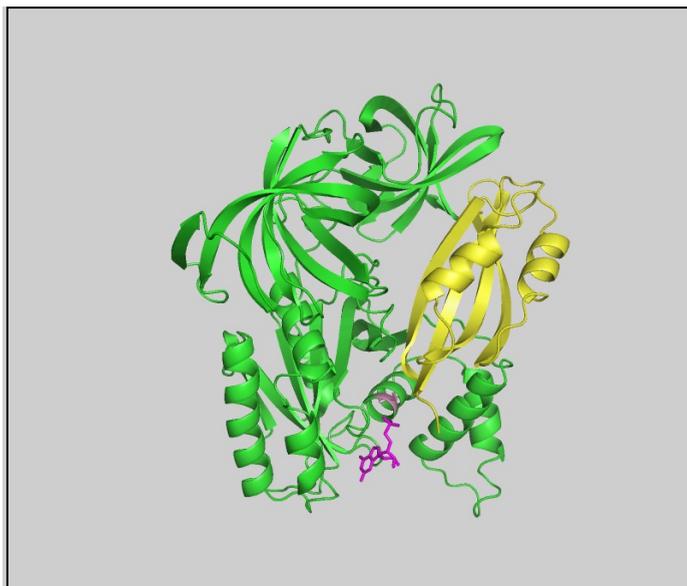
#### **4.3 Does the RAF mediated phosphorylation of EF1A regulate its ability to bind or bundle actin?**

Some years ago, it has been found in *Dictyostelium S.* that EF1A is an actin-binding protein (Yang *et al.*, 1990). Since then, several groups demonstrated that this function is conserved among the species from yeast (Munshi *et al.* 2001) to mammals (Murray *et al.*, 1996). Remarkably, more than 60% of EF1A is associated with the actin cytoskeleton and, moreover, it can bind to microtubules (Moore *et al.*, 1998; Moore *et al.*, 2000) centrosphere and the mitotic apparatus (Kuriyama *et al.*, 1990). It has been shown that EF1A is also implicated in actin bundling and is essential for regulation of actin cytoskeleton and cell morphology (Stephane *et al.*, 2005). As already

## *Discussion*

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mentioned in the introduction section, EF1A1 is connected, directly or indirectly, with several other cellular processes different from the protein biosynthesis (Ejiri, 2002). These processes like apoptosis, transformation and drug resistance are mediated in some cases by the interaction of EF1A with other proteins connected to signaling of the cell. The regulatory mechanism of EF1A in these processes is still not clear but it is conceivable that the association of EF1A to actin is linked to these activities. Recent studies indicate that actin and aa-tRNA binding are mutually exclusive (Liu *et al.*, 1998) and that the EF1B interaction and actin bundling sites on EF1A are overlapping. Interestingly, mutational analysis revealed that EF1B is able to prevent EF1A association to actin bundles (Pittman *et al.*, 2009). Since the C terminus of EF1B interacts with domain I and a distinct pocket of domain II of EF1A, it is likely that the RAF mediated phosphorylation on S21 buries the interface with EF1B $\alpha$  because of an induced conformational change of EF1A that leads also to the lack of the GDP/GTP exchange (Fig. 4.4).



**Fig. 4.4 Co-crystal structure of EF1A•EF1B $\alpha$  C terminus•GDP.** In green elongation factor 1A, in pink serine 21, in hot pink sticks GDP, in yellow the C terminus of the elongation factor 1B $\alpha$ . Structure reviewed by PyMol program.

Remarkably, EF1A was also reported to be an activator of a PI 4-kinase that binds actin and increases actin bundling (Jeganathan, *et al.*, 2008). Regarding this process Kurasawa and coworkers demonstrated F-actin bundling activity of *Tetrahymena* elongation factor 1A investigated with rabbit skeletal muscle actin. This activity depends on a binding ratio of EF1A to skeletal muscle F-Actin in the bundles of 1:1 (Kurasawa *et al.*, 1996). Ten years later, Bunai and colleagues (Bunai *et al.*, 2006) showed, that EF1A of *Tetrahymena* bundles filamentous actin through dimer formation. In their experiments EF1A formed dimers that were able to bundle F-Actin, whereas EF1A monomers were not. Mutations of the RAF mediated phosphorylation sites on EF1A revealed differences in the formation of heterodimers and a different migration pattern. As shown in

Fig. 3.14, the S21 phosphorylation might be a second step after the heterodimerization that seems to be stabilized by the T88 modification. Taken together, these results allow to depict a model to partially explain the role of the RAF-EF1A interaction during the drug resistance process previously observed in H1355 cells treated with IFN $\alpha$ . Lamberti *et al.*, 2007 showed that epidermoid tumor cells counteract the IFN-induced apoptosis through a survival pathway that involves the hyperactivation of the EGF-dependent Ras->Erk signalling. Later it was also found that the elongation factor 1A is involved in this response *via* its overexpression and RAF mediated phosphorylation (Lamberti *et al.*, 2007). Remarkably, both isoforms were found to be expressed and the amount of EF1A2 was increased during a time course following treatment with IFN $\alpha$  until its degradation in 60 minutes. As shown during the course of this work, the heterodimerization of both isoforms is connected to the RAF mediated phosphorylation. In particular, the phosphorylation on T88 seems to stabilize the complex as well as the protein itself. This suggests, that a RAF dependent heterodimerization interferes with the proteasomal degradation of both isoforms. Considering the increased EF1A expression levels which were shown to be correlated with an anti-apoptotic response, these observations indicate that the RAF signal dependent EF1A heterodimerization might be involved in the reported anti-apoptotic answer. In conclusion, B-RAF and C-RAF kinases, regulate the function of elongation factor 1A by phosphorylating both isoforms of the enzyme on a crucial aminoacid residue, Serine 21, important for the binding of GTP/GDP. B-RAF is also responsible for the phosphorylation of EF1A1 on Threonine 88, an exposed aminoacid residue of domain I. These

## *Discussion*

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phosphorylations appear to stabilize the enzyme since mutations in this position increase the instability of the protein through an ubiquitin-mediated degradation mechanism that was confirmed by *in vivo* experiments. In addition, EF1A1 and EF1A2 are able to form a stable heterodimer complex that is strongly destabilized if the S21 of EF1A2 is mutated but not if the same position in EF1A1 is mutated, suggesting that the phosphorylation of this site occurs in a second moment, after the formation of the heterodimer. These data indicate that the S21 phosphorylation might represent a switching mechanism that directs EF1A toward different functional role than protein synthesis. This functional switch for the two EF1A isoforms could be at the root of the H1355 lung cancer cell line survival response during drug resistance process mediated by Ras->Erk signaling.

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