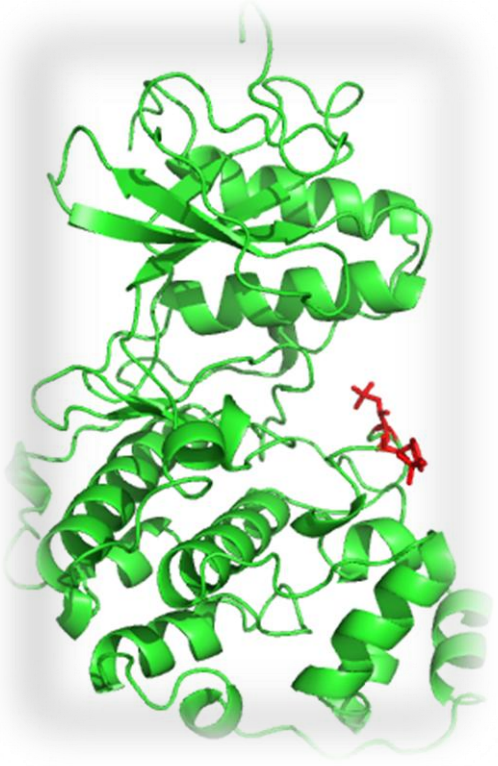
University of Crete
Departments of Biology and Medicine
Post-graduate programme in Molecular Biology and Biomedicine

Master thesis:

Structural study of the interaction between ETS2 Repressor Factor (ERF) and kinase Erk2



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Η ολοκλήρωση της διπλωματικής εργασίας συγχρηματοδοτήθηκε μέσω της Πράξης Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ για Μεταπτυχιακές Σπουδές Πρώτου Κύκλου (Μάστερ) - Οριζόντια Πράξη, *από πόρους του ΕΠ «Εκπαίδευση και Δια Βίου Μάθηση», του Ευρωπαϊκού Κοινωνικού Ταμείου (ΕΚΤ) του ΕΣΠΑ, 2007-2013*.

Ένα μεγάλο ευχαριστώ..

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Summary

ERF (ETS2 Repressor Factor) is a eukaryotic transcriptional repressor exerting tumor suppressor activity and is shown to be implicated in mouse embryonic development as well as in human disease in the case of ERF-related craniosynostosis. The function of ERF in cells is mainly regulated by phosphorylation by ERKs (Extracellular signal- Regulated Kinases), an event that takes place inside nucleus upon mitogenic stimuli, resulting in the rapid export of ERF towards the cytoplasm and the subsequent activation of genes previously suppressed by this factor. Although the association of ERF with Erk2 has been shown to be mediated by two FXF motifs lying in the EID (ERK interaction domain) of ERF, additional regions of this domain seem to participate in intermolecular contacts that confer overall stability to this complex. Therefore, the aim of this study was the purification of the two partners and the subsequent crystallization of the complex they form in order to gain an insight into all those structural elements that determine this particular interaction.

His-tagged rat Erk2, produced in its double- phosphorylated activated form due to its co-expression with the constitutively active mutant of the upstream kinase MEK1, was initially isolated from the bacterial lysate by immobilized metal- affinity chromatography (IMAC) performed on Ni^{+2}-NTA resin. The separation of the double- phosphorylated activated form of the protein from the non-phosphorylated inactive one was accomplished by anion exchange chromatography performed on Mono Q HR5/5 column. GST-tagged human ERF- EID was isolated in the first step of the purification procedure by glutathione S- transferase (GST) - affinity chromatography. Since GST-ERF (EID) was extremely sensitive to proteolysis and GST protein itself has a dimeric tertiary structure, the formation of heterodimers between the full-length protein and the proteolytic fragments of its polypeptide chain could not be prevented. Nevertheless we were successful in the isolation of a population consisting solely of full-length GST-ERF homodimers by gel filtration chromatography performed on Sephacryl S-200 HR16/60 column. Finally, in order to prevent the dimerization event, we performed the replacement of the GST-tag by a 6xHis-tag followed by protein purification by IMAC on Ni^{+2}-NTA column as well as cation exchange chromatography on Mono S HR5/5. We anticipate that this work upon completion will not only shed light on the intricate interaction existing between ERF and kinase Erk2, but it will also pave the way towards the identification of a selective inhibitor of this particular association, with potential therapeutic implication in cases in which an increase in the nuclear ERF levels is required, as is the case of ERF-related craniosynostosis.

Περίληψη

Ο παράγοντας ERF (ETS2 Repressor Factor) είναι ένας ευκαρυωτικός μεταγραφικός ογκοκαταστολέας κι έχει δειχτεί να εμπλέκεται τόσο στην εμβρυική ανάπτυξη του ποντικού όσο και στην ανθρώπινη παθολογία στην περίπτωση της σχετιζόμενης με τον ERF κρανιοσυνόστωσης. Η δράση του ERF στο κύτταρο ρυθμίζεται κυρίως μέσω της φωσφορυλίωσής του από τις κινάσες ERK (Extracellular signal- Regulated Kinases), η οποία λαμβάνει χώρα στον πυρήνα παρουσία εξωκυττάριου μιτογόνου ερεθίσματος και έχει ως αποτέλεσμα την άμεση έξοδο του ERF προς το κυτταρόπλασμα και τη μετέπειτα επαγωγή της έκφρασης των έως τότε ανενεργών γονιδίων- στόχων του παράγοντα αυτού. Παρόλο που η αλληλεπίδραση του ERF με την κινάση Erk2 πραγματοποιείται μέσω δύο μοτίβων FXF που εδράζονται στην ειδική επικράτεια αλληλεπίδρασης του ERF με την ERK (EID- ERK Interaction Domain), επιπλέον περιοχές μέσα στην επικράτεια αυτή φαίνεται να συμμετέχουν σε διαμοριακές επαφές προσδίδοντας ολική σταθερότητα στο σύμπλοκο που δημιουργείται. Συνεπώς, σκοπός της μελέτης αυτής ήταν ο καθαρισμός των δύο αυτών πρωτεϊνών και η μετέπειτα κρυστάλλωση του συμπλόκου που σχηματίζουν με στόχο την βαθύτερη κατανόηση όλων εκείνων των δομικών στοιχείων που καθορίζουν την εν λόγω αλληλεπίδραση.

Η σημασμένη με His-tag κινάση Erk2, ούσα στην ενεργό φωσφορυλιωμένη μορφή της λόγω συν-έκφρασης με ένα συστατικά ενεργό μετάλλαγμα της MEK1 κινάσης, απομονώθηκε σε πρώτη φάση μέσω χρωματογραφίας συγγένειας Ni^{+2} (IMAC- Immobilized Metal- Affinity Chromatography). Ο διαχωρισμός της διπλά φωσφορυλιωμένης, κι άρα πλήρως ενεργούς πρωτεΐνης, από τη μη-φωσφορυλιωμένη, και συνεπώς ανενεργή, επιτεύχθηκε μέσω χρωματογραφίας ανιοντοανταλλαγής στην κολώνα Mono Q HR5/5. Η σημασμένη με GST tag (glutathione S- transferase tag) επικράτεια EID του παράγοντα ERF απομονώθηκε στο πρώτο βήμα καθαρισμού με χρωματογραφία συγγένειας GST. Όμως, η εκτεταμένη πρωτεόλυση της GST-ERF (EID) πρωτεΐνης σε συνδυασμό με το γεγονός ότι η GST αυτή καθ' αυτή είναι διμερής ως προς την τεταρτοταγή της δομή, οδήγησαν στην εμφάνιση ετεροδιμερών συμπλόκων μεταξύ της πρωτεΐνης πλήρους μήκους και των πρωτεολυτικών τμημάτων αυτής. Παρ' όλα αυτά, πετύχαμε την απομόνωση ενός πληθυσμού αποτελούμενου αποκλειστικά από ομοδιμερή της πλήρους μήκους πρωτεΐνης μέσω χρωματογραφίας μοριακής διήθησης στην κολώνα Sephacryl S-200 HR16/60. Τέλος, σε μία προσπάθεια αποφυγής του διμερισμού, προχωρήσαμε στην επανακλωνοποίηση της EID του ERF σε φορέα ειδικό για την προσάρτηση του His-tag στην πρωτεΐνη στόχο. Η νέα πρωτεΐνη απομονώθηκε αρχικά με χρωματογραφία συγγένειας Ni^{+2} και στη συνέχεια καθαρίστηκε περαιτέρω με χρωματογραφία κατιοντοανταλλαγής στην κολώνα Mono S HR5/5. Αναμένουμε ότι το έργο αυτό όταν ολοκληρωθεί, θα αποσαφηνίσει τον τρόπο με τον οποίο συντελείται η αλληλεπίδραση αυτή κι επιπλέον δύναται να ανοίξει το δρόμο προς την ανακάλυψη ενός εκλεκτικού αναστολέα, με πιθανή εφαρμογή στις περιπτώσεις στις οποίες πρέπει να διασφαλισθούν υψηλότερα επίπεδα ERF πρωτεΐνης στον πυρήνα, όπως π.χ. στην περίπτωση της σχετιζόμενης με τον ERF κρανιοσυνόστωσης.

1. Introduction

1.1 MAP kinases

Mitogen- activated protein kinases (MAPKs) are serine/threonine kinases that participate in signal transduction pathways found in almost all eukaryotic organisms [1]. In these pathways, extracellular stimuli lead to the dual phosphorylation of MAP kinases, an event that causes their activation and the subsequent modification of a wide range of cellular substrates. These substrates contain a proline residue in the P+1 site, which is the site next to the phosphorylated amino acid (serine/ threonine), and for this reason, MAP kinases are considered to be proline- directed enzymes [2]. Targets of MAPKs are found in many cellular compartments including nucleus, cytoplasm and plasma membrane and the signaling cascades that are activated each time, seem to regulate many biological processes such as cellular proliferation, cellular differentiation, migration and apoptosis [2, 3]. In vertebrates, there are three best characterized families of MAPKs: the extracellular- signal regulated kinase (ERK) family, the p38 family and the c-Jun N-terminal kinase (JNK) family [4-6]. Each family in mammals includes the following members: ERK1-8 (as far as the ERK family is concerned), p38α/β/γ/δ (p38 family) and JNK1-3 (JNKs) [6].

1.2 RAS/RAF/ERK pathway

The ERK pathway is one of the most studied MAPK pathways probably because of its role in many cellular functions that when dysregulated upon specific mutations, lead to pathological conditions including tumor development [7, 8]. In this pathway, extracellular stimuli such as growth factors, bind to their respective receptors tyrosine kinase (RTKs), an event that leads to receptor dimerization and cross-phosphorylation in tyrosine residues. The phosphorylated tyrosine residues of the receptor are then bound by adaptor proteins such as GRB2, which in turn recruit guanine nucleotide exchange factors (GEFs) like SOS (son of sevenless) to the plasma membrane (Figure 1). The interaction between GEF proteins and RAS leads to a conformational change of the latter that favors the binding of GTP instead of GDP. The GTP-bound RAS is the active form of the protein that promotes the activation of RAF (rapidly accelerated fibrosarcoma), which in turn initiates the phosphorylation cascade resulting in ERK1/2 activation and the subsequent modification of a plethora of cellular targets.

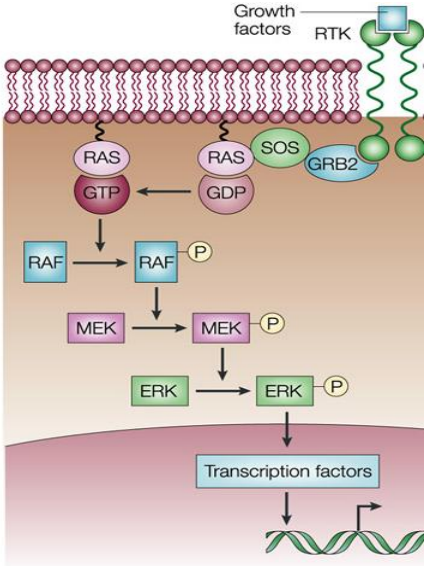


Figure 1: A simplified view of the RAS/RAF/ERK pathway. Chin, L. (2003). The genetics of malignant melanoma: lessons from mouse and man. *Nature reviews. Cancer* 3, 559-570.

MEK1, 2 phosphorylate ERK1, 2 at Thr202 and Tyr204 (for human ERK1), and Thr185 and Tyr187 (for human ERK2). ERKs have been shown to achieve maximum activity only when they are double- phosphorylated. The single phosphorylated forms of ERK2 have less than 1% of the activity of the double- phosphorylated enzyme [9]. ERK1 (p44) and ERK2 (p42) are very similar proteins having 84% sequence identity and seem to share many functions, too [6]. However, while *Erk2* $^{-/-}$ mice are characterized by early embryonic lethality [10], *Erk1* $^{-/-}$ mice are viable, fertile and seem to have normal development, with the exception of a defect observed in thymocyte maturation [11]. Moreover, these mice are considered to be more active in the open field test than their wild- type littermates [12].

1.3 Erk2 Structure and Docking Interactions

The molecule of rat Erk2 consists of two lobes, the N-terminal lobe (the so-called N-terminal domain) with residues 1-109 and 320-358, and the C-terminal lobe (the C-terminal domain) with residues 110-319 [13]. The N-lobe is composed mainly of β-strands (β1-β5), but there are also 2 helices, helix αC and helix αL16 (Figure 2A). The glycine- rich loop between β1- β2 strands (called the P-loop) serves as the ATP- binding site along with the residues Asp104, Asp109, Lys112 and Lys52 that create a hydrogen bonding network upon ATP binding (Figure 3). The C-lobe is formed mainly by α helices, but there are also 4 short β-strands (Figure 2A). This lobe contains a) the phosphorylation lip (the activation loop), which is the site of the phospho-acceptor residues Thr183 and Tyr185, b) the MAP kinase insertion after helix G that consists of 2 helices: α1L14 and α2L14 and c) the P+1 recognition site and the catalytic loop (C-loop) with Asp147 as the putative catalytic residue. The P+1 recognition site is formed by the same loop that forms the phosphorylation lip and Tyr185 is considered to participate in substrate recognition. The P+1 specificity region includes the following conserved sequence: ^{185}YVATRWYR^{192}.

In the non-phosphorylated inactive form of Erk2, Thr183 is located on the surface of the molecule and forms a hydrogen bond with Gly180 in the lip. On the other hand, Tyr185 is inaccessible since it is buried in a hydrophobic pocket comprising residues Val186, Arg192, Ile196, Gly202 and Ile207 [14]. It also forms a hydrogen bond with Arg146. The side chain of Arg192 occupies the P+1 site, excluding substrate from binding there. The site is additionally blocked by Val186. However, upon dual phosphorylation, the lip of the active Erk2 molecule is characterized by a completely different conformation (Figure 2B) [13]. The interactions between the lip and the MAP kinase insertion/ helix G once observed in the inactive form of Erk2 are lost and new contacts are now formed in the active molecule between the lip and L16 helix of the N-lobe. The formation of a 3/10 helix is also observed. All in all, the loss of the old ionic contacts of the lip with the C-lobe and the formation of new ones with the N-lobe of the molecule result in the tighter interaction between the two domains of active Erk2. Phosphorylated Thr183 interacts directly with three Arg residues: Arg68 in helix C, Arg146 in the catalytic loop and Arg170 inside the lip (Figure 4A). Phosphorylated Tyr185 moves to the surface of the molecule and binds to Arg189 and Arg192 which initially blocked substrate binding in the inactive form of Erk2 (Figure 4B). So, P-Tyr185 seems to participate in substrate recognition at the P+1 site. Finally, the overall domain closure observed in active Erk2 brings Lys52 of the ATP- binding site closer to Asp147 (the potential catalytic residue mentioned above).

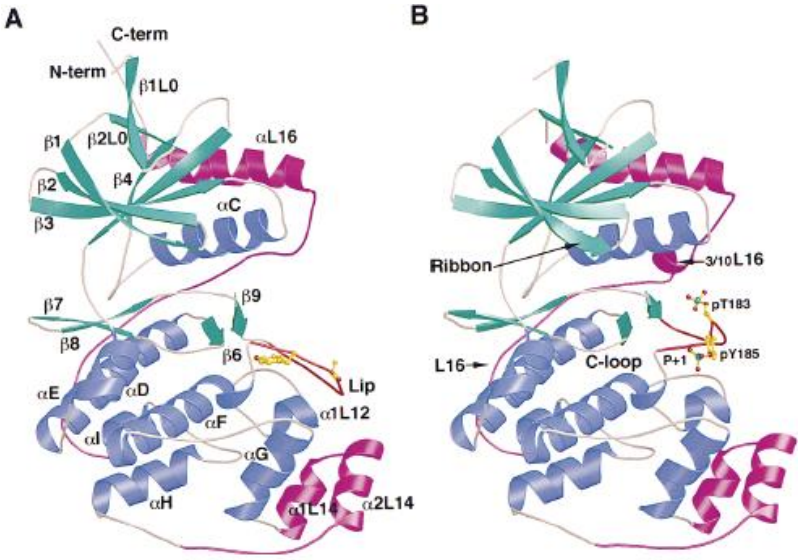


Figure 2: Ribbon diagram of rat A) Erk2 and B) P-Erk2. β-strands are shown in green, helices in blue and magenta. Phosphorylation lip is colored red with side chains of Thr183 and Tyr185 shown. Canagarajah, B.J., Khokhlatchev, A., Cobb, M.H., and Goldsmith, E.J. (1997). Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell* 90, 859-869

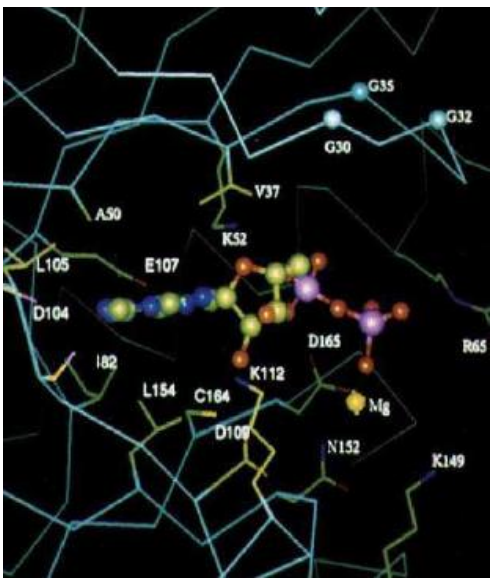


Figure 3: Side chains of residues in contact with Mg-ATP are shown in this stereo diagram. Zhang, F., Strand, A., Robbins, D., Cobb, M.H., and Goldsmith, E.J. (1994). Atomic structure of the MAP kinase ERK2 at 2.3 A resolution. *Nature* 367, 704-711

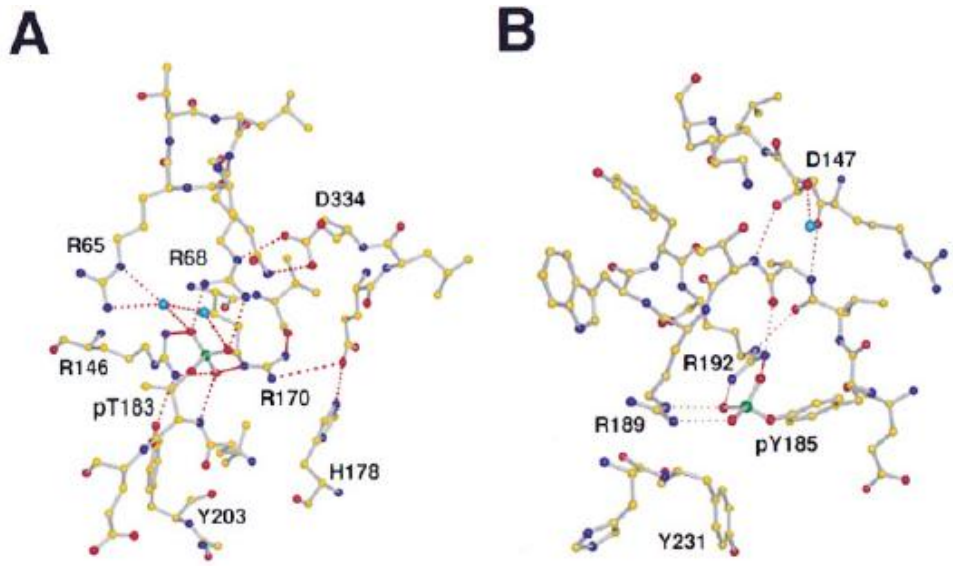


Figure 4: Stereo diagram showing the environment of A) phospho-Thr183 and B) phospho-Tyr185. Ionic interactions of the two phosphorylated residues are depicted. Canagarajah, B.J., Khokhlatchev, A., Cobb, M.H., and Goldsmith, E.J. (1997). Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell* 90, 859-869

The same research group that conducted the above experiments was the one that proposed the model of dimerization between double- phosphorylated active Erk2 molecules. According to this model, phosphorylated Erk2 molecules can form dimers with each other and also with non-phosphorylated ones [15]. The dimer dissociation constant (Kd) between phosphorylated molecules of Erk2 is calculated to be approximately 7,5 nM, 3000 fold lower than that of the non-phosphorylated dimer. The homodimeric population of purified P-Erk2 protein estimated by gel filtration chromatography, increases in size as salt concentration and divalent cations increase too [16]. The dimerization is considered to be performed through a leucine zipper composed of Leu333, Leu336 and Leu344 in the helix L16 from each Erk2 monomer (Figure 5). Two additional residues, His176 of the phosphorylation lip from one molecule and Glu343 in L16 from another Erk2 molecule (partner) contact each other and they seem to be also necessary for the formation of the dimer.

However, there are some contradictory results coming from other research groups regarding Erk2 dimerization that demonstrate that active phosphorylated Erk2 which is tagless- without the His-tag that had been used by the former group for protein purification- is mainly in the monomeric form [17]. As a result, it is still not clear whether double- phosphorylated active Erk2 exists mainly in the monomeric or dimeric state in vivo.

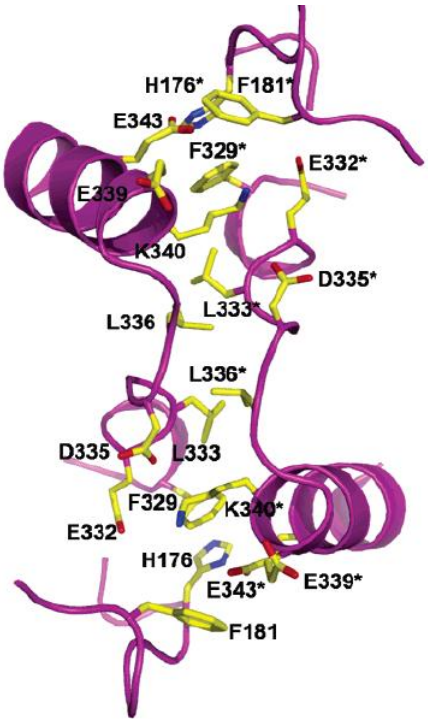


Figure 5: Erk2 residues forming the dimer interface. Asterisks indicate residues of the second Erk2 molecule. Wilsbacher, J.L., et al. (2006). Characterization of mitogen-activated protein kinase (MAPK) dimers. *Biochemistry* 45, 13175-13182

Interactions with targets mediated by the Erk2 active site are characterized by limited specificity, a fact that is true for almost all MAP kinases [18]. However, docking interactions- interactions mediated by distinct sites that are separate from the catalytic site of the kinase- are considered to direct the binding specificity of Erk2 for different substrates. These contacts are performed between the docking motifs lying in the amino acid sequence of various partners (linear motifs) and the respective docking sites present on the surface of the kinase molecule. Two distinct linear docking motifs have been found so far in partners of Erk2: the D-motif or DEJL (docking site for ERK and JNK, LXL), that is found in partners of all three families of MAPKs, and the FXF motif or DEF motif (docking site for ERK, FXF), that exists mainly in ERK substrates [4, 18]. The D-motif is characterized by the following consensus sequence: R/K_{1-2}- X_{2-6}- Φ- X- Φ, where Φ: hydrophobic amino acids and X: any amino acid. The FXF motif consists of 2 phenylalanine residues (F) with a presence of one residue (X) in between them. Some targets of Erk2 contain only one type of docking motifs (either only D-motif, for example RSK1, or only FXF motif, for example c-Fos) and some others contain both of them (ELK-1).

The docking motifs of Erk2 partners mentioned above, bind to separate docking sites on the kinase molecule (Figure 6). D-motif- containing peptides bind to the common docking (CD)/ ΦXΦ groove of Erk2 (the so- called DRS site- D-site recruitment site) [19, 20]. This groove contains the CD site consisting of negatively charged residues (Asp316, Asp319 among them) that interact with the positively charged R/K residues of the D-motif, and the ΦXΦ site consisting of hydrophobic residues. The CD/ΦXΦ groove is located on the opposite side to the catalytic centre of Erk2 and lies at the N-terminus of the loop originating from helix L16, between β7-β8 strands and αD-αE helices. On the other hand, FXF- motif- containing peptides bind to a distinct surface on Erk2, called the FRS site (F-site recruitment site) that is located near the activation loop and the catalytic centre (Figure 6) and it is proposed to involve the following residues: Leu198, Tyr231, Leu232, Leu235 and Tyr261 [19, 21].

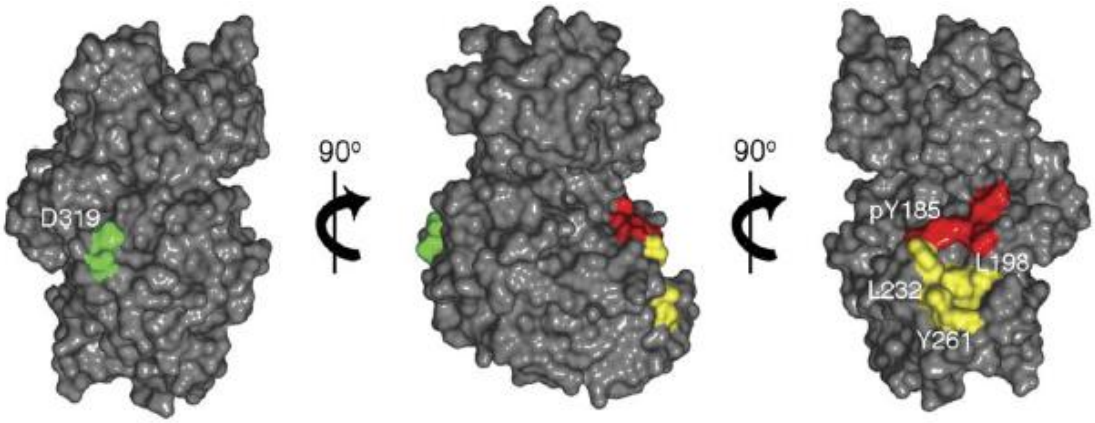


Figure 6: Space-filling model representation of the Erk2 docking sites. CD groove is colored green and FRS site is colored yellow. Phosphorylation lip (activation loop) is shown in red. Dimitri, C.A., Dowdle, W., MacKeigan, J.P., Blenis, J., and Murphy, L.O. (2005). Spatially separate docking sites on ERK2 regulate distinct signaling events in vivo. *Current biology*: CB 15, 1319-1324

1.4 ETS Transcription Factors

ETS proteins (E26 Transformation Specific) are transcription factors characterized by the presence of a common DNA-binding domain, the ETS domain. The family includes 28 members in humans and consists of both transcriptional activators and transcriptional repressors that participate in many cellular functions such as proliferation and differentiation [22, 23]. Although the ETS domain binds DNA sequences of about 12- 15bp in length, it specifically recognizes the following core: 5'-GGA(A/T)-3'. The recognition involves the formation of hydrogen bonds between two arginine residues in H3 helix of the ETS domain and the two guanines of the 5'-GGA(A/T)-3' sequence [22]. Some factors function as monomers while others are reported to bind DNA in a homodimeric form, as is the case of ETS1 upon binding to tandem repeats of the EBS (ETS binding site) [24]. Furthermore, another factor, ETV6 (Yan: ortholog in *D. melanogaster*) is reported to assemble homopolymers through the PNT (Pointed) domain, which is in general found in many members of the ETS family [25]. The PNT domain seems to serve as a mediator of protein- protein contacts and is also considered to participate in the docking interactions observed between ETS1 and the kinase ERK2 [26]. Finally, another feature that is common between many ETS factors is the presence of intrinsically disordered regions into their polypeptide chains [22, 27, 28]. These regions are normally unstructured and are thought to function either as linkers or as regulatory linear motif elements facilitating interactions with various partners due to their increased flexibility.

1.5 ERF (ETS2 Repressor Factor)

ERF belongs to the family of *ets* genes and is responsible for the production of a 548 amino acid polypeptide chain that folds into the 75kDa ERF protein present in almost all cellular types [29]. Like other ETS transcription factors, ERF recognizes and binds to ets binding sites (EBS) lying in the regulatory regions of a variety of genes, through the ETS domain located at the N- terminus of the protein. A repression domain present at the C-terminus of ERF is responsible for its transcriptional suppressor activity [29].

The cellular function of ERF has been shown to be mainly regulated by the phosphorylation status of the protein that changes during the cell cycle in response to mitogenic stimuli thus controlling ERF subcellular localization. The phosphorylation events are attributed to the direct interaction of ERF with ERKs (ERK1/2), and the residues of ERF that are shown to be phosphorylated both in vitro and in vivo by ERKs are: Ser161, Ser246, Ser251 and Thr526 [30, 31]. While in resting cells ERF localizes mainly in the nucleus, upon extracellular stimuli that activate RAS/ERK pathway, ERKs enter the nucleus and phosphorylate ERF at the fore-mentioned sites, an event that leads to the immediate export of the latter from the nucleus and the subsequent transcriptional activation of genes that were previously suppressed by ERF (Figure 7) [31]. These genes are involved in cell cycle progression and cellular proliferation, since phosphorylation- defective ERF mutants (phosphorylation residues substituted by alanines) exhibit constitutive nuclear localization, arrest normal cells at G1 phase and have the ability to block the oncogenic transformation of NIH3T3 cells by the *Ras* oncogene, too [30, 31]. Indeed, ERF, in association with E2F factor and Retinoblastoma protein (Rb), has been shown to repress the transcription of *c-Myc* [32]. Beyond its role in the regulation of cell cycle progression, ERF seems to affect differentiation processes since *Erf* -null mice die at embryonic day E10,5 due to defects in trophoblast stem cell differentiation [33]. Finally, apart from that, recent scientific research revealed that reduced dosage of ERF causes complex craniosynostosis in humans and mice [34], a disorder characterized by the premature fusion of cranial sutures, implicating additional emerging roles for this factor that remain to be further examined.

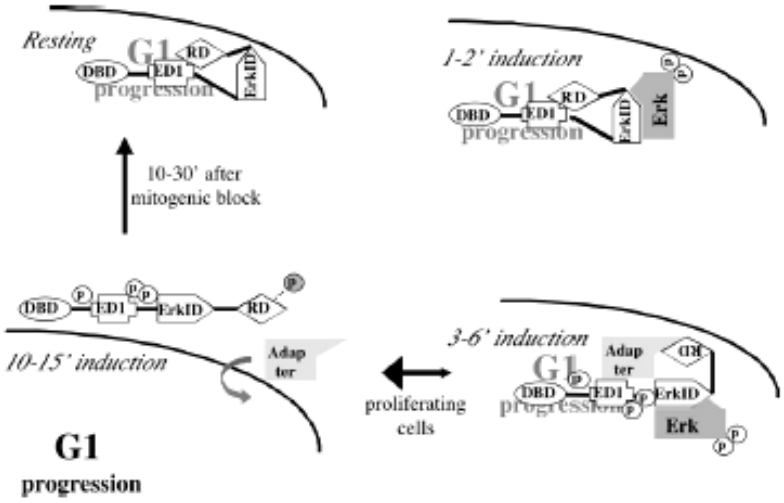


Figure 7: ERK phosphorylation- dependent ERF subcellular localization. In resting cells (upper left), ERK is inactive, ERF exhibits nuclear localization and suppresses genes-targets involved in cell-cycle progression. Upon mitogenic stimulus, ERK is phosphorylated and active and translocates into the nucleus (upper right). Active ERK phosphorylates ERF at multiple sites leading to its conformational change and the subsequent interaction with export machinery (lower right). ERF is further phosphorylated at Thr526 and is released into the cytoplasm (lower left). Transcription of cell-cycle progression genes is activated. Phosphorylated ERF can both enter and exit the nucleus. However once dephosphorylated upon ERK inactivation, it accumulates in the nucleus. DBD: DNA binding domain, ED1: export domain 1, ErkID: ERK interaction domain, RD: repressor domain. Le Gallic, L., Virgilio, L., Cohen, P., Biteau, B., and Mavrothalassitis, G. (2004). ERF nuclear shuttling, a continuous monitor of Erk activity that links it to cell cycle progression. *Molecular and cellular biology* 24, 1206-1218

1.6 Structural insight into ERF- Erk2 Interaction

As mentioned above, association of MAP kinases with partners is mediated by both catalytic domain interactions and docking interactions through distinct docking grooves present on the kinase molecule. In the case of Erk2 MAP kinase, substrates have been shown to bind either through the D-docking motif (DEJL) or through the FXF docking motif (DEF), while others are reported to use both of these linear peptides for successful binding. ERF is shown to interact both with inactive and active Erk2 molecules and possesses four FXF motif sequences and an inverted D- motif sequence located at the repression domain of the protein (Figure 8) [35]. The region of Erk2 that mediates interaction with ERF is a pocket consisting of αF, αG helices and the activation loop.

Experiments, in which truncated forms of ERF had been tested for their ability to bind to active and inactive Erks, showed that the minimal region of ERF that is necessary for this interaction is the area spanning amino acids 294-385 [35]. In particular, the region 294-313 seems to be important for the association with active Erk2 whereas it appears to be dispensable for the association with inactive Erk2 molecules. On the other hand, the region 352-385 seems to mediate interaction with inactive Erk2, but it also contributes to the association with active one. These regions (294-313 and 352-385) contain respectively two of the four FXF motifs identified in ERF sequence as mentioned above. The first FXF motif (position: 303-305, FSF) is considered to be utilized by active Erks, since mutations of phenylalanines to alanines result in decreased association with active Erk2, whereas the second FXF motif (position: 375-377, FKF) seems to be utilized by inactive Erks but also contributes to the contacts

made with active ones. Substitutions of phenylalanines by alanines here, lead to decreased association with inactive Erk2, but once both motifs become mutated (ASA, AKA), even greater decrease is observed in the interaction with active Erk2 only, compared to the single ASA mutation. When the above mutants were tested for their ability to become phosphorylated by Erk2, it was revealed that phosphorylation of the double- mutant ASA/AKA was impaired, since a 10-fold higher Erk2 concentration was used in order to accomplish phosphorylation at levels similar to that of wild-type ERF protein [35]. Last but not least, both ASA and ASA/AKA mutants were characterized by increased nuclear localization in comparison to the wild- type protein.

However, it was observed that mitogenic stimuli during serum induction in cells result in the successful export of the afore-mentioned mutants of ERF from the nucleus, indicative of the fact that ERF-Erk interaction is not completely abolished [35]. Furthermore, peptides carrying the FXF motifs derived from ERF were unable to totally block this interaction even when added at a molar excess of 100-fold. On the contrary, a larger peptide carrying both FXF motifs and their surrounding sequences derived from the ERK interaction domain of ERF (region: 294- 425), was able to significantly increase nuclear concentration of ERF and block Erk2 activity as evident by the decrease in the transcription of an Elk1-regulated serum response element and by the inhibition of Rsk2 activation (both Elk1 and Rsk2 are known Erk2 substrates). In conclusion, the two FXF motifs present in ERF sequence mediate its specific association with Erks, but additional regions of the interaction domain, either through their sequence or through their overall 3-D conformation, determine also the formation of the stable ERF-Erk2 complex.

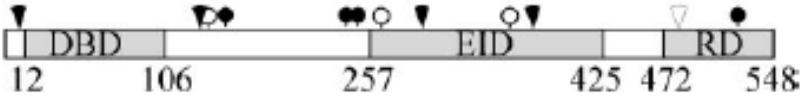


Figure 8: Schematic representation of ERF protein. DBD is the ETS DNA binding domain, EID is the Erk interaction domain and RD is the repression domain. FXF motifs are depicted as closed triangles and the inverted D motif is shown as open triangle. Circles represent possible MAPK phosphorylation sites while closed circles indicate sites found to be phosphorylated by Erks in vivo. The numbers correspond to the boundaries of the three domains of ERF. Polychronopoulos, S., Verykokakis, M., Yazicioglu, M.N., Sakarellos-Daitsiotis, M., Cobb, M.H., and Mavrothalassitis, G. (2006). The transcriptional ETS2 repressor factor associates with active and inactive Erks through distinct FXF motifs. *The Journal of biological chemistry* 281, 25601-25611

1.7 Scientific Aim/ Biological Significance

Although ERF- Erk2 interaction is shown to be mediated by two FXF motifs lying in the amino acid sequence of ERF protein, additional regions in the Erk interaction domain (EID) seem to play important roles in the formation of those contacts that offer overall stability to the ERF- Erk2 complex. As a result, the aim of this work is the purification of the two partners -Erk2 and EID of ERF- and the subsequent crystallization of the complex they form in order to gain a deeper view into all those structural features that determine the specific binding of ERF factor to the Erk2 kinase molecule. This work intends to offer a better understanding of the mechanism of this interesting interaction as well as an insight into the way in which ERKs recognize particular cellular targets. Finally, the achievement of the above goal may also have therapeutic implications since the knowledge of structural elements underlying the association can lead to the identification of a potent inhibitor of this particular interaction that would ensure sustained ERF localization in the nucleus even upon Erk pathway activation. Such a substance could be examined for its ability to alleviate the decreased nuclear presence of ERF in the case of ERF-related craniosynostosis or even be tested against the tumorigenic proliferation in various types of human cancer cell lines.

2. Materials and Methods

2.1 Materials

2.1.1 Expression vectors

- pGEX-4T-1 vector:

This vector is used for the introduction of the GST tag (Glutathione S- transferase tag) upstream of the gene encoding the protein of interest to be cloned. The expression of the GST-tagged protein of interest is driven by the tac promoter which is a hybrid promoter derived from the *tryptophane* (*trp*) and *lactose* (*lac*) promoters [36]. Thus, expression can be repressed by the *lac* repressor and activated when needed by the addition of IPTG (isopropyl-β-D-thiogalactoside). This vector carries also the *lacI* gene for the prevention of leaky expression and the amp^R gene that confers resistance to ampicillin. A thrombin cleavage site is present at the C-terminus of the gene encoding GST for the removal of the tag after protein expression and purification (Figure 1).

The GST- ERF fusion protein used in this study was created previously in our laboratory by inserting the 502bp- Sau3AI/ Sau3AI fragment of ERF coding for amino acids 257-425 (the Erk interaction domain- EID), into the BamHI site of the pGEX-4T-1 vector [35].

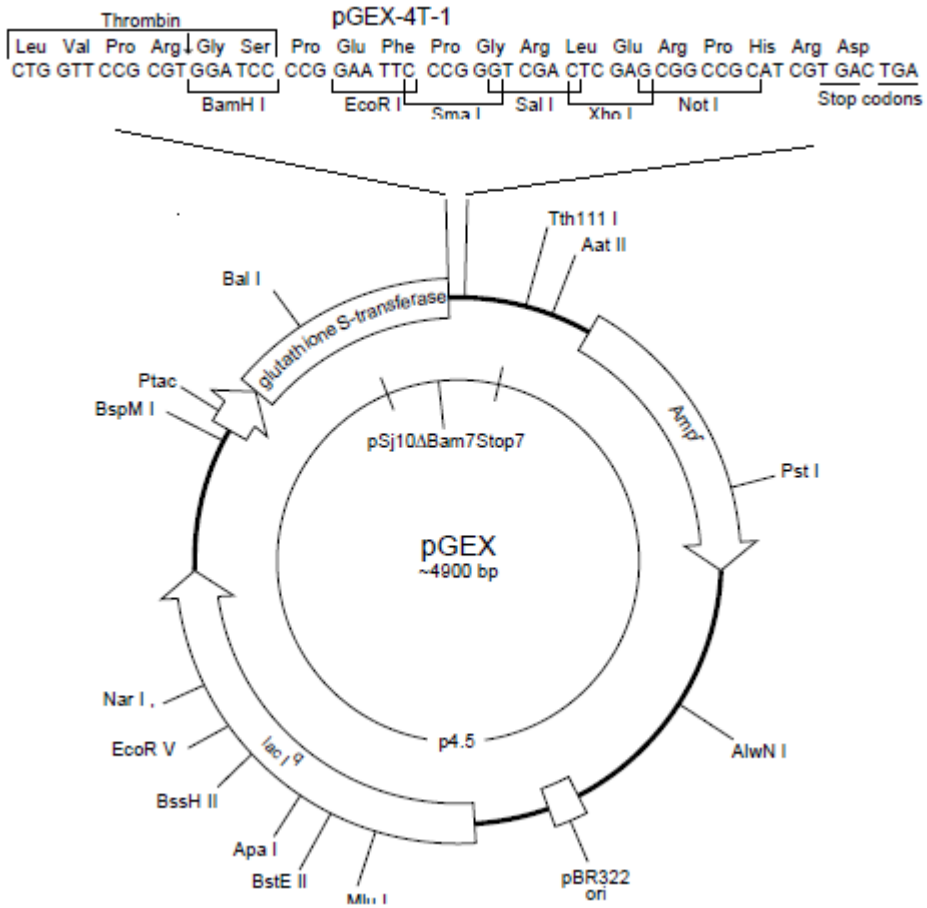


Figure 1: Schematic representation of pGEX-4T-1 vector. Pharmacia Biotech.

- pET-28a (+) vector:

This vector is used for the production of proteins tagged with 6 consecutive residues of histidine (6xHis tag) at the N- terminus. The vector carries also an optional C-terminal His- tag sequence. Expression is driven by the T7 promoter, a promoter recognized by T7 RNA polymerase. The gene coding for T7 RNA polymerase is located at the chromosome of the bacterial strains belonging to T7 system and its expression is regulated by the *lac* promoter [37]. Addition of IPTG results in the expression of T7 RNA polymerase gene and the subsequent activation of the T7 promoter of the vector. The vector carries also the *lacI* gene and the gene conferring resistance to kanamycin (Figure 2).

The 526bp- BamHI/XhoI fragment of ERF was removed from pGEX-4T-1/502 ERF plasmid DNA and cloned into the pET-28a vector digested with BamHI and XhoI restriction enzymes provided by Minotech.

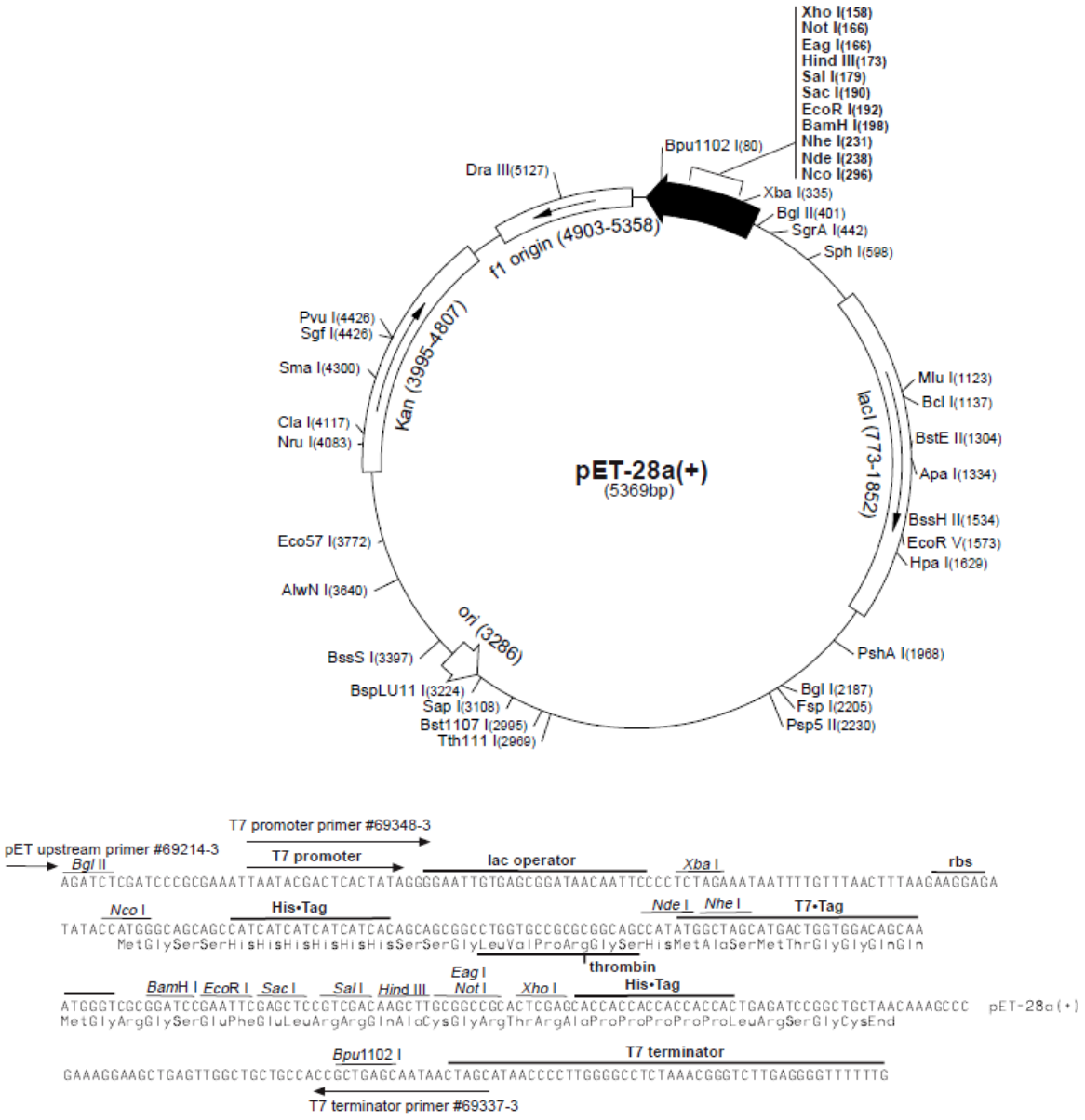


Figure 2: Circular map of pET-28a (+) vector. Novagen.

- pET-MEK1/R4F- His_6-ERK2

This plasmid DNA was kindly provided by Melanie Cobb, University of Texas. It is employed for the production of His-tagged, activated double phosphorylated rat Erk2 due to the simultaneous expression of the constitutively active mutant of MEK1 kinase (ΔN3 S218D S222D, referred to as MEK1-R4F), whose gene is located upstream of the gene coding for Erk2 in the pET vector [38]. An internal ribosomal site lies between the two genes (Figure 3) and the transcription is controlled by a T7 promoter.

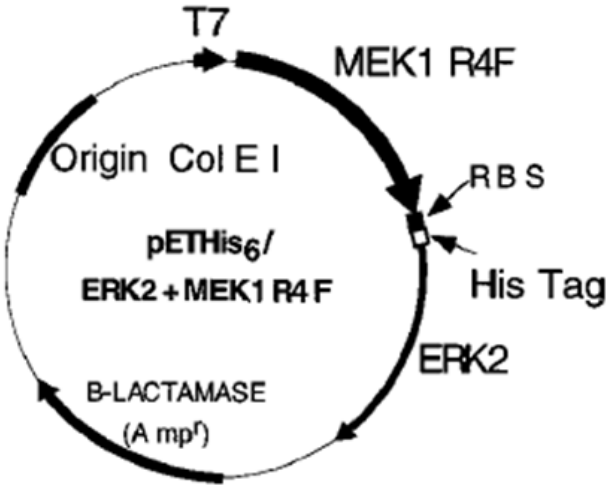


Figure 3: Schematic representation of pET-MEK1/R4F- His_6-ERK2 plasmid DNA. Khokhlatchev, A., Xu, S., English, J., Wu, P., Schaefer, E., and Cobb, M.H. (1997). Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria. Efficient synthesis of active protein kinases. *The Journal of biological chemistry* 272, 11057-11062

2.1.2 Bacterial strains

Plasmids were grown in the following strains:

- XL1-blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *pro*AB *lacI*^{q} ZΔM15 Tn10 (Tet^r)]. These cells are endonuclease (*endA1*) and recombination (*recA1*) deficient and the *hsdR17* mutation they carry protects cloned DNA from cleavage by the EcoK endonuclease system. This strain provides also blue-white color screening facilitating cloning applications.
- DH10b: F– *mcr*A Δ(*mrr- hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ–. This strain was also employed for DNA cloning.

Protein expression was tested in the following strains:

- Origami 2 (DE3): Δ(*ara–leu*)*7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$ *lacI*^{q} *pro*] (DE3) *gor522*::Tn*10 trxB* (Str^R, Tet^R). The mutations in *thioredoxin reductase* (*trxB*) and *glutathione reductase* (*gor*) enhance the formation of disulfide bonds in the cytosol.
- BL21 (DE3) RP: F$^-$ *ompT hsdS*(r_B– m_B–) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [*argU proL* Cam^r]. The cells lack both the Lon and OmpT proteases, thus minimizing degradation during protein expression and purification. This strain carries also extra copies of the genes *argU* and *proL* responsible for the synthesis of tRNAs that recognize the arginine codons AGA, AGG and the proline codon CCC respectively. (DE3: carries the gene for T7 RNA polymerase under control of lacUV5)

2.1.3 Matrices/ Columns for protein purification

The following matrices/ columns were used for protein purification experiments:

- Ni $^{+2}$ -NTA (nitriloacetic acid) resin (by Qiagen): Ni^{+2} is immobilized onto agarose beads through the chelating ligand NTA. The immobilized nickel ion is then bound by the polyhistidine tag of the protein to be purified. This chromatography is referred to as immobilized metal- affinity chromatography (IMAC) [39].

- Glutathione Sepharose 4B (by GE Healthcare): glutathione ligand (GSH) is coupled to cross-linked 4% agarose and is employed for the purification of GST- tagged proteins [40].

- Mono Q HR 5/5 (Pharmacia- GE Healthcare): strong anion exchanger with particle size of 10μm and $-CH_2-N^+-(CH_3)_3$ as the charged group of the gel.

- Mono S HR 5/5 (Pharmacia- GE Healthcare): strong cation exchanger with $-CH_2-SO_3$ as the charged group.

- Superdex 200 HR 10/30 (Amersham Biosciences): gel filtration column with internal diameter of 10mm and bed height of 30cm. It is produced by covalent bonding of dextran to highly cross-linked porous agarose beads. Total bed volume is approximately 24ml. Optimal separation is evident for proteins within the molecular weight range of 10.000- 600.000 daltons. Exclusion limit MW: $1{,}3 \times 10^6$

- Superdex 75 HR 10/30 (Amersham Biosciences): gel filtration column with diameter of 10mm, bed height of 30cm and optimal resolution at molecular weights between 3.000 and 70.000 daltons. Exclusion limit MW: 100.000

- Sephacryl S 200 HR 16/60 (GE Healthcare): gel filtration column with diameter of 16mm, bed height of 60cm and total bed volume of approximately 120ml. It is constructed by covalently cross-linking allyl dextran with N,N' methylene bisacrylamide. Separation is achieved for proteins within the molecular weight range of 5.000- 250.000 daltons. Exclusion limit MW: 400kDa.

2.1.4 Media- Solutions- Buffers

Media for bacterial cultures

LB (Luria Broth):	10g Tryptone	
	5g Yeast Extract	
	10g NaCl	per 1 liter
LB * (electrocompetent cells):	10g Tryptone	
	5g Yeast Extract	
	2g NaCl	per 1 liter

Solutions/ buffers for DNA extraction and analysis

P1 (resuspension buffer):	50mM Tris-HCl, pH=8.0 10mM EDTA 100µg/ml RNase A
P2 (lysis buffer):	200mM NaOH 1% w/v SDS
P3 (neutralization buffer):	3M CH_3COOK pH=5.5 (adjusted with CH_3COOH)
QBT (Qiagen equilibration):	750mM NaCl 50mM MOPS, pH=7.0 15% v/v isopropanol 0.15% v/v Triton X-100
QC (Qiagen wash buffer):	1M NaCl 50mM MOPS, pH=7.0 15% v/v isopropanol
QF (Qiagen elution buffer):	1.25M NaCl 50mM Tris-HCl, pH=8.5 15% v/v isopropanol
TE buffer:	10mM Tris-HCl, pH=8.0 1mM EDTA
TAE buffer (50X):	2.5M Tris- acetate, pH=8.0 50mM EDTA, pH=8.0
DNA loading buffer (6X):	30% v/v glycerol 60mM Tris-HCl, pH=8.0 6mM EDTA 0.6% SDS Orange G dye

Solutions/ buffers for protein extraction, purification and analysis

Lysis buffer (Ni-NTA Binding buffer):	50mM NaH_2PO_4	
	300mM NaCl	pH=8.0
Elution buffer (Ni-NTA resin):	50mM NaH_2PO_4	
	300mM NaCl	pH=7.0
	250mM Imidazole	
Regeneration buffer (Ni-NTA):	6M GuHCl	
	0.2M CH_3COOH (glacial acetic acid)	
Elution buffer (GSH resin):	50mM Tris	
	100mM Glutathione	pH=8.0
PBS (10X):	1.37M NaCl	
	27mM KCl	
	100mM Na_2HPO_4	
	18mM KH_2PO_4	pH=7.4
Mono Q A buffer:	20mM Tris- HCl, pH=8.0	
	50mM NaCl	
	1mM EDTA	
	1mM DTT	
	1mM benzamidine	
	(1mM glycerophosphate,	
	10% glycerol for Erk2 purification)	
Mono Q B buffer:	20mM Tris- HCl, pH=8.0	
	400mM NaCl	
	1mM EDTA	
	1mM DTT	
	1mM benzamidine	
	(1mM glycerophosphate,	
	10% glycerol, for Erk2 purification)	

Mono S A buffer:	50mM HEPES, pH=7.5
	100mM NaCl
	1mM DTT
	1mM EDTA
	1mM benzamidine
Mono S B buffer:	50mM HEPES, pH=7.5
	500mM NaCl
	1mM DTT
	1mM EDTA
	1mM benzamidine
Protease inhibitors:	1mM benzamidine
	1µM pepstatin
	1µM bestatin
	0.1mM AEBSF (Pefabloc)
	10µg/ml aprotinin
	10µg/ml leupeptin

(Different combinations of the above inhibitors were used in purification experiments, as described in 'Methods' section.)

Kinase reaction buffer:	20mM Tris-HCl, pH=8.0
	10mM $MgCl_2$
	1mM DTT
	0.5mM ATP
SDS-gel loading buffer (4X):	200mM Tris-HCl, pH=6.8
	8% w/v SDS
	40% v/v glycerol
	4% v/v mercaptoethanol
	0.04% w/v bromophenol blue
Tris- Glycine Running buffer (10X):	250mM Tris
	2M glycine
	1% w/v SDS pH=8.3

Protein Transfer buffer (1X):	20mM Tris
	160mM glycine
	0.08% w/v SDS
	20% v/v methanol
TBS (10X):	0.5M Tris-HCl, pH=8.0
	1.38M NaCl
	27mM KCl
TBS-T (1X):	50mM Tris-HCl, pH=8.0
	138mM NaCl
	2.7mM KCl
	0.05% v/v Tween20
Coomassie staining solution:	0.25% w/v Coomassie R-250
	45% v/v methanol
	10% v/v CH_3COOH (glacial acetic acid)
De-staining solution:	20% v/v methanol
	10% v/v CH_3COOH (glacial acetic acid)

2.1.5 Antibodies

The following antibodies were used:

Mouse monoclonal anti- MAP kinase, activated (diphosphorylated) ERK1/2, Sigma M8159

Rabbit monoclonal anti- p44/p42 MAPK (ERK1/2), Cell Signaling #4695

Goat polyclonal anti- GST, Pharmacia Biotech 27-4577-01

Mouse anti- His (kindly provided by G. Garinis laboratory)

2.2 Methods

2.2.1 Preparation of electrocompetent bacterial cells

For the preparation of electrocompetent cells, 20ml LB were inoculated with the desired bacterial strain and the cells were grown O/N at 37oC with vigorous shaking. 2ml from this O/N culture were used for the inoculation of 200ml of LB* and the cells grew at 37oC until an optical density (600nm) of 0.6- 0.7 (OD_{600}= 0.6- 0.7). The culture was then placed on ice for 15-30min and finally bacteria cells were harvested by centrifugation at 4000rpm for 15min at 4oC (Sorvall Rotor SLA3000). The supernatant was discarded and the cells were resuspended in 200ml of ice-cold nanopure water. Centrifugation took place followed by additional consecutive washes with 100ml, 20ml, 10ml and finally 4ml of ice-cold nanopure water. In the end, cells were resuspended with 1.5ml of 10% glycerol, dispensed into aliquots of 50μl in 1.5ml pre-chilled tubes and snap frozen in dry ice. The aliquots were stored at -80oC.

2.2.2 Transformation by Electroporation

A cuvette was placed under a UV lamp for 10min. Meanwhile a 50μl aliquot of electrocompetent cells was removed from -80oC and kept for 5 min on ice. 1μl of DNA (1ng/μl) was added to the tube containing the electrocompetent cells and then the mix was transferred into the UV- irradiated pre-chilled cuvette. An electrical pulse of 1800 volts was applied for 5msec. During recovery, cells were incubated in 1ml of LB at 37oC for 45min. Following centrifugation, cells were plated onto LB- agar plates in presence of the appropriate antibiotic and incubated either at 37oC or at RT depending on the requirements of each experiment.

2.2.3 DNA preparations/ cloning

Small- scale plasmid DNA preparation (Mini-prep)

Single colonies were placed into tubes containing 2ml of LB in presence of the antibiotic used for selection and the cultures were grown O/N at 37oC. Next day, 1ml from each culture was transferred into 1.5ml tubes followed by centrifugation at 13000rpm for 1min at RT. Supernatant was discarded and the bacterial pellets were resuspended in 200μl of cold P1 buffer. Cell lysis took place by the addition of 200μl of P2 lysis buffer and incubation for 4min at RT. Finally, 200μl of cold neutralization buffer P3 were added and the lysate was incubated for 5min on ice so as to allow the precipitation of genomic DNA, proteins, cell debris and KDS. Centrifugation at maximum speed for 10min at RT took place and the resulting supernatant containing plasmid DNA was transferred into a new 1.5ml tube. The DNA was precipitated by addition of 0.8 volumes of room-temperature isopropanol followed by incubation for 10min at RT. The tubes containing DNA were centrifuged at max. speed for 10min, the supernatant was discarded and the DNA pellets were washed with 70% ethanol. Finally pellets were air-dried and resuspended in 30-50 μl of TE buffer.

Large-scale plasmid DNA preparation (Maxi-prep)

For large- scale plasmid DNA preparation, 250ml LB containing the appropriate antibiotic were inoculated with the desired bacterial strain and the cells were grown O/N at 37oC. Next day, cells were harvested by centrifugation at 4000rpm for 15min at 4oC (Sorvall Rotor SLA3000) and the pellet was resuspended in 10ml of cold P1 buffer. Lysis was performed by the addition of 10ml of P2 lysis buffer and incubation for 5min at RT. Finally, 10ml of cold P3 neutralization buffer were added, followed by incubation on ice for 20min and centrifugation at 15000xg for 30min at 4oC. The supernatant was then filtered and applied onto a Qiagen-tip 500 column equilibrated with 10ml QBT buffer. The column was washed twice with 30ml of QC buffer and the plasmid DNA was eluted from the column with 15ml of QF buffer. Finally DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol, mixing well and spinning at 15000xg for 30min at 4oC. The DNA pellet was

washed with 5ml of 70% ethanol, air-died after centrifugation and resuspended in 200μl of TE buffer. This protocol is described in detail in Plasmid Purification Handbook provided by Qiagen.

Digests with restriction enzymes

All digests were performed using 10 units of restriction enzyme per 1 μg of DNA. The conditions of each reaction concerning optimal temperature, buffer and duration of digest were selected according to the suppliers' instructions.

Gel extraction of DNA using Phenol

The piece of agarose gel containing the DNA fragment of interest was cut into small slices under a UV lamp and placed into a 1.5ml tube. After centrifugation at maximum speed for 1min at RT, equal volume of phenol was added to the tube containing the gel and mixed by vortexing. The tube was frozen at -80oC for 30min. Then, it was removed from -80oC and centrifuged while frozen at maximum speed for 20min at RT. In case agarose was not melted, the gel was vortexed, frozen again at -80oC and centrifuged again while frozen. The supernatant containing DNA was transferred into a new 1.5ml tube and equal volume of phenol/chloroform (1:1) was added to the tube followed by centrifugation at maximum speed for 10min at RT. The supernatant was in turn transferred into another 1.5ml tube and the DNA was precipitated by the addition of 2 volumes of 100% ethanol and 0.1 volumes of 3M CH_3COONa pH=5.3. The sample was mixed, frozen at -80oC for 20min and centrifuged at 13000rpm for 10min at 4oC. DNA pellet was washed with 70% ethanol, air-dried and resuspended in 5-10μl of TE buffer.

Ligation

100ng of vector DNA were mixed with 30ng of insert DNA according to the following equation:

Insert mass (ng) = 3 x (MW of insert/ MW of vector) x vector mass (ng),

in which a ratio of 1 molecule vector to 3 molecules of insert DNA (1:3) was used, since the DNA fragments to be ligated, had incompatible sticky ends. The reaction was catalyzed by 2.5 units of T4 DNA ligase (Minotech biotechnology, #202) and performed for 4 hrs at 16oC.

2.2.4 Protein Expression/ Purification

Expression experiments using 1lt bacterial cultures

100ml LB medium with the appropriate antibiotic were inoculated with the bacterial strain of interest (either Origami 2 or BL21- DE3- RP) containing the protein expression construct, and the cells were grown O/N at RT (25oC) with vigorous shaking. Next day, OD_{600} of the O/N culture was measured and 1lt LB supplemented with the appropriate antibiotic was inoculated with such a quantity so that the initial OD_{600} of the 1lt culture would be 0.13- 0.15. The cells were then grown at RT (25oC) until an OD_{600} of 0.5- 0.6 was reached. At this time-point, 1ml was removed from the culture, centrifuged and resuspended in 50μl of 1x SDS- protein loading buffer (kept as uninduced control sample). Induction of expression was performed by the addition of either 0.1mM or 0.3mM IPTG (see 'Results' section for details) to the culture and the cells were further incubated for either 2 or 3 hrs at RT (see 'Results' section, too). Finally, 1ml was removed from the culture, centrifuged and resuspended in 100μl of 1x SDS- protein loading buffer (this sample is referred to as the total extract induced sample). The whole culture was then centrifuged at 4000rpm for 15min at 4oC (Sorvall Rotor SLA3000), the supernatant was discarded and the bacterial pellet was either frozen at -20oC or immediately resuspended for cell lysis and subsequent protein purification according the requirements of each experiment ('Results' section).

Expression experiments using 10lt bacterial cultures

As a pre- culture here, 1lt LB medium with the appropriate antibiotic was inoculated with the bacterial strain carrying the protein expression construct and the cells were grown O/N at RT (25oC). Next day, the OD_{600} of the O/N culture was measured and 10lt of LB inside a fermentor- apparatus were inoculated as described above. Cells were grown at RT (25oC) until an OD_{600} of 0.5- 0.6 was reached. The rest of the procedure was exactly the same as this described above for the experiments of 1lt culture. Finally, cells were harvested by centrifugation at 4500rpm for 20min at 4oC (JLA-8.1000 Beckman Rotor). The bacterial pellet was either frozen at -20oC or immediately transferred into a plastic beaker and resuspended in the appropriate amount of cold sonication buffer. In particular, the weight of the pellet was measured and 5ml of cold sonication buffer were added per 1 gr.

Protein Extraction for IMAC and GST- affinity chromatography

Bacterial pellet (from 1lt culture) was resuspended in 40ml of cold sonication buffer: either Ni-NTA Binding buffer (for IMAC) or PBS (for GST- affinity chromatography) containing the protease inhibitors listed in detail in each experiment (see 'Results' section). In the case of His_6- Erk2, lysozyme was added to the binding buffer at a final concentration of 2mg/ml and the sample was incubated for 45min on ice. Cell lysis was completed by sonication. In the case of GST- ERF and His_6-tagged ERF, sonication was also followed by the addition of 1% Triton X-100 and incubation of the sample on a rotating platform at 4oC for 30min. The sample was then transferred into specific pre-chilled polycarbonate tubes and centrifuged at 12.000xg for 30min at 4oC (10.000rpm in Sorvall Rotor SS-34). Finally, supernatant was transferred into a new pre-chilled 50ml- tube for the subsequent purification procedure.

Immobilized Metal- Affinity Chromatography (IMAC)

IMAC was used as the first step of purification for the 6xHis- tagged proteins in our experiments. Ni-NTA resin was loaded onto gravity- flow columns, let to settle and then equilibrated with at least 10 column volumes of cold Binding buffer (50mM NaH_2PO_4, 300mM NaCl, pH=8.0). Meanwhile, the protein sample was centrifuged at 12.000xg for 30min at 4oC (10.000rpm in Sorvall Rotor SS-34) so as to be free of any proteins that had possibly precipitated. The supernatant was transferred into a new tube and 100μl were then removed and kept as a load/input control sample while the rest of the sample was loaded onto the equilibrated Ni-NTA column. The flow- rate was adjusted at approximately 0.5ml/min and the flow-through sample was collected in a separate 50ml- tube. Column was then washed with 10 column volumes of cold Washing buffer (50mM NaH_2PO_4, 300mM NaCl, 50-70mM imidazole, pH=7.0- see 'Results' section), the initial volumes passing through the column were collected into separate fractions, and finally, protein elution was performed by the addition of 4- 5 column volumes of cold Elution buffer (50mM NaH_2PO_4, 300mM NaCl, 140-250mM imidazole pH=7.0). This procedure was performed either at RT (in the case of His-tagged Erk2) or at 4oC (in the case of His-tagged ERF protein). Ni-NTA resin can be reused following wash with 0.5M NaOH for 30min.

GST- Affinity Chromatography

A bed volume of 2ml of Glutathione sepharose beads, equilibrated with PBS, were added to the centrifuged cell lysate, from which a sample of 100μl had been earlier removed and kept as a load/input control sample. The incubation with the GSH beads was performed O/N at 4oC with gentle agitation. Next day, the beads were sedimented by centrifugation at 500xg for 5min at 4oC, transferred into a gravity- flow column and allowed to settle. The lysate was loaded onto the column and the flow-through was collected in a separate 50ml-tube. The whole procedure took place at 4oC. Column was washed with 20 column volumes of cold PBS containing 1mM PMSF. Finally, elution was performed at 4oC by the addition of 3 column volumes of cold Elution buffer (50mM Tris, 100mM GSH pH=8.0) containing 1mM benzamidine. The column was tapped and incubated for at least 15min with

the elution buffer. The eluted protein was collected in fractions of 1ml. GSH beads can be reused following extensive wash with PBS.

Ion Exchange Chromatography

Ion exchange chromatography was performed using Pharmacia FPLC system with two pumps allowing for gradient elution. For anion exchange chromatography, Mono Q HR 5/5 was connected to the system, while Mono S HR 5/5 column was used respectively for cation exchange chromatography. The pressure limit was set to 3.5 MPa. All chromatographic runs took place at room temperature (RT). Before each run, pump A and pump B were washed with degassed nanopure water and then respectively with Buffer A (Mono Q A or Mono S A) and Buffer B (Mono Q B or Mono S B). All buffers were filtered through a 0.22μm filter. For column equilibration, 5 column volumes (5ml) of Buffer A were pumped, followed by 10 column volumes (10ml) of Buffer B and finally, re-equilibration was performed by washing with 10 column volumes (10ml) of Buffer A. The protein sample dialyzed against Buffer A, was either centrifuged or filtered and then applied onto the column by injection. The flow-rate was adjusted at 0.5ml/min. The FPLC programs used for gradient elution in different experiments are demonstrated in 'Results' section. Fractions of 1ml were collected and analyzed by SDS-PAGE. At the end of each experiment, column was washed with 5ml of Buffer B, 10ml of 2M NaCl so as to remove any protein left bound, and then re-equilibrated with Buffer A. Both pumps were washed with nanopure water. For long-term storage, the column was also washed with water and finally, stored in presence of 20% ethanol.

Gel Filtration Chromatography

For gel filtration chromatography, either Superdex 75 HR10/30 or Superdex 200 HR10/30 columns were connected to Pharmacia FPLC, while Sephacryl S 200 HR16/60 column was connected to the AKTA FPLC system. Superdex columns were equilibrated with 4 column volumes (100ml) of gel filtration buffer (50mM Tris-HCl pH=8, 250mM NaCl, 1mM EDTA, 1mM DTT) and Sephacryl column was equilibrated by addition of 2 column volumes (240ml) of buffer. The pressure limits were set as follows: 1.5MPa for Superdex 75, 1MPa for Superdex 200 and 0.15MPa for Sephacryl S200. The protein samples were concentrated by dialysis against gel filtration buffer containing 50% glycerol, centrifuged at maximum speed and applied onto columns by injection. For gel filtration chromatography through Superdex columns, the flow-rate was adjusted at 0.4ml/min and fractions of 0.4ml were collected, while for chromatography through Sephacryl S200 column, a flow-rate of 0.2ml/min was applied and fractions of 2ml were collected.

SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western blot

Protein samples were mixed with SDS-loading buffer and heated for 5min at 100oC. They were then loaded onto an acrylamide gel consisting of a 4% upper part (stacking gel) and a 12% separating gel and subjected to electrophoresis. At the end of the electrophoresis, the gel was either stained with Coomassie R-250 or used for protein transfer into a nitrocellulose membrane (western blot). The transfer of proteins was performed using the semi-dry method in a SEMI-PHOR transfer apparatus for 90min at 40mA. Finally the membrane was removed, air-dried and incubated for 30min into blocking solution containing 5% nonfat milk dissolved into TBS-T. Then the membrane was incubated O/N in the primary antibody solution (antibody diluted in TBS-T) at 4oC with gentle agitation. Next day, three washes of 5min each, were performed with TBS-T followed by incubation of the membrane with the secondary antibody solution (diluted also in TBS-T) for 1 hour at RT. The secondary antibody was conjugated with HRP (horseradish peroxidase). Finally, after three washes with TBS-T proteins of interest were detected on the membrane by the addition of chemiluminescent substrates like ECL system provided by GE Healthcare.

Coomassie staining

The gel was incubated with Coomassie staining solution for 45-60min at RT with gentle agitation. De- staining was finally performed by the addition of De-staining solution and incubation of the gel for 3-5 hours at RT. Usually 3-4 changes of De- staining solution were needed during this time.

Bradford assay

For the estimation of protein concentration, 5-10μl of protein sample were added into 800μl of dH_2O and then mixed with 200μl of 5X Protein Assay Dye Reagent (Bio-Rad). Absorbance at 595nm was measured using a spectrophotometer and finally protein concentration was estimated by a standard curve of BSA solutions of known concentration. Each measurement was performed in duplicates.

In vitro kinase assay

2μg of either GST-ERF or His-tagged ERF protein were incubated with 200ng of His-tagged Erk2 kinase in presence or absence of 0.5mM ATP in kinase reaction buffer (20mM Tris-HCl pH=8, 10mM $MgCl_2$, 1mM DTT). The reactions were performed at 30oC for 45- 60min and in final volume of 20μl. Finally they were stopped by the addition of 4x SDS-loading buffer. 13μl from each reaction were loaded onto an SDS-PAGE gel.

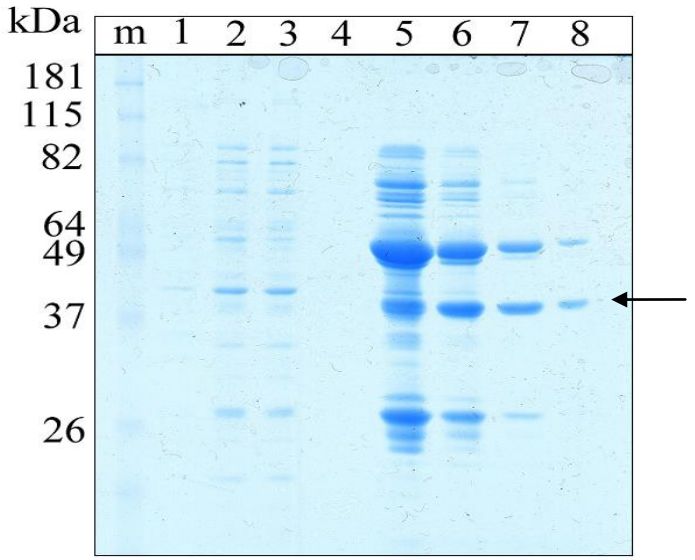
3. Results

3.1 Purification of 6xHis-tagged Erk2

3.1.1 Testing purification conditions on Ni-NTA resin- 1st Step of His-Erk2 purification.

In this initial experiment, 1lt LB medium was inoculated with Origami 2 (DE3) bacterial strain transformed with pET-MEK1/R4F- His_6-ERK2 plasmid DNA. The cells were grown at RT until an OD_{600} of 0.5-0.6 was reached and then induced by addition of 0.3mM IPTG for 3 hours at RT. Cells were finally harvested by centrifugation and pellet was stored O/N at -20oC. Next day, bacterial pellet was resuspended in 40ml of cold Binding buffer containing 10μg/ml leupeptin, 1mM PMSF, 1mM β-glycerophosphate and 2mg/ml lysozyme and cells were incubated for 45min on ice. Lysis was further performed by sonication (10 pulses, each of 30sec, with intervals of 30sec) and cell lysate was finally centrifuged at 12.000xg for 30min at 4oC. Supernatant was transferred into a 50ml-tube and incubated with 1ml of Ni-NTA resin for 60min at 4oC on a rotating platform. The beads were extensively washed with Washing buffer (50mM NaH_2PO_4, 300mM NaCl, 2mM Imidazole pH=7.0) and finally, incubated 3 times with 1ml of Elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM Imidazole pH=7.0) each time for 10min at 4oC for protein elution. Fractions collected at key points of the purification procedure were analyzed by SDS-PAGE followed by western blot and Coomassie staining (Figure 1).

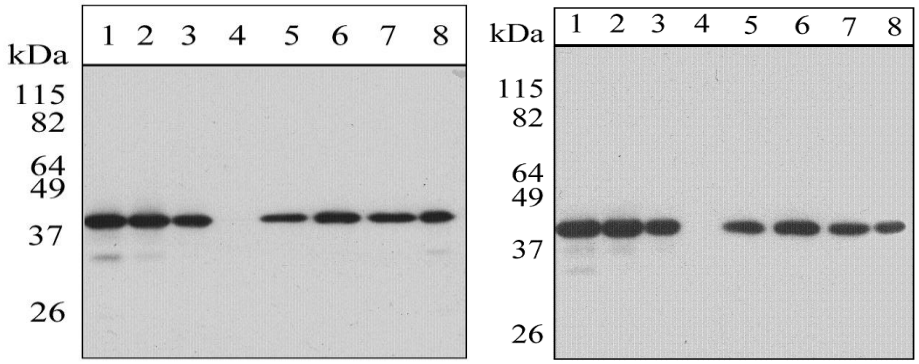
A) Coomassie staining- 12% SDS-PAGE gel



Lanes:

1. Total cell extract (1/20000)
2. Load- Input (1/10000)
3. Flow through (1/10000)
4. Wash (1/10000)
5. Elution 1 (1/100)
6. Elution 2 (1/100)
7. Elution 3 (1/100)
8. Beads- Pellet

B) Western blot- 12% SDS-PAGE gels



Lanes:

1. Total cell extract (1/20000)
2. Load- Input (1/20000)
3. Flow through (1/20000)
4. Wash (1/20000)
5. Elution 1 (1/10000)
6. Elution 2 (1/10000)
7. Elution 3 (1/10000)
8. Beads- Pellet

Mouse anti- phospho p44/p42 (1:8000) Rabbit anti- p44/p42 (1:2000)

Figure 1: Initial purification experiment showing a plethora of bacterial proteins eluted along with His-tagged Erk2 from Ni-NTA beads. The arrow indicates the bands of Erk2. The number beside each sample in parenthesis is referred to the fraction of the bacterial pellet (the fraction of the sample) that was loaded onto the gel.

Looking at lane 3 of western blot results (Figure 1B) we can see that a considerable amount of protein is not bound by the beads (it flows through the column away). The black arrow in Figure 1A shows the bands of Erk2 protein in elution samples (phosphorylated and non-phosphorylated Erk2 proteins that are hardly distinguishable from one other). The presence of Erk2 protein in lane 8 of western blot (Figure 1B) indicates that a fraction of protein is still not eluted (it is still bound by the beads). Consequently, additional steps of elution are needed next time. More importantly however, a plethora of different proteins are bound by the beads and eluted along with Erk2 as indicated by staining of the gel with Coomassie (Figure 1A). In order to remove the non-specifically bound proteins from elution fractions and achieve a higher grade of Erk2 purity from the first step of purification, elution samples were dialyzed against Binding buffer, loaded onto the Ni-NTA column and eluted using buffers with increasing imidazole concentration. The first 2 fractions from each elution step were loaded onto 12% SDS-PAGE gels for analysis.

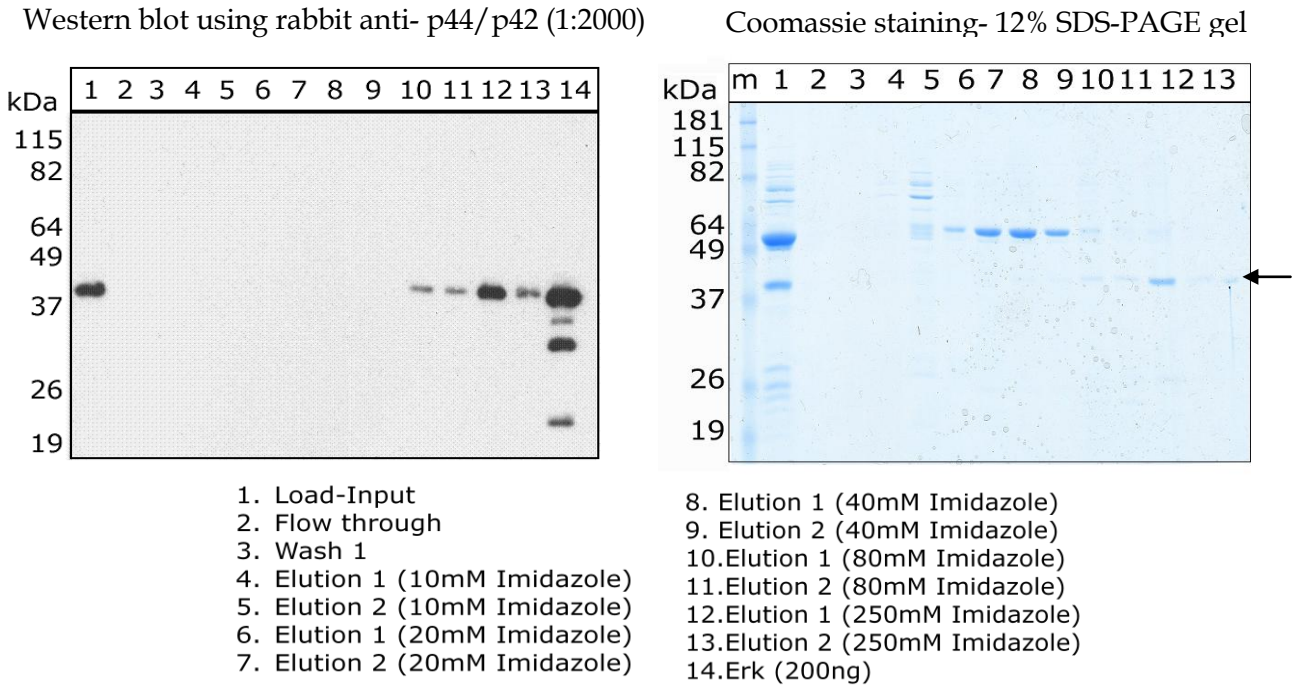
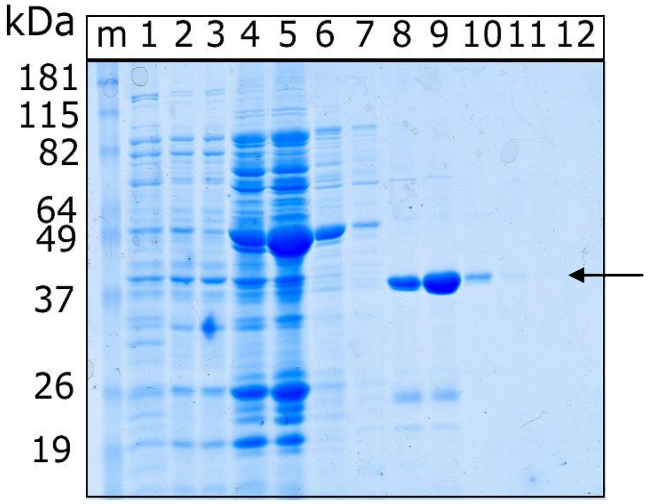


Figure 2: Purification experiment with step-wise elution using buffers with increasing imidazole concentration. His-tagged Erk2 starts to elute at concentrations ≥ 80mM. The arrow indicates the band of Erk2.

The non-specifically bound proteins seem to be eluted from the beads at a lower concentration of imidazole, whereas His-tagged Erk2 starts to get washed off of the column at concentrations ≥ 80mM (Figure 2). As a result, a washing buffer of 50mM imidazole and an elution buffer of 250mM imidazole were tested in the following experiment of Erk2 purification. Indeed, a higher degree of purity was achieved from this first step of purification (Figure 3A). Furthermore, using 1.5ml Ni-NTA resin instead of 1ml, the majority of His-tagged Erk2 protein is now bound by the beads instead of flowing through the column away (see lane 3 in western blot- Figure 3B).

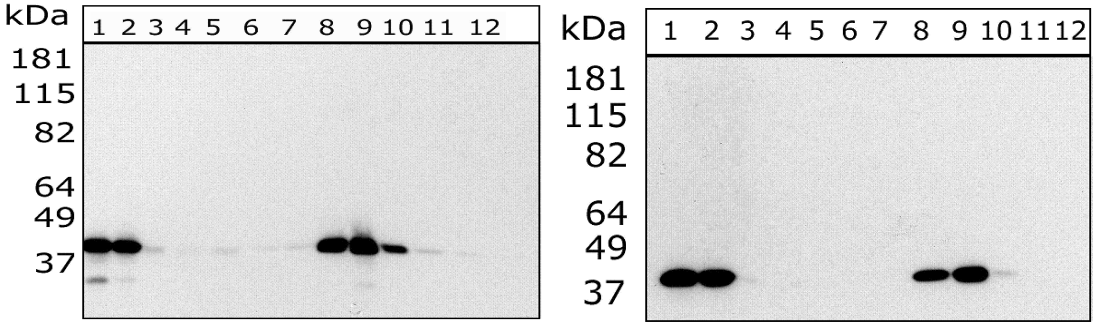
A) Coomassie staining- 12% SDS-PAGE gel



Description of lanes:

1. total cell extract (1/10000 for Coomassie, 1/20000 for Western blot)
2. load- input (1/10000 Coomassie, 1/20000 Western blot)
3. flow-through (1/10000 Coomassie, 1/20000 Western blot)
4. wash 1 (1/100 Coomassie, 1/10000 Western blot)
5. wash 2 (1/100 Coomassie, 1/10000 Western blot)
6. wash 3 (1/100, 1/10000)
7. wash 4 (1/100, 1/10000)
8. elution 1 (1/100, 1/10000)
9. elution 2 (1/100, 1/10000)
10. elution 3 (1/100, 1/10000)
11. elution 4 (1/100, 1/10000)
12. elution 5 (1/100, 1/10000)

B) Western blot- 12% SDS-PAGE gels



Mouse anti- phospho p44/p42 (1:8000)

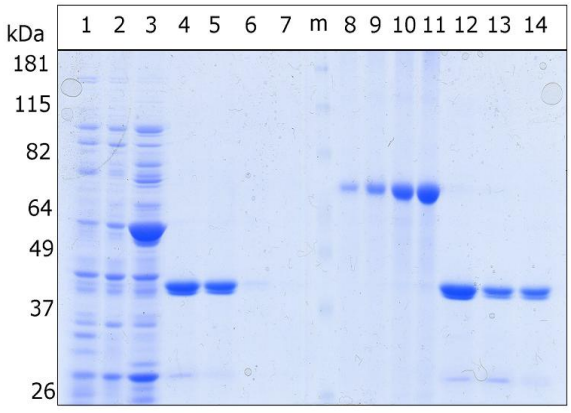
Rabbit anti- p44/p42 (1:2000)

Figure 3: Final conditions selected for His-tagged Erk2 purification include a wash step at 50mM imidazole. A) The arrow indicates the bands of Erk2 (phosphorylated and non-phosphorylated) which are in close proximity to each other. B) The samples of panel A were tested for the presence of total and phosphorylated Erk2 by western blot using the indicated antibodies.

3.1.2 Testing storage conditions- Protein precipitates upon storage in elution buffer at -20oC.

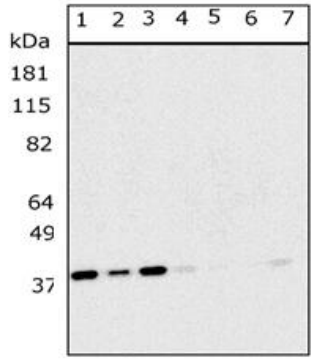
All elution samples containing His-tagged Erk2 protein from the previous experiment were initially stored at -20oC in elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM Imidazole pH=7.0). However, after removal from -20oC and analysis by SDS-PAGE, lower levels of protein were observed in the supernatant after centrifugation of the above samples indicative of the fact that a considerable amount of His-tagged Erk2 had precipitated (Figure 4A, B).

A) Coomassie staining- 12% SDS-PAGE gel



1. Total extract
2. Load
3. Wash 1
4. Elution 1
5. Elution 2
6. Elution 3
7. Elution 4
8. BSA 0,5μg
9. BSA 1μg
10. BSA 2μg
11. BSA 3μg
12. His-Erk2 (Exp1)
13. His-Erk2 (Exp1 after centrifugation)
14. Elution 2 (after centrifugation)

B) Western blot- 12% SDS-PAGE gel



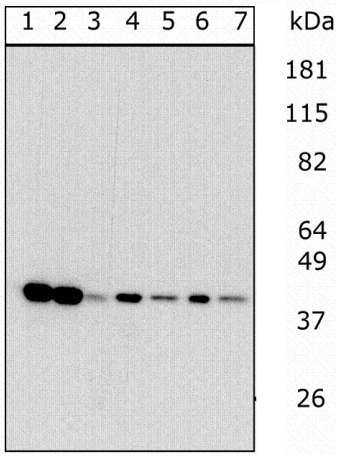
1. Elution 2
2. Elution 2 after centrifugation
3. His-Erk2 (Exp1)
4. His-Erk2 (Exp1, 1/3 dilution)
5. His-Erk2 (Exp1, 1/9 dilution)
6. His-Erk2 (Exp1, 1/27 dilution)
7. His-Erk2 (Exp1, after centrifugation)

Rabbit anti- p44/p42 (1:2000)

Figure 4: His-tagged Erk2 protein precipitates when stored at -20oC in elution buffer. A) Coomassie staining showing samples before and after centrifugation. Compare lane 12 with lane 13, and lane 5 with lane 14. BSA standards were used in order to aid in the determination of the amount of Erk2 protein. B) Detection of the total Erk2 protein in the indicated samples by western blot: compare lane 1 with 2, and lane 3 with 7.

In the following experiment, 50% glycerol was added to the elution samples of His-Erk2 and tested for its ability to prevent protein precipitation. However, immediately upon glycerol addition fiber-like particles appeared in the samples and a lower amount of protein was evident in the supernatant after centrifugation of that samples followed by SDS-PAGE analysis (Figure 5).

Western blot- 12% SDS-PAGE gel



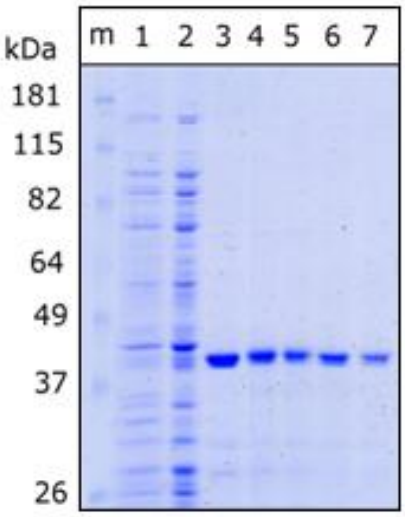
1. Total extract
2. Load- Input
3. Flow-through
4. Elution 1 initial, before adding glycerol
5. Elution 1 + 50% glycerol, after centrifugation
6. Elution 2 initial, before adding glycerol
7. Elution 2+ 50% glycerol, after centrifugation

Rabbit anti- p44/p42 (1:2000)

Figure 5: Addition of 50% glycerol causes a decrease in the solubility of His-tagged Erk2. Total Erk2 protein was detected by western blot. Compare lane 4 with 5 as well as lane 6 with lane 7.

Apart from that, even in the absence of glycerol, it seems that protein tends to aggregate and precipitate while being stored at 4oC in the elution buffer (Figure 6), suggesting that this same buffer is not the appropriate one for long- term storage of the protein perhaps due to its high salt concentration or due to its pH (pH=7) that approximates the isoelectric point of His-Erk2.

Coomassie staining- 12% SDS-PAGE gel



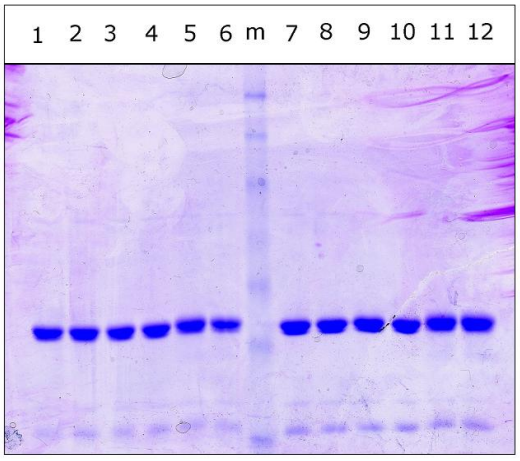
1. Total cell extract
2. Load- Input
3. Elution 1 (initial, before adding glycerol)
4. Elution 1 + 50% Glycerol
5. Elution 1 + 50% Glycerol after centrifugation
6. Elution 2 (initial, immediately after elution process)
7. Elution 2 without glycerol after centrifugation

Figure 6: Protein tends to precipitate while being stored in Elution buffer at 4oC. See lane 6 and compare it with lane 7 which represents the protein existing in the supernatant after centrifugation of sample of lane 6.

For this reason, protein was finally dialyzed against the buffer of the next chromatographic step (Mono Q A buffer) and tested in a series of different storage conditions while being in this buffer: a) at 4oC, b) at -80oC without glycerol (by flash- freezing using dry ice- ethanol bath), c) at -80oC with 50% glycerol (by flash- freezing using dry ice- ethanol bath), d) at -20oC with 10% glycerol and e) at -20oC with 50% glycerol. Protein does not precipitate in any of the conditions listed above (Figure 7). The experiment was performed with two different samples of His-tagged Erk2, Elution 1 aliquot 2 sample and Elution 1 aliquot 3 sample coming respectively from the second and the third batch of the 10lt- culture purification experiment conducted in a fermentor apparatus.

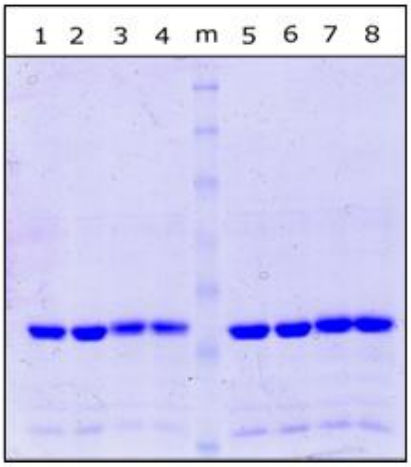
Coomassie staining- 12% SDS-PAGE gels

Testing freezing at -80oC



1. Elution 1 al.2, 4 oC
2. Elution 1 al.2, 4 oC after centrifugation
3. Elution 1 al.2, -80 oC
4. Elution 1 al.2, -80 oC after centrifugation
5. Elution 1 al.2, +50% glycerol -80 oC
6. Elution 1 al.2, +50% glycerol -80 oC after centrifugation
7. Elution 1 al.3, 4 oC
8. Elution 1 al.3, 4 oC after centrifugation
9. Elution 1 al.3, -80 oC
10. Elution 1 al.3, -80 oC after centrifugation
11. Elution 1 al.3, +50% glycerol -80 oC
12. Elution 1 al.3, +50% glycerol -80 oC after centrifugation

Testing freezing at -20oC



1. Elution 1 al.2, +10% glycerol -20 oC
2. Elution 1 al.2, +10% glycerol -20 oC after centrifugation
3. Elution 1 al.2, + 50% glycerol -20 oC
4. Elution 1 al.2, +50% glycerol -20 oC after centrifugation
5. Elution 1 al.3, +10% glycerol -20 oc
6. Elution 1 al.3, +10% glycerol -20 oC after centrifugation
7. Elution 1 al.3, +50% glycerol -20 oC
8. Elution 1 al.3, +50% glycerol -20 oC after centrifugation

Figure 7: No precipitation detected in any of the conditions tested after protein dialysis into Mono Q A buffer. Gel on the left includes samples tested for storage at -80oC and gel on the right includes those tested at -20oC. The amount of protein in supernatant after centrifugation was evaluated in comparison to total protein (before centrifugation).

As a result, we decided to store the protein in Mono Q A buffer (20mM Tris-HCl, pH=8.0, 50mM NaCl, 1mM DTT, 1mM EDTA, 1mM benzamidine, 1mM glycerophosphate) in presence of 10% glycerol also utilized in a relevant publication [38]. The protein was dispensed into aliquots, flash- frozen using dry ice- ethanol bath and stored finally at -80oC until the next chromatographic step.

3.1.3 Separation of the double- phosphorylated, active form of Erk2 from the non-phosphorylated one by ion exchange chromatography- 2nd Step of His-Erk2 purification.

The double- phosphorylated, active form of Erk2 kinase was isolated by anion exchange chromatography performed on a Mono Q HR 5/5 column [38]. Separation here is based on the difference existing in the pI values between the two forms of His-tagged Erk2. The theoretical pI value of the non-phosphorylated form is 6.7 whereas that of the double- phosphorylated form is 6.4. The 6xHis tag was included in the calculation of the above pI values. Protein was loaded onto Mono Q column previously equilibrated with Mono Q A buffer (20mM Tris-HCl, pH=8.0, 50mM NaCl, 1mM DTT, 1mM EDTA, 1mM benzamidine, 1mM glycerophosphate, 10% glycerol), the column was extensively washed with this buffer (25ml- 25 column volumes) and finally elution was performed running the following Mono Q FPLC program:

00.0 CONC % B 0.0

00.0 ML/MIN 0.5

05.0 CONC % B 25.0

05.0 ML/MIN 0.5

55.0 CONC % B 75.0

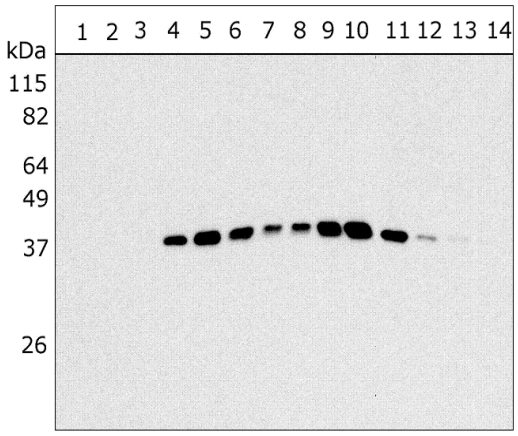
55.0 ML/MIN 0.5

60.0 CONC % B 100

60.0 ML/MIN 0.5

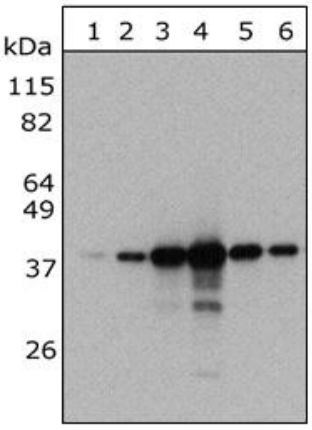
Mono Q B buffer was exactly the same as Mono Q A except that the concentration of NaCl was 400mM instead of 50mM. Fractions of 1ml were collected. Peak fractions were analyzed by SDS- PAGE followed by Coomassie staining and Western blot (Figure 8A, B). Two consecutive peaks were observed. The first peak (fraction 14) was shown to belong to the non-phosphorylated form of Erk2, while the second (fraction 19) was shown to belong to the double- phosphorylated active form of Erk2 (Figure 8A). Fractions containing only the active form were pooled, dialyzed into Mono Q A buffer and flash- frozen at -80oC.

A) Western blot- 12% SDS-PAGE gels



1. Fraction 3 (0,5 µl)
2. Fraction 11 (0,5 µl)
3. Fraction 12 (0,5 µl)
4. Fraction 13 (0,5 µl)
5. Fraction 14 (0,5 µl)
6. Fraction 15 (0,5 µl)
7. Fraction 16 (0,5 µl)
8. Fraction 17 (0,5 µl)
9. Fraction 18 (0,5 µl)
10. Fraction 19 (0,5 µl)
11. Fraction 20 (0,5 µl)
12. Fraction 21 (0,5 µl)
13. Fraction 22 (0,5 µl)
14. Fraction 23 (0,5 µl)

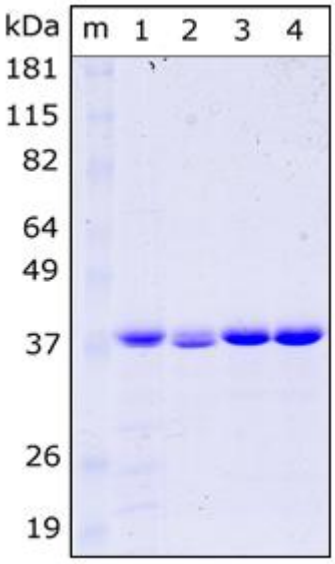
Rabbit anti- p44/p42 (1:10000)



1. Fraction 13 (0,5 µl)
2. Fraction 15 (0,5 µl)
3. Fraction 17 (0,5 µl)
4. Fraction 19 (0,5 µl)
5. Fraction 21 (0,5 µl)
6. Fraction 23 (0,5 µl)

Mouse anti- phospho p44/p42 (1:20000)

B) Coomassie staining- 12% SDS-PAGE gel



Lanes:

1. Load (10µl)
2. Fraction 13 (10µl)
3. Fraction 17 (10µl)
4. Fraction 18 (10µl)

Figure 8: Separation of the double- phosphorylated active form of His-tagged Erk2 from the inactive non-phosphorylated one by anion exchange chromatography. A) Total Erk2 and diphosphorylated Erk2 were detected in the indicated fractions by western blot. B) Coomassie staining of the peak fractions.

Finally, we performed an in vitro kinase assay to check whether His-tagged Erk2 protein was still active at the end of the purification procedure. 2µg of GST-ERF fusion protein were incubated with 200ng of purified double- phosphorylated His-tagged Erk2 in kinase reaction buffer at 30oC in presence or absence of 0.5mM ATP. The final volume of each reaction was 20µl. After 45min reactions were ceased by the addition of 6.6µl of 4x SDS loading buffer. 13µl and 6µl of each reaction (26.6µl new total volume) were loaded onto a 12% SDS-PAGE gel followed by Coomassie staining of the gel. The shifted band of GST-ERF indicates its successful phosphorylation by His-tagged Erk2 (Figure 9). The lower bands belong to the proteolytic fragments of GST-ERF and are also shifted in lanes 3 and 4 indicating possibly that these fragments carry residues- sites of phosphorylation. Consequently, Erk2 being expressed and purified according to the protocols mentioned above is fully active since it is able to interact with and phosphorylate its substrate in vitro.

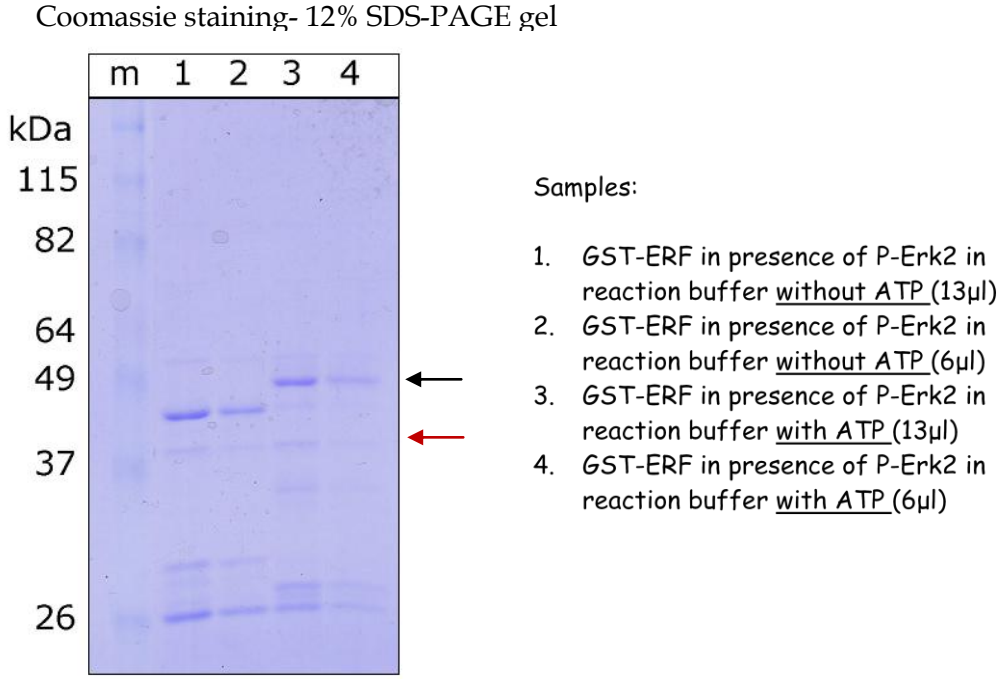


Figure 9: In vitro kinase assay showing that bacterially expressed His-tagged Erk2 is fully active at the end of two chromatographic steps. The black arrow shows the band of GST-tagged ERF that exhibits mobility shift in lanes 3 and 4 (compare lane 1 with 3 and lane 2 with lane 4). The red arrow shows the band of His-tagged Erk2.

3.2 Purification of GST-tagged ERF

3.2.1 Testing purification conditions on GSH- resin- 1st Step of GST-ERF purification

For the production of GST-ERF fusion protein, 1lt LB medium was inoculated with BL21 (DE3) RP bacteria transformed with pGEX-4T-1/ ERF 502 plasmid DNA and the cells were grown at room temperature (RT) until an OD_{600} of 0.5- 0.6 was reached. At this time point, induction of expression was performed by the addition of IPTG at a final concentration of 0.1mM and incubation for 2 hours at RT (as was determined by previous experimental data in our laboratory). Cells were finally harvested by centrifugation and the pellet was stored at -20oC. Next day, the pellet was resuspended in 40ml of cold PBS buffer containing 1mM PMSF and 10μg/ml leupeptin and the cells were lysed by sonication (10 pulses, each of 30sec, with intervals of 30sec). Triton X-100 was added at a final concentration of 1% followed by incubation of the lysate at 4oC for 30min on a rotating platform. Finally the sample was centrifuged, the supernatant was transferred into a new 50ml- tube and 1ml of Glutathione (GSH) resin equilibrated with PBS was added to the sample followed by incubation for 30min at RT with gentle agitation. After centrifugation, resin was transferred into a gravity- flow column and the whole sample was loaded again onto it. Column was washed with 20ml of cold PBS buffer and the protein was eluted by the addition of 1ml of GSH elution buffer 3 times (elution 1, 2, 3). Fractions collected at key points of the purification procedure were analyzed by SDS-PAGE.

Apart from the fact that almost half of the GST-ERF protein produced by the cells flows through the column away (Figure 10A), load/input sample and consequently elution samples seem to be enriched with the lower bands that belong to the proteolytic fragments of the GST-ERF fusion protein (Figure 10A, B). Even though proteolysis is also evident inside cells (Figure 10A), it seems that the purification procedure through sonication- dependent cell lysis affected somehow the stability of the protein.

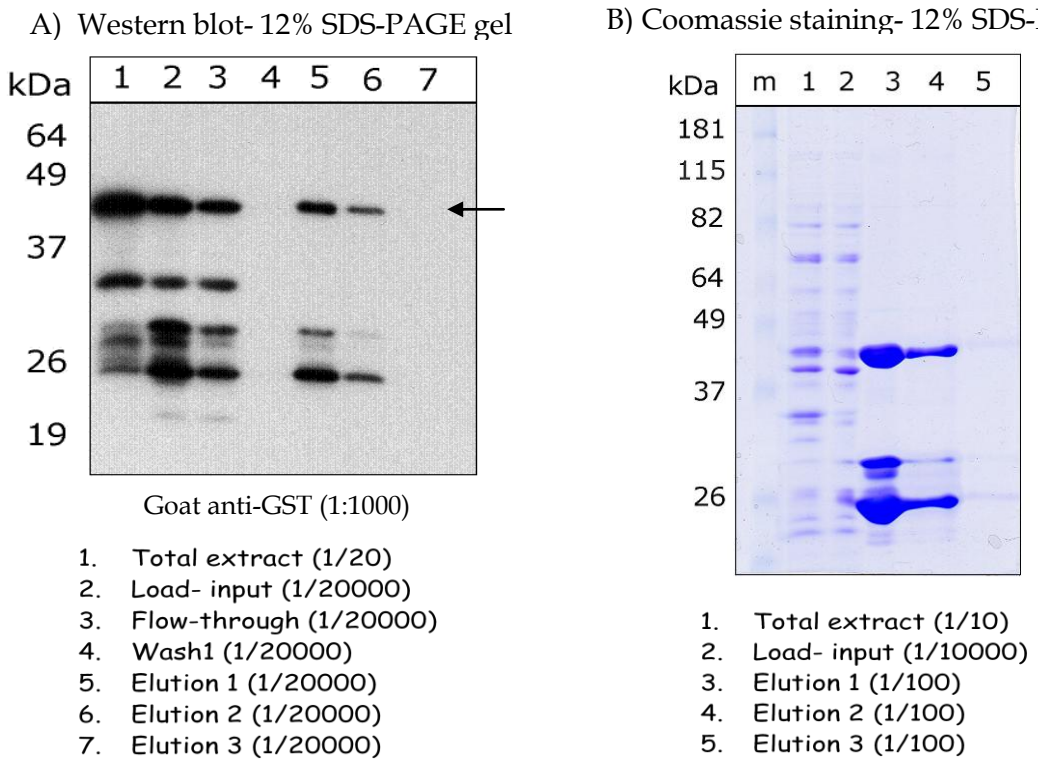


Figure 10: Initial purification experiment of GST-tagged ERF showing increased sensitivity to proteolysis. A) Anti-GST antibody was used to detect the GST-ERF fusion protein. The black arrow indicates the band of the full- length polypeptide chain. B) Total protein in samples was visualized by Coomassie staining.

The first problem was solved upon incubation of the lysate with 2ml of GSH resin O/N at 4oC (with gentle agitation). In order to tackle the problem of proteolysis however, we performed an extensive series of experiments in which a variety of factors were tested. As a result, protein degradation was remarkably reduced upon the following changes we made in the purification procedure protocol. Since storage of the bacterial pellet at -20oC causes increased proteolysis (data not shown), immediately after cell harvesting, the bacterial pellet was resuspended in cold PBS buffer containing a combination of protease inhibitors (0.1mM Pefabloc, 1mM benzamidine, 1mM EDTA, 50μg/ml leupeptin and 10μg/ml aprotinin) and the cells were lysed by mild sonication (5 pulses, each of 30sec, with intervals of 3min). Additional sonication pulses (> 5) were shown to have a negative effect in the stability of GST-ERF protein (data not shown). Following incubation with 1% Triton X-100, the lysate was centrifuged, the supernatant was transferred into a 50ml-tube and then placed at 4oC in a container filled with ice for the O/N incubation with the GSH resin. Next day the sample was centrifuged, the resin was transferred into a gravity- flow column and the whole lysate was loaded again onto it. This time column wash was performed by the addition of cold PBS containing 1mM PMSF. Elution buffer supplemented with 1mM benzamidine was finally added to the column for protein elution. All steps described above were strictly executed at 4oC. The proportion of the full- length GST-ERF protein was notably increased (Figure 11).

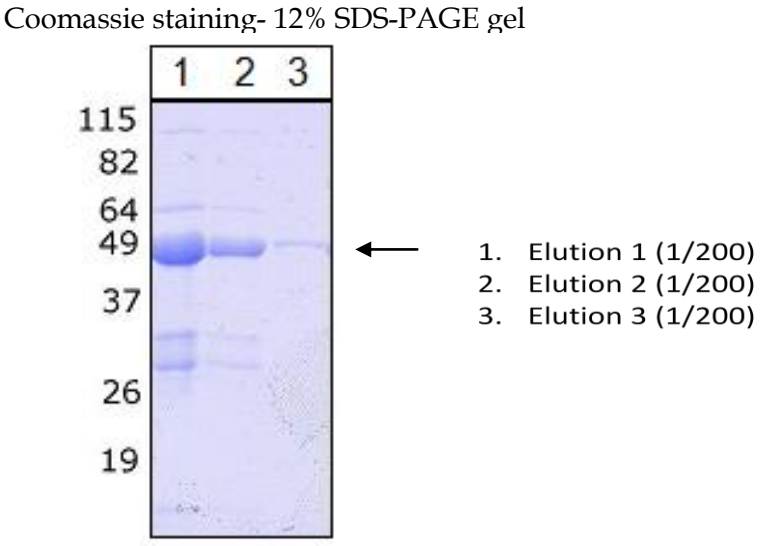
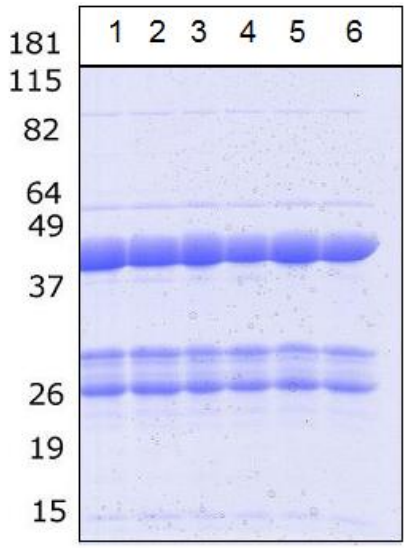


Figure 11: Final conditions selected for lysate preparation and GST- affinity chromatography result in minimization of protein degradation. Total protein content of elution samples was analyzed by SDS-PAGE followed by Coomassie staining of the gel.

Finally, the following experiment was conducted in order to check the stability of the protein purified by the procedure mentioned above. A certain quantity of GST-ERF protein in elution buffer (50mM Tris-HCl, pH=8.0, 100mM GSH, 1mM benzamidine) was removed and kept at 4oC, while the rest of the elution sample was divided into two parts: the first part was dialyzed against 20mM Tris-HCl, pH=8.8, 50mM NaCl, 1mM DTT, 1mM benzamidine and the second part was dialyzed against 20mM Tris-HCl, pH=6.8, 50mM NaCl, 1mM DTT, 1mM benzamidine. After dialysis both protein samples (parts) were stored at 4oC and each day the situation of the protein in the three samples was evaluated by removing a small quantity, adding SDS-protein loading buffer and finally running all samples collected on SDS-PAGE gels for Coomassie staining. Although protein seems to be stable for the first two days at 4oC in all buffers tested, its condition deteriorates notably as time goes by in buffers with pH=8.0 and pH=6.8 (Figure 12- middle gel). After ten days at 4oC protein in the buffer with pH=6.8, appears to be totally cleaved into peptides in contrast to the protein that had been dialyzed into the buffer with pH=8.8 which seems to be more stable (Figure 12-lower gel).

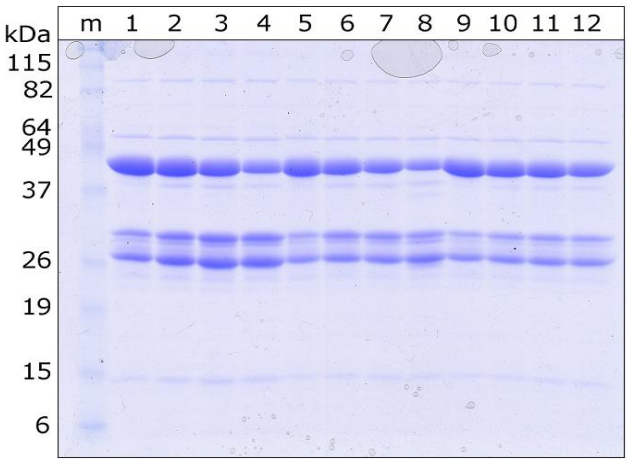
Coomassie staining- 12% SDS-PAGE gels

2 days at 4oC



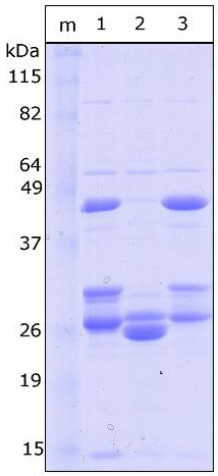
1. GST-ERF in 50mM Tris, pH=8, 100mM GSH, 1mM Benzamidine, Day 1 at 4oC
2. GST-ERF in 50mM Tris, pH=8, 100mM GSH, 1mM Benzamidine, Day 2 at 4oC
3. GST-ERF dialyzed in 20mM Tris, pH=6.8, 50mM NaCl, 1mM DTT, 1mM benzamidine, Day 1 at 4oC
4. GST-ERF dialyzed in 20mM Tris, pH=6.8, 50mM NaCl, 1mM DTT, 1mM benzamidine, Day 2 at 4oC
5. GST-ERF dialyzed in 20mM Tris pH=8.8, 50mM NaCl, 1mM DTT, 1mM benzamidine, Day 1 at 4oC
6. GST-ERF dialyzed in 20mM Tris pH=8.8, 50mM NaCl, 1mM DTT, 1mM benzamidine, Day 2 at 4oC

7 days at 4oC



1. GST-ERF in pH=8, Day 1
2. GST-ERF in pH=8, Day 3
3. GST-ERF in pH=8, Day 4
4. GST-ERF in pH=8, Day 7
5. GST-ERF in pH=6.8 Day 1
6. GST-ERF in pH=6.8 Day 3
7. GST-ERF in pH=6.8 Day 4
8. GST-ERF in pH=6.8 Day 7
9. GST-ERF in pH=8.8 Day 1
10. GST-ERF in pH=8.8 Day 3
11. GST-ERF in pH=8.8 Day 4
12. GST-ERF in pH=8.8 Day 7

10 days at 4oC



Samples:
1. ERF in pH=8 (elution buffer), Day 10
2. ERF in pH=6.8 Day 10
3. ERF in pH=8.8 Day 10

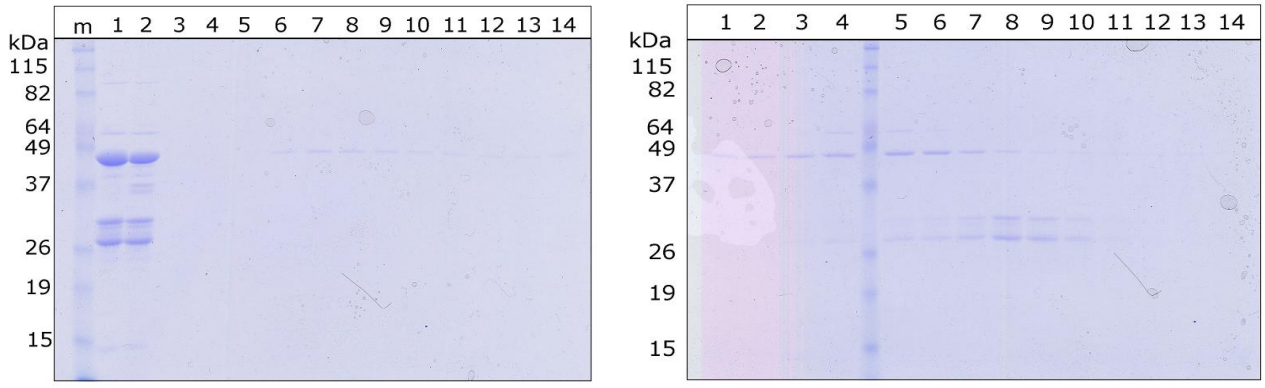
Figure 12: GST-ERF fusion protein is more stable in a slightly basic environment. Protein condition was checked at the indicated time points by SDS-PAGE and Coomassie staining. After 10 days at 4oC, the majority of GST-ERF protein in buffer with pH=6.8 was degraded (lower gel).

3.2.2 Gel filtration chromatography as 2nd Step of purification- Dimerization of GST-ERF protein

In order to isolate the full- length GST-ERF protein from its proteolytic fragments, an experiment of gel filtration chromatography was performed on Superdex 200 HR10/30 column. 100μl of protein concentrated at approximately 7.5μg/μl were injected onto the column which was previously equilibrated with gel filtration buffer (50mM Tris-HCl, pH=8.0, 250mM NaCl). The flow-rate was adjusted at 0.4ml/min and fractions of 400μl were collected. Those fractions containing protein as indicated by the chromatogram (Figure 13A) were analyzed by SDS-PAGE and Coomassie staining of the gel. The results show that GST-ERF protein exists in dimeric forms (Figure 13B). We speculate that this dimerization event is mediated by the tag of the protein, since Glutathione S- Transferases (GSTs) are shown to be dimeric [41]. However, what we observe is that a considerable amount of the full-length GST-ERF protein forms heterodimers with the proteolytic fragments carrying also the GST tag (Figure 13B), a fact that hinders its successful purification. Moreover, the peaks are so close to each other that the isolation of the homodimeric form by this column is impossible.

A) Chromatogram of GST-ERF on Superdex 200 HR10/30

B) Coomassie staining- 12% SDS-PAGE gels



1. Load/input
2. RT control sample
3. Fraction 17
4. Fraction 18
5. Fraction 19
6. Fraction 20
7. Fraction 21
8. Fraction 22
9. Fraction 23
10. Fraction 24
11. Fraction 25
12. Fraction 26
13. Fraction 27
14. Fraction 28

1. Fraction 29
2. Fraction 30
3. Fraction 31
4. Fraction 32
5. Fraction 33
6. Fraction 34
7. Fraction 35
8. Fraction 36
9. Fraction 37
10. Fraction 38
11. Fraction 39
12. Fraction 40
13. Fraction 41
14. Fraction 42

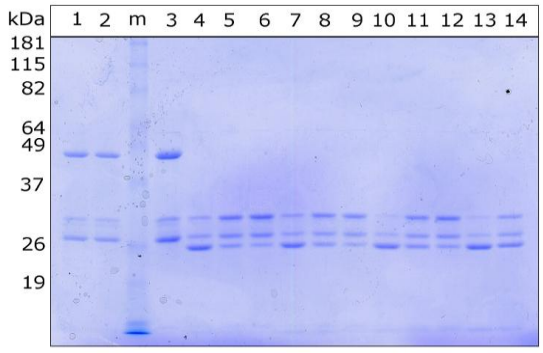
Figure 13: Full-length GST-ERF protein forms heterodimeric complexes with the proteolytic fragments of its polypeptide chain. A) Chromatogram of GST-ERF on Superdex 200 gel filtration column: protein absorbance at 280nm (y axis) in fractions listed below (x axis). B) SDS-PAGE analysis and Coomassie staining of the eluted fractions.

In order to prevent dimerization of full-length ERF protein with its proteolytic fragments through the GST moiety, one option is to cleave the GST tag using thrombin. The vector used for the production of the fusion protein carries a specific sequence recognized by thrombin- a thrombin recognition motif- between the GST tag and the protein to be cloned into the vector. In the following experiments, a variety of different conditions were tested with the aim to find the appropriate combination for a successful enzymatic cleavage: enzyme- substrate ratio, incubation time, temperature and buffer of reaction. In the first experiment, the following parameters were tested:

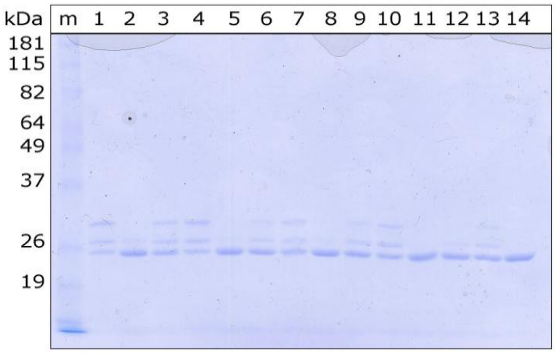
- Enzyme (units) to substrate (μg) ratio:
  - 1:30
  - 1:100
  - 1:300
- Temperature of reaction:
  - RT
  - 37^{o}C
- Incubation times:
  - 15min
  - 1 h
  - 3 hrs
  - 6 hrs
  - O/N

Each reaction included 12μg of GST-ERF protein in PBS as cleavage buffer and in final volume of 20μl. At each time point, 2μg of protein were removed and the reaction was stopped by the addition of SDS-protein loading buffer. Before thrombin experiments, all protease inhibitors were removed from ERF samples by dialysis. Samples were finally analyzed by SDS-PAGE followed by Coomassie staining. Full-length GST-ERF band (at about 46kDa) was disappeared at the earliest time-point (15min after the addition of thrombin), while a band of ~26kDa that belongs to GST arises (Figure 14A). However ERF band (~18-20kDa) does not appear to be anywhere, probably due to the cleavage of the protein into small peptides by thrombin.

A) Coomassie staining- 12% SDS-PAGE gels (Exp.1)



1. ERF control RT (2μg)
2. ERF control 37oC (2μg)
3. ERF initial control sample
4. Reaction 1:30 RT after 15min
5. Reaction 1:100 RT after 15min
6. Reaction 1:300 RT after 15min
7. Reaction 1:30 37oC after 15min
8. Reaction 1:100 37oC after 15min
9. Reaction 1:300 37oC after 15min
10. Reaction 1:30 RT after 1h
11. Reaction 1:100 RT after 1h
12. Reaction 1:300 RT after 1h
13. Reaction 1:30 37oC after 1h
14. Reaction 1:100 37oC after 1h



1. Reaction 1:300 37oC after 1h
2. Reaction 1:30 RT after 3h
3. Reaction 1:100 RT after 3h
4. Reaction 1:300 RT after 3h
5. Reaction 1:30 37oC after 3h
6. Reaction 1:100 37oC after 3h
7. Reaction 1:300 37oC after 3h
8. Reaction 1:30 RT after 6h
9. Reaction 1:100 RT after 6h
10. Reaction 1:300 RT after 6h
11. Reaction 1:30 37oC after 6h
12. Reaction 1:100 37oC after 6h
13. Reaction 1:300 37oC after 6h
14. Reaction 1:30 RT O/N incubation

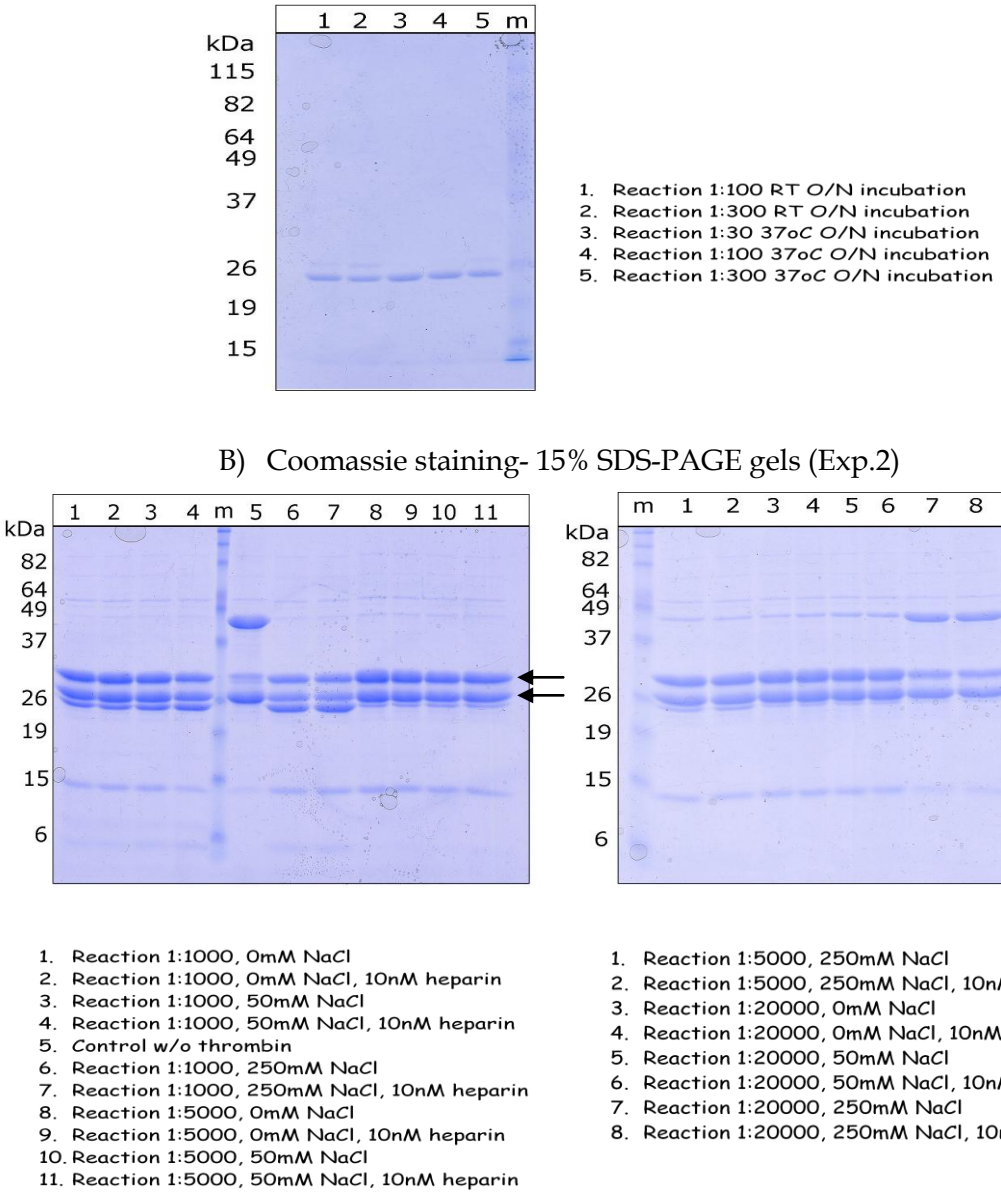


Figure 14: Efforts to specifically remove the GST tag by thrombin digest resulted in the complete cleavage of ERF protein. A) GST band arises at ~26kDa. ERF band (~19-20kDa) is not detected in any of the conditions tested. B) Ionic strength and presence of heparin were tested for their ability to control thrombin specificity. The two black arrows show the pre- existing bands that become more intense upon addition of thrombin.

In the next experiment, we wanted to check whether ionic strength and presence of heparin can affect thrombin specificity [42, 43]. Our aim was to direct specificity towards the GST-specific cleavage site and decrease affinity for putative sites existing in ERF sequence. Each reaction included 10μg of ERF protein in elution buffer, in final volume of 10μl (total volume of reaction). All reactions took place at 25oC for 11 hours and the combinations of the conditions tested are listed in Table 1. Reactions were stopped by the addition of 4x SDS loading buffer and totally loaded onto 15% SDS-PAGE gels for Coomassie staining. Instead of recognizing the GST-specific cleavage site, thrombin seems to initially cut at internal sites of ERF protein resulting in the production of peptides similar to those occurring by the pre-existing proteolytic activity towards GST-ERF protein (Figure 14B). Neither changes in ionic strength nor changes in heparin presence are able to modify the affinity of thrombin for these sites. As

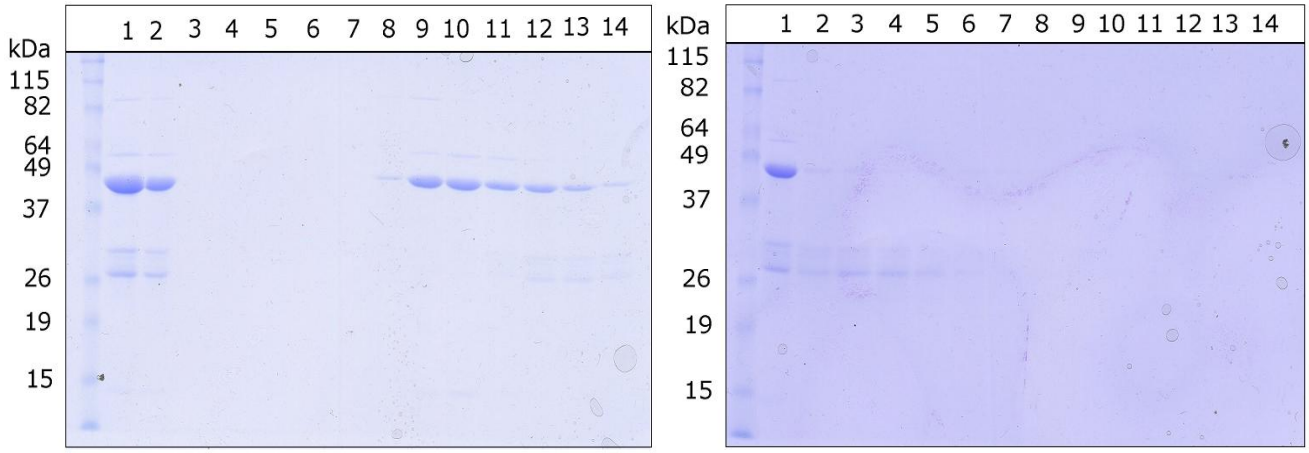
a result selective enzymatic removal of GST tag is not possible in our case and consequently dimerization cannot be prevented.

Enzyme- Substrate Ratio (Units/μg)	Reaction buffer	Heparin presence (10nM)
1. 1:1000	50mM Tris, pH=8	-
2. 1:1000	50mM Tris, pH=8	+
3. 1:1000	50mM Tris, 50mM NaCl, pH=8	-
4. 1:1000	50mM Tris, 50mM NaCl, pH=8	+
5. 1:1000	50mM Tris, 250mM NaCl, pH=8	-
6. 1:1000	50mM Tris, 250mM NaCl, pH=8	+
7. 1:5000	50mM Tris, pH=8	-
8. 1:5000	50mM Tris, pH=8	+
9. 1:5000	50mM Tris, 50mM NaCl, pH=8	-
10. 1:5000	50mM Tris, 50mM NaCl, pH=8	+
11. 1:5000	50mM Tris, 250mM NaCl, pH=8	-
12. 1:5000	50mM Tris, 250mM NaCl, pH=8	+
13. 1:20000	50mM Tris, pH=8	-
14. 1:20000	50mM Tris, pH=8	+
15. 1:20000	50mM Tris, 50mM NaCl, pH=8	-
16. 1:20000	50mM Tris, 50mM NaCl, pH=8	+
17. 1:20000	50mM Tris, 250mM NaCl, pH=8	-
18. 1:20000	50mM Tris, 250mM NaCl, pH=8	+

Table 1: Conditions tested in thrombin experiment number 2. A total of 18 reactions were performed.

Since the separation of the full-length GST-ERF homodimers was not achieved by Superdex 200 HR10/30 column, Sephacryl S 200 HR16/60 was utilized in this gel filtration chromatography experiment. 2ml of GST-ERF protein at a concentration of 1.8mg/ml (in 20mM Tris-HCl pH=8.8, 50mM NaCl, 1mM DTT, 1mM EDTA, 1mM benzamidine and 50% glycerol) were loaded onto the column previously equilibrated with the following buffer: 50mM Tris-HCl, pH=8.8, 250mM NaCl, 1mM DTT, 1mM EDTA, 1mM benzamidine. The flow-rate was adjusted at 0.2ml/min and fractions of 2ml were collected. All fractions containing protein were analyzed by SDS-PAGE and Coomassie staining of the gel. Despite the fact that a considerable amount of full-length GST-ERF protein forms heterodimers with the proteolytic fragments, the isolation of a population consisting solely of full-length GST-ERF homodimers was accomplished (Figure 15).

Coomassie staining- 12% SDS-PAGE gels



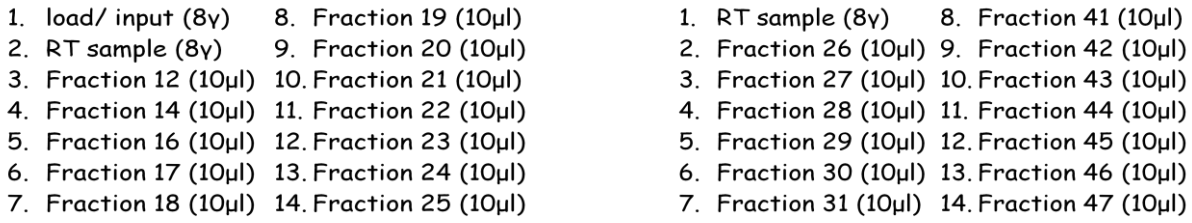


Figure 15: Isolation of full-length GST-ERF homodimeric complexes by gel filtration chromatography on Sephacryl S 200 HR16/60. The first peak (fractions 20, 21- lanes 9, 10 of the gel on the left) is the one containing the homodimeric population of the full-length GST-ERF protein.

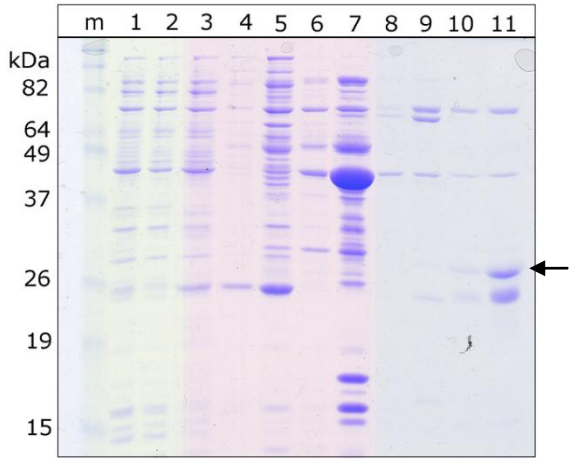
3.3 Purification of 6xHis-tagged ERF

3.3.1 Testing purification conditions on Ni-NTA resin- 1st Step of His-tagged ERF purification

Another solution to the problem of dimerization observed in the case of GST-ERF protein, would be to substitute the GST tag by another one, e.g. the His tag. For this purpose, we cloned ERF EID (ERK Interaction Domain) into a vector carrying a His-tag located upstream of the gene encoding the protein of interest. For the expression of His-tagged ERF protein, 1lt LB was inoculated with BL21 (DE3) RP bacteria transformed with pET-28a/His-ERF plasmid DNA. The cells were grown at RT until an OD_{600} of 0.5-0.6 was reached, and then induced with 0.1mM IPTG for 2 hours at RT. Although GST- ERF protein was efficiently expressed in this bacterial strain, His-tagged ERF was in general expressed at low levels in all strains and under all conditions tested (data not shown). Cells were finally harvested by centrifugation and pellet was either immediately resuspended in sonication buffer or stored O/N at -20oC. The resuspension was performed by addition of 40ml of cold Binding buffer (50mM NaH_2PO_4, 300mM NaCl pH=8.0) containing the following protease inhibitors: 10μg/ml leupeptin, 10μg/ml aprotinin, 0.1mM Pefabloc (AEBSF), 1μM bestatin and 1μM pepstatin. Cell lysis was performed by sonication (6x30sec with intervals of 2min) and the lysate was finally cleared from cellular debris by centrifugation at 11.000xg for 30min at 4oC. Supernatant was transferred into a new 50ml-tube and then loaded onto a gravity-flow Ni-NTA column previously equilibrated with 10 column volumes of cold Binding buffer. The chromatography took place at 4oC because of the sensitivity that had been exhibited by ERF protein towards proteolysis in our previous experiments. Following column washing, elution in this initial experiment was performed using buffers containing increasing imidazole concentration (10mM, 40mM, 80mM and 250mM of imidazole) in order to be able to select the proper conditions for the purification of the protein. Samples collected at key points of the purification procedure were analyzed by SDS-PAGE followed by Western blot and Coomassie staining of the gel.

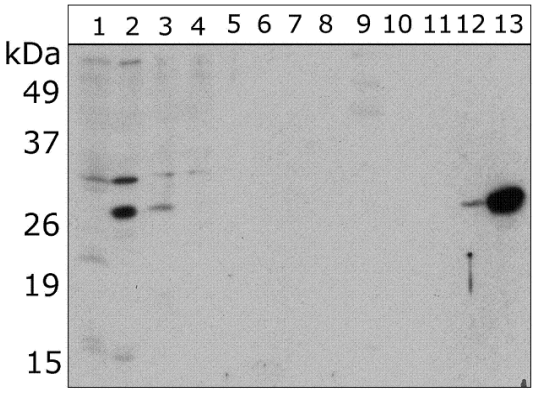
The majority of the bacterial proteins bound to the resin are washed off of the column at a lower concentration of imidazole (40-80mM) whereas His-tagged ERF starts to be eluted at concentrations ≥ 80mM (Figure 16A). Consequently, a wash step at 70mM imidazole and an elution step at 140mM imidazole were selected for the purification experiments that came next. Unfortunately, a variety of bacterial proteins bind the resin with high affinity and cannot be removed from our sample in any of the conditions tested in this step since they are eluted along with His-ERF as shown in this 10lt culture purification experiment conducted in a fermentor apparatus (Figure 16B). Moreover, the protein that was loaded onto the column in our initial experiment seems to be lower in amount in comparison to the total protein produced by the cells (Figure 16C- compare lanes 2, 3), which means either that sonication- dependent cell lysis was incomplete or that the majority of His-tagged ERF protein localizes in pellet after centrifugation.

A) Coomassie staining- 15% SDS-PAGE gel



Lanes:
1. Input (1/10000)
2. Flow-through (1/10000)
3. Wash- 2mM Imidazole (1/100)
4. Elution fraction 1- 10mM Imidazole (1/100)
5. Elution fraction 2- 10mM Imidazole (1/100)
6. Elution 1- 40mM Imidazole (1/100)
7. Elution 2- 40mM Imidazole (1/100)
8. Elution 1- 80mM Imidazole (1/100)
9. Elution 2- 80mM Imidazole (1/100)
10. Elution 1- 250mM Imidazole (1/100)
11. Elution 2- 250mM Imidazole (1/100)

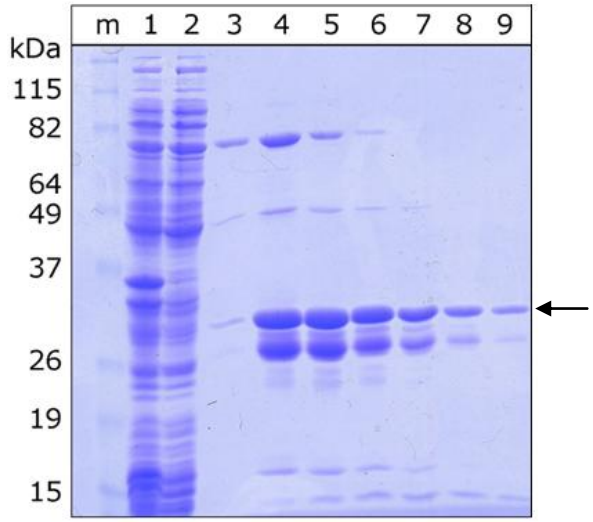
C) Western blot- 15% SDS-PAGE gel



Mouse anti-His (1:1000)

Lanes:
1. Uninduced extract (1/5)
2. Induced total extract (1/10)
3. Input (1/10000)
4. Flow-through (1/10000)
5. Wash- 2mM Imidazole (1/500)
6. Elution 1- 10mM Imidazole (1/500)
7. Elution 2- 10mM Imidazole (1/500)
8. Elution 1- 40mM Imidazole (1/500)
9. Elution 2- 40mM Imidazole (1/500)
10. Elution 1- 80mM Imidazole (1/500)
11. Elution 2- 80mM Imidazole (1/500)
12. Elution 1- 250mM Imidazole (1/500)
13. Elution 2- 250mM Imidazole (1/500)

B) Coomassie staining- 12% SDS-PAGE gel



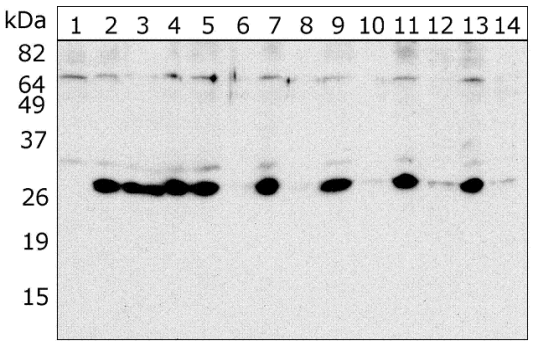
1. Total extract (1/10)
2. Load/ Input (1/100.000)
3. Elution fraction 1 (1/200)
4. Elution fraction 2 (1/200)
5. Elution fraction 3 (1/200)
6. Elution fraction 4 (1/200)
7. Elution fraction 5 (1/200)
8. Elution fraction 6 (1/200)
9. Elution fraction 7 (1/200)

Figure 16: Purification experiment on Ni-NTA resin showing that His-tagged ERF is eluted along with a variety of contaminants. Arrows show the band of His-ERF. A), C) Initial experiment in which step-wise elution was performed using buffers with increasing imidazole concentration. His-ERF is eluted at imidazole concentration > 80mM. B) 10lt culture purification experiment with an elution step at 140mM imidazole. C) His-tagged ERF protein was detected in samples by western blot using anti-His antibody.

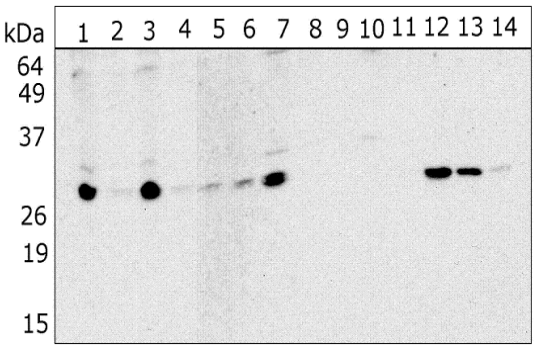
Consequently, in the next experiment, samples of 30μl were removed from the lysate after each pulse during sonication procedure in order to evaluate the amount of His-tagged ERF protein that comes out in supernatant following centrifugation. Interestingly, only traces of protein were detected in supernatant despite the fact that no degradation was observed even after 9 pulses (Figure 17A). This suggests that the majority of the protein is in the post-sonicate pellet. Indeed, this is exactly what happens, as shown in the following experiment in which protein localizes in supernatant upon incubation of the post-sonicate lysate with 1% Triton X-100 (Figure 17B).

A) Western blot- 15% SDS-PAGE gels

Mouse anti-His (1:1000)

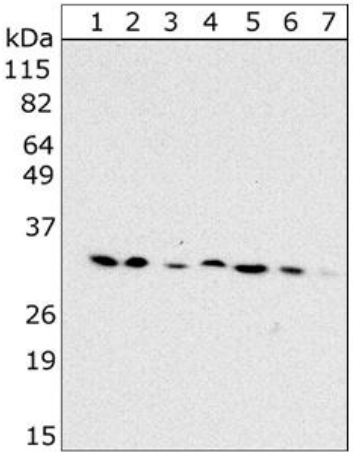


1. Uninduced total extract (1/5)
2. Induced extract- 0,1mM IPTG (1/10)
3. Induced extract- 1mM IPTG, 1h (1/10)
4. Induced extract- 1mM IPTG, 2hrs (1/10)
5. Total extract 3 pulses (1/10000)
6. Supernatant 3 pulses (1/10000)
7. Total extract 4 pulses (1/10000)
8. Supernatant 4 pulses (1/10000)
9. Total extract 5 pulses (1/10000)
10. Supernatant 5 pulses (1/10000)
11. Total extract 6 pulses (1/10000)
12. Supernatant 6 pulses (1/10000)
13. Total extract 7 pulses (1/10000)
14. Supernatant 7 pulses (1/10000)



1. Total extract 8 pulses (1/10000)
2. Supernatant 8 pulses (1/10000)
3. Total extract 9 pulses (1/10000)
4. Supernatant 9 pulses (1/10000)
5. Supernatant (load) before freeze (1/10000)
6. Supernatant (load) after freeze and centrifugation- Input (1/10000)
7. Pellet (1/10000)
8. Flow- through (1/10000)
9. Wash 1 (1/500)
10. Wash 2 (1/500)
11. Elution 1 (1/500)
12. Elution 2 (1/500)
13. Elution 3 (1/500)
14. Elution 4 (1/500)

B) Western blot- 12% SDS-PAGE gel



Lanes:

1. Total extract (1/10)
2. Total extract after sonication before Triton addition (1/100.000)
3. Supernatant before Triton addition (1/100.000)
4. Pellet before Triton addition (1/100.000)
5. Total extract after Triton (1/100.000)
6. Supernatant after Triton (1/100.000)
7. Pellet after Triton (1/100.000)

Mouse anti-His (1:1000)

Figure 17: Addition of Triton X-100 is required for the solubilization of His-tagged ERF protein during extraction from bacteria. Protein levels in supernatant are compared to those of the total protein (total extract) by western blot using anti-His antibody. B) The presence of His-ERF in the supernatant of the post-sonicate lysate was examined in samples before and after addition of Triton X-100 by immunoblotting.

3.3.2 Cation exchange chromatography- 2nd Step of His-ERF purification

Since the theoretical isoelectric point of His-tagged ERF protein is approximately 10.1, cation exchange chromatography was selected as the second step of purification. An aliquot of protein purified by Ni-NTA affinity chromatography was dialyzed O/N against the following buffer (Mono S A buffer): 50mM HEPES pH=8.0, 50mM NaCl, 1mM DTT, 1mM EDTA, 1mM benzamidine. Interestingly, next day, a part of the protein sample appeared to have precipitated. Total protein sample, sample from supernatant after centrifugation and pellet sample were removed and analyzed by SDS-PAGE followed by Coomassie staining of the gel. A part of the total protein sample had indeed precipitated (Figure 18). However, the ratio of the bacterial proteins to His-ERF protein in pellet was completely different upon comparison with the respective ratio of the proteins in our initial sample, suggestive of the fact that probably some bacterial proteins are the ones that precipitate, affecting our own protein too. Examination of the conditions that led to this precipitation event revealed that precipitation occurs when NaCl concentration is below 100mM while pH of the buffer does not seem to affect the solubility of the proteins (data not shown). Consequently, in the following experiments His-ERF protein samples purified by Ni-NTA affinity chromatography were dialyzed against Mono S A buffer containing 100mM NaCl, for further purification by cation exchange chromatography.

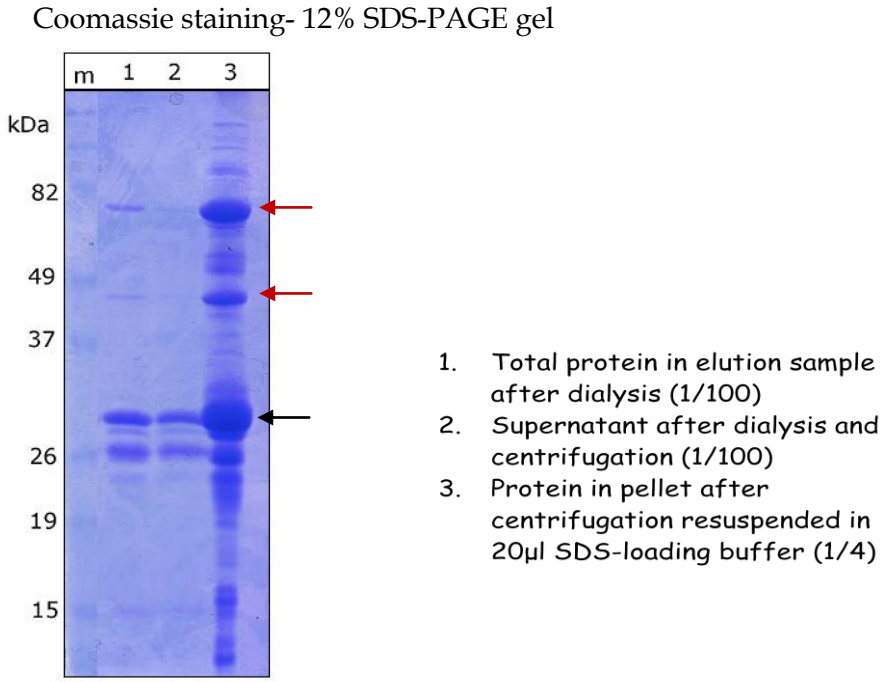


Figure 18: Bacterial proteins eluted along with His-ERF from Ni-NTA column precipitate in Mono S A buffer containing 50mM NaCl. Black arrow shows the band of His-ERF while red arrows show the bands of two bacterial proteins that precipitate. Numbers in parentheses indicate the portion of the sample loaded onto the gel.

A quantity of ~3.36mg of His-ERF protein sample (eluted from Ni-NTA resin) was dialyzed against Mono S A buffer (50mM HEPES pH=7.5, 100mM NaCl, 1mM DTT, 1mM EDTA, 1mM benzamidine) and loaded onto Mono S HR 5/5 column previously equilibrated with the same buffer. Wash was completed by addition of 10 column volumes of Mono S A buffer and finally elution was performed by a salt concentration gradient according to the following Mono S FPLC program:

00.0 CONC % B 0.0

00.0 ML/MIN 0.5

01.0 CONC % B 5.0

01.0 ML/MIN 0.5

51.0 CONC % B 80.0

51.0 ML/MIN 0.5

55.0 CONC % B 100

55.0 ML/MIN 0.5

The following buffer was used for pump B (Mono S B buffer): 50mM HEPES pH=7.5, 500mM NaCl, 1mM DTT, 1mM EDTA, 1mM benzamidine. Fractions of 1ml in size were collected and analyzed by SDS-PAGE followed by Coomassie staining of the gels. The majority of the bacterial proteins previously existing in His-ERF sample, flow directly through the column away while His-ERF protein is bound by the resin and eluted at approximately 220mM NaCl (Figure 19). However, the peak appears to be broad enough resulting in a considerable protein loss and the two bands lying just below His-ERF band seem not to be able to be separated from our protein even though a long shallow gradient was used for elution.

Coomassie staining- 12% SDS-PAGE gels

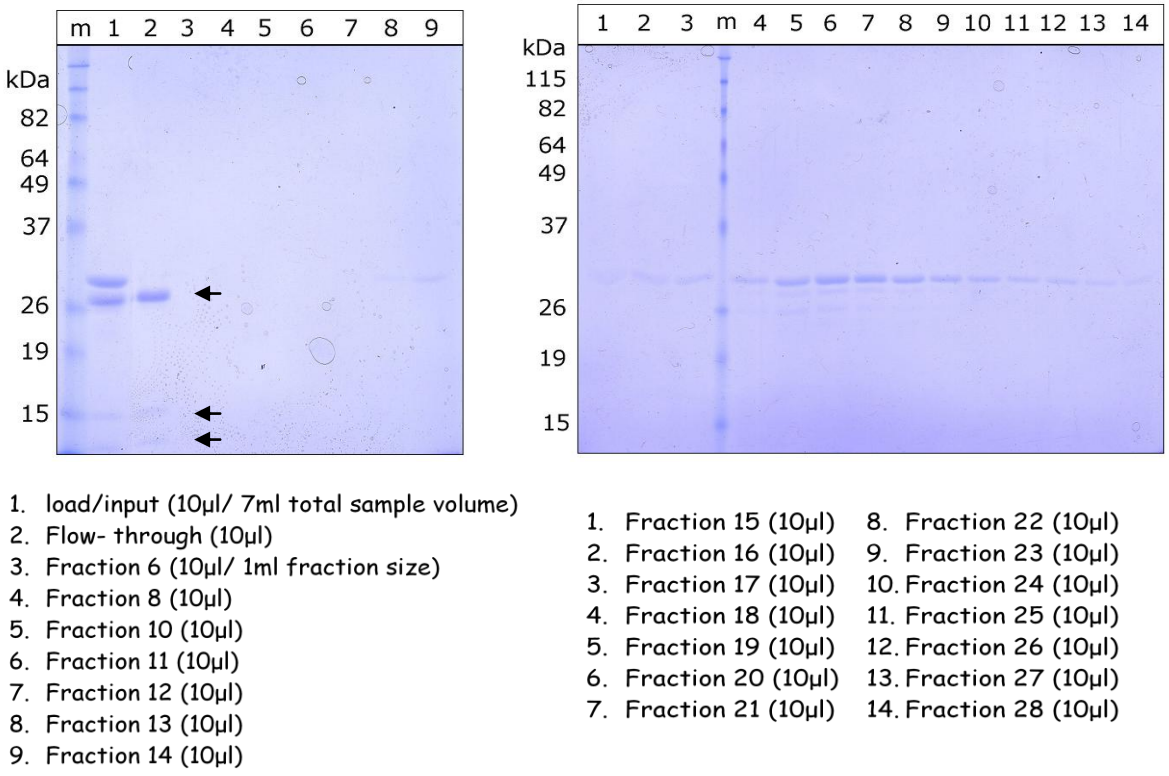


Figure 19: Separation of His-tagged ERF protein from contaminants by cation exchange chromatography. Arrows show the bands of the contaminating proteins that flow directly through the column away (lane 2, gel on the left). His-ERF protein is eluted, producing the broad peak shown (gel on the right).

4. Conclusions/ Discussion

The intention of this work was to offer an insight into all those structural elements that determine the association of ERK2 kinase with its substrate, ERF. Despite the fact that this association has been shown to be mediated by two FXF motifs lying in the EID (ERK Interaction Domain) of ERF protein, additional regions in this domain seem to contribute to the contacts made between the two molecules [35]. For this reason, our aim was to purify both partners -Erk2 kinase and the EID of ERF protein- and to subsequently achieve the crystallization of the complex they form. Even though we did not manage to complete this work and perform our structural study, the results of the experiments presented in this master thesis are promising enough for the future integration of this particular project.

Activated double- phosphorylated rat His-Erk2 was produced using a system in which co-expression of the Erk2 kinase with a constitutively active mutant of the upstream kinase, MEK1, takes place. This system was initially developed in order to overcome the difficulties existed in obtaining sufficient quantities of pure activated MAP kinases [38]. However, toxicity in bacteria resulting in limited protein amounts was another problem we faced during our expression experiments. His-tagged Erk2 was purified in the first step using Ni-NTA affinity chromatography, in which column wash was performed with buffer containing 50mM imidazole followed by elution with buffer containing 250mM imidazole. In contrast to other published work where a washing buffer of 10mM imidazole was used since Erk2 was getting washed off of the column at higher imidazole concentrations [38], we successfully performed a wash step at 50mM imidazole resulting in the removal of most of the contaminant proteins existing in our protein sample. The separation of the double- phosphorylated active form of Erk2 from the non- phosphorylated inactive one was accomplished by anion exchange chromatography on Mono Q HR5/5 column as previously determined by other research groups [13, 38], resulting in the isolation of activated His-tagged Erk2 protein of sufficient purity. However, additional steps of purification including isoelectric focusing and hydrophobic interaction chromatography have been utilized in the past for the obtainment of active rat Erk2 of highest purity for crystallization experiments [13]. Finally, the problem of precipitation during storage was solved by changing the buffer of the protein, since either the ionic strength or the pH value of the previous buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole) possibly disfavored protein's solubility.

GST-tagged ERF protein was isolated from bacterial lysates by GSH affinity chromatography and further purified by gel filtration chromatography on Sephacryl S 200 HR16/60 column. One of the greatest problems we faced in the purification of GST-ERF was the extensive degradation of the protein that was finally prevented following a strategy combining specific protease inhibitors and manipulations in very low temperatures (working in constant presence of ice). It is not clear whether this extended proteolysis is attributed to increased protease activity or increased structural instability or even to both of them. What is true however, is that the FXF motifs lying in the ERF EID (ERK interaction domain) are located in a region characterized by increased structural flexibility -an intrinsically disordered region- as indicated by predictive data created by the FoldIndex tool (data not shown) [44]. This is in consistence with the fact that many linear motifs are in general found in natively disordered regions of proteins [45, 46]. The degradation of the protein was followed by another problem observed in the case of GST-ERF too. Since GST protein itself has a dimeric tertiary structure [41], GST-ERF fusion protein exists in a dimeric form. The problem in our case was that, a considerable amount of protein was found in heterodimeric complexes formed with the proteolytic fragments of the GST-ERF polypeptide chain and therefore unable to be further purified. Efforts to remove the GST tag from the protein using thrombin were not successful since the enzyme was hydrolyzing ERF protein into small peptides. However we were finally able to separate full-length GST-ERF homodimers by gel filtration chromatography on Sephacryl S 200 HR5/5.

In order to prevent the dimerization and the subsequent protein loss described above, we decided to replace the GST tag by a His_6-tag. ERF EID was cloned into pET-28a vector and the isolation of the His-tagged ERF protein from the bacterial lysate was performed using Ni-NTA affinity chromatography (IMAC). A wash step at 70mM imidazole and an elution step at 140mM imidazole were selected as final conditions of purification. The problem of the low levels of His-tagged ERF solubilization during its extraction from bacteria was solved by the addition of 1% Triton X-100. The majority of the contaminants were removed by cation exchange chromatography on Mono S HR5/5 column. However, we were not successful in separating His-tagged ERF from the two bands of lower molecular weight lying just below ERF band, despite the fact that a very long and shallow gradient had been used in our experiments. A hypothesis is that these two bands may belong to proteolytic fragments of our own protein, exhibiting similar pI values. One option would be to test our protein sample across a wide range of pH for both cation and anion exchange chromatography and select the conditions that offer sufficient separation.

This work constitutes the first step needed for the acquisition of an insight into all those structural elements that determine the association of ERF with MAP kinase ERK2. We hope that we will be able to reveal the secrets of this intricate interaction following completion of our experiments as soon as possible.

5. References

1. Robinson, M.J., and Cobb, M.H. (1997). Mitogen-activated protein kinase pathways. Current opinion in cell biology *9*, 180-186.
2. Davis, R.J. (1993). The mitogen-activated protein kinase signal transduction pathway. The Journal of biological chemistry *268*, 14553-14556.
3. Seger, R., and Krebs, E.G. (1995). The MAPK signaling cascade. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *9*, 726-735.
4. Jacobs, D., Glossip, D., Xing, H., Muslin, A.J., and Kornfeld, K. (1999). Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. Genes & development *13*, 163-175.
5. Whitmarsh, A.J., and Davis, R.J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med (Berl) *74*, 589-607.
6. Roskoski, R., Jr. (2012). ERK1/2 MAP kinases: structure, function, and regulation. Pharmacological research : the official journal of the Italian Pharmacological Society *66*, 105-143.
7. Kim, E.K., and Choi, E.J. (2010). Pathological roles of MAPK signaling pathways in human diseases. Biochimica et biophysica acta *1802*, 396-405.
8. Chin, L. (2003). The genetics of malignant melanoma: lessons from mouse and man. Nature reviews. Cancer *3*, 559-570.
9. Robbins, D.J., Zhen, E., Owaki, H., Vanderbilt, C.A., Ebert, D., Geppert, T.D., and Cobb, M.H. (1993). Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. The Journal of biological chemistry *268*, 5097-5106.
10. Aouadi, M., Binetruy, B., Caron, L., Le Marchand-Brustel, Y., and Bost, F. (2006). Role of MAPKs in development and differentiation: lessons from knockout mice. Biochimie *88*, 1091-1098.
11. Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P., and Pouyssegur, J. (1999). Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science *286*, 1374-1377.
12. Selcher, J.C., Nekrasova, T., Paylor, R., Landreth, G.E., and Sweatt, J.D. (2001). Mice lacking the ERK1 isoform of MAP kinase are unimpaired in emotional learning. Learn Mem *8*, 11-19.
13. Canagarajah, B.J., Khokhlatchev, A., Cobb, M.H., and Goldsmith, E.J. (1997). Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell *90*, 859-869.
14. Zhang, F., Strand, A., Robbins, D., Cobb, M.H., and Goldsmith, E.J. (1994). Atomic structure of the MAP kinase ERK2 at 2.3 A resolution. Nature *367*, 704-711.
15. Khokhlatchev, A.V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M.H. (1998). Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell *93*, 605-615.
16. Wilsbacher, J.L., Juang, Y.C., Khokhlatchev, A.V., Gallagher, E., Binns, D., Goldsmith, E.J., and Cobb, M.H. (2006). Characterization of mitogen-activated protein kinase (MAPK) dimers. Biochemistry *45*, 13175-13182.
17. Kaoud, T.S., Devkota, A.K., Harris, R., Rana, M.S., Abramczyk, O., Warthaka, M., Lee, S., Girvin, M.E., Riggs, A.F., and Dalby, K.N. (2011). Activated ERK2 is a monomer in vitro with or without divalent cations and when complexed to the cytoplasmic scaffold PEA-15. Biochemistry *50*, 4568-4578.
18. Remenyi, A., Good, M.C., and Lim, W.A. (2006). Docking interactions in protein kinase and phosphatase networks. Current opinion in structural biology *16*, 676-685.
19. Burkhard, K.A., Chen, F., and Shapiro, P. (2011). Quantitative analysis of ERK2 interactions with substrate proteins: roles for kinase docking domains and activity in determining binding affinity. The Journal of biological chemistry *286*, 2477-2485.
20. Tanoue, T., Maeda, R., Adachi, M., and Nishida, E. (2001). Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. The EMBO journal *20*, 466-479.

21. Dimitri, C.A., Dowdle, W., MacKeigan, J.P., Blenis, J., and Murphy, L.O. (2005). Spatially separate docking sites on ERK2 regulate distinct signaling events in vivo. Current biology : CB *15*, 1319-1324.
22. Hollenhorst, P.C., McIntosh, L.P., and Graves, B.J. (2011). Genomic and biochemical insights into the specificity of ETS transcription factors. Annual review of biochemistry *80*, 437-471.
23. Mavrothalassitis, G., and Ghysdael, J. (2000). Proteins of the ETS family with transcriptional repressor activity. Oncogene *19*, 6524-6532.
24. Lamber, E.P., Vanhille, L., Textor, L.C., Kachalova, G.S., Sieweke, M.H., and Wilmanns, M. (2008). Regulation of the transcription factor Ets-1 by DNA-mediated homo-dimerization. The EMBO journal *27*, 2006-2017.
25. Kim, C.A., Phillips, M.L., Kim, W., Gingery, M., Tran, H.H., Robinson, M.A., Faham, S., and Bowie, J.U. (2001). Polymerization of the SAM domain of TEL in leukemogenesis and transcriptional repression. The EMBO journal *20*, 4173-4182.
26. Callaway, K., Waas, W.F., Rainey, M.A., Ren, P., and Dalby, K.N. (2010). Phosphorylation of the transcription factor Ets-1 by ERK2: rapid dissociation of ADP and phospho-Ets-1. Biochemistry *49*, 3619-3630.
27. Dyson, H.J., and Wright, P.E. (2005). Intrinsically unstructured proteins and their functions. Nature reviews. Molecular cell biology *6*, 197-208.
28. Minezaki, Y., Homma, K., Kinjo, A.R., and Nishikawa, K. (2006). Human transcription factors contain a high fraction of intrinsically disordered regions essential for transcriptional regulation. Journal of molecular biology *359*, 1137-1149.
29. Sgouras, D.N., Athanasiou, M.A., Beal, G.J., Jr., Fisher, R.J., Blair, D.G., and Mavrothalassitis, G.J. (1995). ERF: an ETS domain protein with strong transcriptional repressor activity, can suppress ets-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. The EMBO journal *14*, 4781-4793.
30. Le Gallic, L., Sgouras, D., Beal, G., Jr., and Mavrothalassitis, G. (1999). Transcriptional repressor ERF is a Ras/mitogen-activated protein kinase target that regulates cellular proliferation. Molecular and cellular biology *19*, 4121-4133.
31. Le Gallic, L., Virgilio, L., Cohen, P., Biteau, B., and Mavrothalassitis, G. (2004). ERF nuclear shuttling, a continuous monitor of Erk activity that links it to cell cycle progression. Molecular and cellular biology *24*, 1206-1218.
32. Verykokakis, M., Papadaki, C., Vorgia, E., Le Gallic, L., and Mavrothalassitis, G. (2007). The RAS-dependent ERF control of cell proliferation and differentiation is mediated by c-Myc repression. The Journal of biological chemistry *282*, 30285-30294.
33. Papadaki, C., Alexiou, M., Cecena, G., Verykokakis, M., Bilitou, A., Cross, J.C., Oshima, R.G., and Mavrothalassitis, G. (2007). Transcriptional repressor erf determines extraembryonic ectoderm differentiation. Molecular and cellular biology *27*, 5201-5213.
34. Twigg, S.R., Vorgia, E., McGowan, S.J., Peraki, I., Fenwick, A.L., Sharma, V.P., Allegra, M., Zaragkoulias, A., Sadighi Akha, E., Knight, S.J., et al. (2013). Reduced dosage of ERF causes complex craniosynostosis in humans and mice and links ERK1/2 signaling to regulation of osteogenesis. Nature genetics *45*, 308-313.
35. Polychronopoulos, S., Verykokakis, M., Yazicioglu, M.N., Sakarellos-Daitsiotis, M., Cobb, M.H., and Mavrothalassitis, G. (2006). The transcriptional ETS2 repressor factor associates with active and inactive Erks through distinct FXF motifs. The Journal of biological chemistry *281*, 25601-25611.
36. de Boer, H.A., Comstock, L.J., and Vasser, M. (1983). The tac promoter: a functional hybrid derived from the trp and lac promoters. Proceedings of the National Academy of Sciences of the United States of America *80*, 21-25.
37. Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Applied microbiology and biotechnology *72*, 211-222.
38. Khokhlatchev, A., Xu, S., English, J., Wu, P., Schaefer, E., and Cobb, M.H. (1997). Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria. Efficient synthesis of active protein kinases. The Journal of biological chemistry *272*, 11057-11062.

39. Bornhorst, J.A., and Falke, J.J. (2000). Purification of proteins using polyhistidine affinity tags. Methods in enzymology *326*, 245-254.
40. Harper, S., and Speicher, D.W. (2011). Purification of proteins fused to glutathione S-transferase. Methods Mol Biol *681*, 259-280.
41. Sheehan, D., Meade, G., Foley, V.M., and Dowd, C.A. (2001). Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. The Biochemical journal *360*, 1-16.
42. Wells, C.M., and Di Cera, E. (1992). Thrombin is a Na(+)-activated enzyme. Biochemistry *31*, 11721-11730.
43. Adams, T.E., and Huntington, J.A. (2006). Thrombin-cofactor interactions: structural insights into regulatory mechanisms. Arteriosclerosis, thrombosis, and vascular biology *26*, 1738-1745.
44. Prilusky, J., Felder, C.E., Zeev-Ben-Mordehai, T., Rydberg, E.H., Man, O., Beckmann, J.S., Silman, I., and Sussman, J.L. (2005). FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics *21*, 3435-3438.
45. Neduva, V., and Russell, R.B. (2005). Linear motifs: evolutionary interaction switches. FEBS letters *579*, 3342-3345.
46. Gould, C.M., Diella, F., Via, A., Puntervoll, P., Gemund, C., Chabanis-Davidson, S., Michael, S., Sayadi, A., Bryne, J.C., Chica, C., et al. (2010). ELM: the status of the 2010 eukaryotic linear motif resource. Nucleic acids research *38*, D167-180.