Antibody Production to Human Recombinant ERK1: Comparison of Human ERK1 and Sea Urchin ERK1

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I. Abstract

Mitogen-activated protein (MAP) kinases (also called extracellular-signal-regulated kinases [ERKs]) are activated in response to a wide array of extracellular stimuli in many different cell types (Gardner et al., 1993). ERKs are serine/threonine kinases that are important signal transducers during the G_O to G_1 transition point where the cell in a resting state of the cell cycle enters the first growth stage before DNA synthesis and also during the G_2 to M phase where the cell continues to grow to the point where the nucleus and cytoplasm divide (Ruderman, 1993). ERKs are important regulators of such cellular processes as cell growth, cell division, and cell differentiation. My study compares ERK1 from a human recombinant GST/ERK1 fusion protein and ERK1 from sea urchin (*Lytechinus variegatus* or *Strongylocentrotus purpuratus*) egg extracts with the use of mouse antibodies produced against the recombinant protein. The major finding in this study was that epitope cross-reactivity occurred in sea urchin extracts in the molecular weight range expected for the recombinant GST/ERK1 fusion protein on a Western blot.

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II. Introduction

Mitogen-activated protein (MAP) kinases (also called extracellular-signal-regulated kinases [ERKs]) play a key role in relaying extracellular signals generated at the plasma membrane to the cell nucleus. Crucial to the study of cell biology is the understanding of the signal transmission mechanisms which cause cell growth, division, or differentiation (Anderson, 1992; Boulton et al., 1991). For many years, it has been known that hormones and growth factors, upon binding their receptor, produce changes in the phosphorylation states of a number of intracellular proteins (Edelman et al., 1987). Characteristic of these receptors are either an intrinsic protein tyrosine kinase activity or the ability to promote cellular tyrosine phosphorylation by interacting with an associated tyrosine kinase. The most probable sites for protein phosphorylation in activated cells are on serine and threonine residues. However, an important thought is how the initial tyrosine specific signal can be converted into a signal which targets serine and threonine residues. Key advances in this area have come about through the discovery and characterization of the serine/threonine protein kinase, MAP2-kinase (Anderson, 1992; Ray and Sturgill, 1987).

MAP2-kinase was first discovered by Ray and Sturgill (1987) in cytosolic extracts of 3T3-L1 adipocytes as a serine/threonine kinase which

was stimulated by insulin and which also phosphorylated microtubule-associated protein (MAP-2) as a selective substrate (Anderson, 1992). MAP-kinase (MAPK) has become the term for this cytosolic serine/threonine protein kinase which utilizes microtubule-associated protein (MAP-2) and myelin basic protein (MBP) *in vitro* and which itself is phosphorylated on tyrosine (Hoshi et al., 1991; Rossomando et al., 1989). MAPK is universally activated in quiescent fibroblasts by such mitogens as platelet-derived growth factor (PDGF), epidermal-derived growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor, insulin, and 12-O-tetradecanoyl-phorbol 13-acetate (TPA) (Rossomando et al., 1989). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed the kinase to migrate with a molecular mass of 40-42 kDa and to become phosphorylated on tyrosine during activation of the cells by insulin (Ray and Sturgill, 1988). Since pp^{42} contains these properties, the enzyme is often called $p42^{mapk}$, a major tyrosine kinase target protein (Anderson, 1992; Rossomando et al., 1989).

In order for MAP-kinases to be fully active, they must be phosphorylated on both a threonine and a tyrosine, which are separated by one amino acid in the protein (Ray and Sturgill, 1988). MAP-kinase is phosphorylated by a 45 kDa MAP-kinase-kinase (MEK) (Pelech, 1993;

Ruderman, 1993). MEK itself is phosphorylated and activated by a serine/threonine protein kinase, Raf (MAP-kinase-kinase-kinase) (Gardner et al., 1993). The upstream activator of Raf in this regulatory cascade is a monomeric GTP-binding protein, Ras (Ruderman, 1993; Davis 1993). In turn, Ras is activated by tyrosine kinase receptor complexes at the plasma membrane. This class of plasma membrane receptors is commonly used in growth factor regulation of cell growth and proliferation. There are several known MAP-kinase-kinase-kinases including Raf1, which is found downstream of tyrosine kinase receptors in this regulatory cascade. MAP-kinase has different downstream targets in this cascade which include the rsk family of S6 kinase, the EGF receptor, and c-myc and c-jun, which code for nuclear transcription factors (Gardner et al., 1993; Hartley et al., 1994; and Sturgill et al., 1988).

MAP-kinases are important signal transducers during the G_0 to G_1 and G_2 to M, as well as during other potential cell cycle transition points (Ruderman, 1993). Evidence that MAP kinases are involved in the aforementioned transitions comes from different experiments. In the presence of tissue-specific growth factors or other mitogens, G_0-arrested somatic cells activate a complex cascade of transcriptional changes, which eventually trigger the activation of DNA synthesis. G_2-arrested oocytes

respond to such elements as sperm, hormones, or other species-specific mitogens by activating buildups of proteins and pre-existing mRNAs which rapidly lead to shutdown of transcription and reorganization of most cellular structures as cells enter M-phase of Meiosis I (Smith, 1989). Although activation of both somatic cells and oocytes leads to different end points, early signalling pathways found during cell cycle reentry are in many ways very similar. Activation of both cell types leads to a rise in intracellular pH, alteration of the cytoskeleton, changes in phospholipid metabolism, transient elevation of free intracellular calcium, and profound pattern changes in gene transcription and mRNA translation (Berridge, 1993; Ruderman, 1993; Smith, 1989; and Whitaker and Swann, 1993). MAP-kinase activation is found early in cell cycle activation, as well. In clams, fertilization of oocytes induces release of the cell from G_O-arrest (Shibuyo et al., 1992). Activation of MAP-kinase appears quickly and transiently here, but the failure of MAP-kinase to reappear during the next three cell cycles suggests that MAP-kinase activation is an off-on event linked with release from cell cycle arrest rather than a continuous event necessary for progression through each cell cycle (Ruderman, 1993).

An important target of MAP-kinase is the serine/threonine kinase called S6 kinase II or pp^{90RSK}. This kinase has the ability to phosphorylate

the ribosomal protein S6 *in vitro* (Erikson, 1991; Ruderman, 1993). Several growth factor-stimulated S6 kinases have been identified, and cloning experiments with a number of sources for S6 kinase genes show the existence of two distinct families, p70 S6 kinase and $p90^{rsk}$ (Alcorta et al., 1989; Bannerjee et al., 1990; and Kozma et al., 1990). Both $p42^{mapk}$ and $p44^{mapk}$ can phosphorylate and reactivate $p90^{rsk}$ but not p70 S6 kinase (Sturgill et al., 1988). Since $pp90^{rsk}$ has been found to be a physiological substrate of MAP-kinase, then this kinase along with MAP-kinase might be participating in a protein kinase cascade in which protein synthesis could be promoted by growth factors. During activation of both G_0- and G_2-arrested cells, there appears to be an increase in S6 phosphorylation although direct evidence indicating physiological importance of S6 phosphorylation for cell cycle activation is lacking. An entirely different enzyme p70S6K appears to phoshorylate intact S6, and this protein is part of a different signaling pathway than MAP kinase, with MAP kinase playing no role in activation (Ballou et al., 1991; Chung et al., 1992; Price et al., 1992; and Ruderman, 1993). Further studies on the effects of protein phosphorylation on ribosomal protein S6 function hope to give rise to better understanding of the significance of such a pathway to the control of growth and cell division (Anderson, 1992; Ruderman, 1993).

MAP-2 and myelin basic protein (MBP) have shown to be important *in vitro* substrates for MAP-kinases (Shaw et al., 1988; Tsao et al., 1990). Both of these proteins can be phosphorylated *in vivo*, and MAP-2 undergoes increased phosphorylation in response to growth factors. Furthermore, MAP-kinase phosphorylates MBP *in vitro* at threonine 97, and this site is not a target for a number of other protein kinases (Anderson, 1992). Hoshi et al. (1991) found in a study that MAP-kinase-catalyzed phosphorylation of MAP-2 decreases its ability to polymerize tubulin *in vitro*. This decrease of MAP-2 tubulin-polymerizing activity was roughly correlated to the extent that the microtubule-binding domain of MAP-2 was phosphorylated by MAP-kinase. Perhaps, therefore, when MAP-kinase phosphorylates the microtubule-binding domain of MAP-2, MAP-2 undergoes reduction in tubulin-polymerizing activity. Hoshi et al. (1991) also proposed that phosphorylation of MAP-2 by MAP-kinase may occur in neuronal tissues and may also cause functional changes of MAP-2 *in vivo*.

Mitosis is characterized by major changes in higher eukaryotes which affect chromatin condensation, the nuclear envelope, and reorganization of the microtubule network. The protein kinase called $p34^{cdc2}$ and cyclins play a key role in control of such events, and these proteins act as major regulators of the eukaryotic cell cycle (Lee and Nurse, 1987; Nurse, 1990;

and Peter et al., 1992). In theory, $p34^{cdc2}$ may promote mitotic entry via two separate mechanisms. It may either directly phosphorylate certain structural targets, or it may promote protein phosphorylation cascades by first activating other protein kinases. In the first case, $p34^{cdc2}$ has a number of physiological substrates which include vimentin, histone H1, nuclear lamins, caldesmon, and high-mobility-group proteins (Moreno and Nurse, 1990). In the second case, M-phase cell extracts were studied and shown to have possessed several distinct protein kinase activities (Cicirelli et al., 1988). Although many of the protein kinases found at M-phase remain unidentified, recent studies from frog and maturing sea star oocytes indicate the inclusion of different MAP-kinase members at this phase (Peter et al., 1992).

The activity of $p34^{cdc2}$ kinase is regulated in the cell cycle and peaks during mitosis. To exit from mitosis, $p34^{cdc2}$ kinase must be inactivated by degradation of the cyclin B regulatory subunit and the dephosphorylation of the kinase at threonine 161. During mitosis, a $p34^{cdc2}$ kinase subpopulation localizes to the mitotic apparatus. This localization probably plays a key role in the function of $p34^{cdc2}$, and it may also play a role its regulation. Moreover, $p34^{cdc2}$ kinase localizes to the mitotic spindle during mitotic exit, and this localization may be important to the inactivation of the kinase. In order for efficient exit from mitosis to occur, $p34^{cdc2}$ maintains an

association with centrosomal microtubules. This localization suggests that if the $p34^{cdc2}$ kinase is to be inactivated, then it must be present on microtubules. The correct components allowing for exit from mitosis are probably present on the mitotic spindle, and the assembly of microtubules may play a key role in the regulation of mitotic exit (Andreassen and Margolis, 1994). Interestingly, since different members of the MAP-kinase family have overlapping substrate specificity with $p34^{cdc2}$, then perhaps MAP-kinases play a key physiological role in mediating the disassembly of the nuclear lamina during mitosis. For example, both MAP-kinases and $p34^{cdc2}$ kinase phosphorylate lamin (Peter et al., 1990). Perhaps, cdc2-like kinase substrates during the G_1 to S transition of the cell cycle may be important physiological substrates of MAP-kinase (Peter et al., 1992).

In a previous study, Walker et al. (in press) identified MAP-kinase in sea urchin cytoskeleton fractions. They found that MAP-kinase in their isolated samples of dividing eggs possessed high MBP kinase activity, low histone H1 phosphorylation, and no $p34^{cdc2}$ immunoreactivity by Western blotting. All of these activities are characteristic of MAP-kinase (Anderson, 1992; Pelech et al., 1988, 1990; Shaw et al., 1988; and Tsao et al., 1990). Walker et al. found Western blot bands in the ~42 kDa region which may indicate the presence of MAP-kinase in one of these bands. However, since

bands were also seen in other molecular weight ranges on the blot, primarily a band in the ~49-50 kDa region, then further study was needed to characterize which band(s) represented MAP-kinase.

The goal of my research was to determine if the human MAP-kinase, ERK1, was homologous in structure to sea urchin *Lytechinus variegatus* or *Strongylocentrotus purpuratus* MAP-kinase. The human recombinant glutathione-S-transferase (GST)/ERK1 protein was used to immunize mice. These antibodies were tested for binding against purified ERK1 from the recombinant protein and sea urchin proteins through a series of immuno-binding techniques. Western blot analysis was used to confirm whether these antibodies recognized the GST portion or the ERK1 portion of the recombinant protein by the presence and/or absence of defined bands appearing in either protein molecular weight region on the blots.

III. Materials and Methods

The Recombinant ERK1 Plasmid

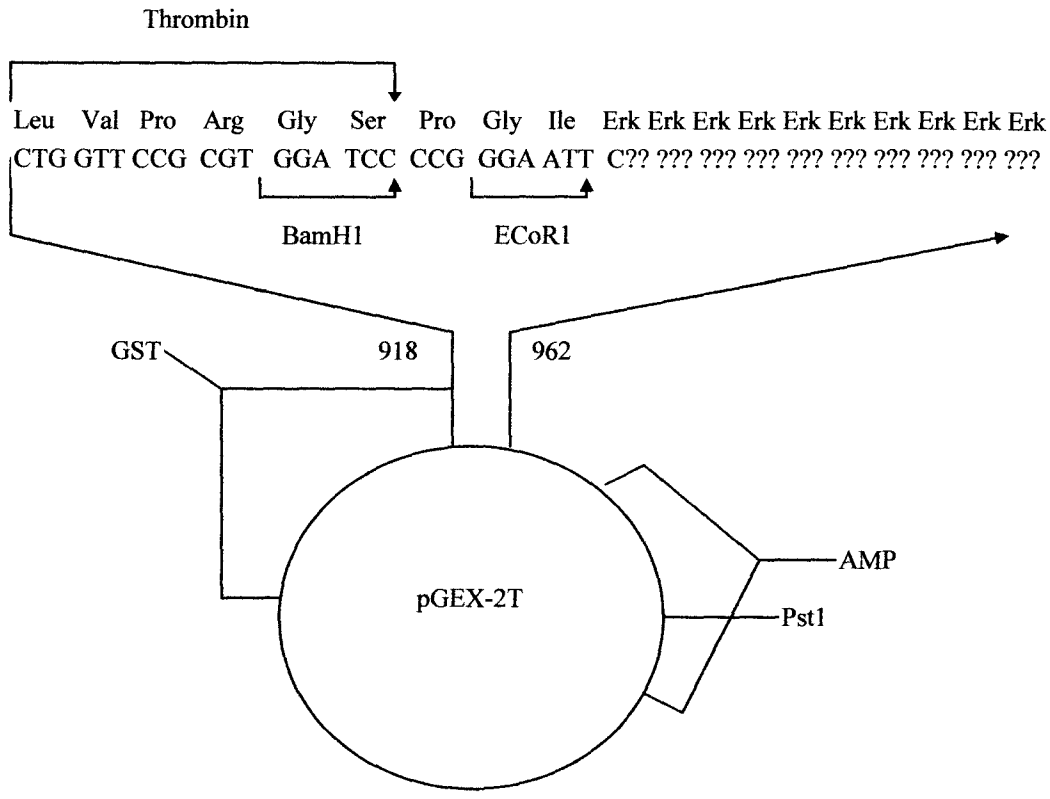
The recombinant ERK1 plasmid (pGEX-ERK1) is derived from a pGEX-2T vector (Pharmacia, Inc.) that has a glutathione-S-transferase (GST) gene which is under the control of a lac promoter. Also, pGEX-ERK1 has an ampicillin resistance gene to allow for selection of the plasmid-containing bacteria (Figure 1). The C-terminal end of the GST gene has a polylinker region with a unique ECoR1 restriction site. The ERK1 gene was inserted into the plasmid at this ECoR1 restriction site. The GST/ERK1 fusion protein derived from plasmid DNA can be purified by affinity chromatography using glutathione-agarose as the chromatographic matrix.

Plasmid Preparation

One tube (X4) which contained ampicillin and 3 ml of LB broth was inoculated with the non-recombinant *E. coli* containing the pGEX-2T vector. A similar tube was inoculated with the pGEX-2T vector containing human ERK1, and the plasmid DNA was purified from overnight cultures by use of the PERFECT prep (PERFECT prep^{TM}). The cultures were microcentrifuged for 2 minutes at 7,000 X g, and the resulting supernatant

was discarded. After adding 100 µl of solution I to the tube and vortexing, 100 µl of solution II was added and the tube inverted. Next, 100 µl of solution III was added and mixed by inversion. Solution III acted as a neutralizing agent. Microcentrifugation at 7,000 X g for 4 minutes was performed to remove debris. The supernatant was transferred to a spin column inside a collection tube, and 450 µl of DNA binding matrix was added to the column. The tube was capped and inverted vigorously. The column was then microcentrifuged at 7,000 X g for 1 minute, and the filtrate was discarded. The column was washed with diluted purification solution and centrifuged at 7,000 X g for 2 minutes. The column was then placed in a new collection tube and microcentrifuged at 7,000 X g for 2 minutes. After centrifugation, the column was placed in a new collection tube, and DNA was eluted from the column by adding 70 µl TE (pH 8) and microcentrifuged at 7,000 X g for 2 minutes. The eluted DNA was stored in the refrigerator.

Figure 1. The pGEX-2T vector containing a polylinker sequence where ERK1 is inserted. ERK1 is inserted at the ECoR1 site as indicated by an arrow. Thrombin cleaves between serine and proline in this vector as also indicated by an arrow.


Thrombin
Leu Val Pro Arg Gly Ser Pro Gly Ile Erk Erk Erk Erk Erk Erk Erk Erk Erk Erk
CTG GTT CCG CGT GGA TCC CCG GGA ATT C?? ??? ??? ??? ??? ??? ??? ??? ??? ???
BamH1
ECoR1
GST
918
962
pGEX-2T
AMP
Pst1

Restriction Mapping

The purified plasmid DNA was analyzed on an ECoR1 restriction digest. The following components were assembled on ice in order: 20 µl DNA from above, 1 µl distilled water, 2.5 µl buffer H, and 1.5 µl ECoR1. The tubes were transferred to a 37° C water bath for 1 hour, and 2 µl stop buffer was added. The uncut plasmid DNA, λ DNA, and digested samples (12.5 µl DNA + 2 µl stop buffer) were loaded onto a 1% agarose/TAE gel and were run until the dye front was 2/3 the way through the gel. The gel was then stained with ethidium bromide for 30 minutes and viewed on a U. V. box.

Induction of ERK1

The bacteria containing the plasmid were treated with IPTG resulting in synthesis of ERK1. IPTG turns on the lac promoter resulting in transcription of the GST gene. Bacterial protein were later analyzed by SDS-PAGE. First, 3 ml of overnight cultures were grown and 100 µl aliquots were transferred to 500 ml LB/ampicillin at 100 µg/ml. The 500 ml cultures were grown overnight, and IPTG was then added to 0.4 mM and grown for 1.5 hours at 37° C. Bacteria were recovered by centrifugation at 2000 x g for 20 minutes. Cells were then washed by resuspension in 500 ml

TBS and centrifuged at 1,800 x g again. The pellets were resuspended in 10 ml TBS and point sonicated (Branson, Inc.) at 50% power for 1.5 minutes. Triton X100 (1.3 ml of 10%) was added, and the pellets were sonicated for another 30 seconds on ice. Debris was removed by centrifugation at 4,600 x g for 30 minutes. The supernatant was mixed with 4 ml of glutathione-agarose 4B (Sigma Chem. Co.) on ice for 0.5-1 hour. The slurry was next poured into a 20 ml syringe column. The column was then washed with 75-100 mls of TBS to remove unbound protein. The bound protein was eluted by carefully overlaying the gel bed and washing with 15 mM glutathione/50 mM tris pH 8.28 (Sigma Chem. Co.). Twenty-five fractions (0.5 ml each) were taken, and samples from each fraction were run on SDS-PAGE mini gels to determine fractions containing peak protein.

Fusion Protein Digest

The ERK1 fragment was then separated from the GST fragment by thrombin digestion of the fusion protein. Thrombin, has been shown to cleave pGEX-2T fusion proteins in a number of cases (Bill et al., 1995). Thrombin cleaves between the GST and ERK1 regions of the fusion protein. The thrombin-digested ERK1 was then run on an SDS-PAGE mini-gel to visualize the ERK1 and GST fragments.

Protein Analysis

Once the correct protein was collected, it became important then to quantitate the purified recombinant protein in order to properly immunize the mouse with it. The method used here to quantitate was the Bradford method (Bradford, 1976).

The assay was carried out as follows. BSA standards were made up at 100, 250, 500, 750, and 1,000 μg/ml water concentrations. These standards were used in order to generate a curve to which my unknown peak recombinant ERK1 fractions could be compared. The standard assay contained 790 μl water, 200 μl concentrated BioRad dye, and 10 μl sample. The unknown samples and the BSA standards were vortexed briefly and allowed to stand 5 minutes. After this time point, the samples were read with a spectrophotometer at O.D. 595 nm. A graph of BSA standards (μg/ml) versus O.D. was generated by use of a statistical program (Cricket Graphics). The program allowed for generation of an equation for the standard curve which allowed for calculation of unknown quantities.

Qualitative analysis of proteins was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE protocol used for this research involved the use of a 10% acrylamide resolving gel and a 5% acrylamide stacking gel. The gels were run at 150

volts (constant voltage) for 1.5-2 hours until the dye front was within 1 cm of the bottom of the gel. Protein was visualized by Coomassie blue staining of the gels.

Antibody Production to GST/ERK1 Fusion Protein

Antibodies to the human recombinant GST/ERK1 fusion protein were produced by immunizing mice with the antigen mixed with Freund's adjuvant. Freund's adjuvant is a water-in-oil emulsion prepared with nonmetabolizable oils. Complete Freund's adjuvant (CFA) contains killed *M. tuburculosis*, while incomplete Freund's adjuvant (IFA) does not contain the bacteria. The adjuvants were used to stimulate a strong and prolonged immune response. The initial injection was performed with CFA, and subsequent injections were performed with IFA. The final injection before bleeding the spleen for hybridoma studies was an intravenous injection, and this injection used 0.85% saline instead of any adjuvants (Harlow and Lane, 1988). *Mycobacterium tuburculosis* was resuspended by vortexing or shaking when CFA was used. Next, an emulsion was formed by mixing equal amounts of the adjuvant with the protein antigen using a double hub needle with syringes. The emulsion was tested by placing a drop of the antigen mixture on ice water. Dispersal of the emulsion should not occur.

The emulsion was transferred to a syringe, and the samples were now ready for injection (Harlow and Lane, 1988).

The mouse in this study was initially injected with 200 to 400 μl of antigen (10 μg), and the mouse was anesthetized with Metofane (Pitman-Moore, Inc.) before injection. The mouse was injected (21-gauge needle) intraperitoneally once a week with the first injection being the only one where CFA was used with the antigen. Each week, the mouse was injected with antigen and IFA using a 21-gauge needle. After five antigen boosts (10 μg each), the mouse was bled from the retroorbital socket to test for antibodies. After collecting blood for antibody titer analysis, the blood was clotted on ice for 30 minutes. Serum was removed by centrifugation at ~1,800 X g for 1 minute. Serum was stored at 4° C.

Hybridoma Production

The myeloma cell line , P3X, was grown at 37° C in RPMI 1640 media containing 80% RPMI, 20% FCS, and 1 ml L-glutamine. When the mouse was determined by ELISA to be producing antibodies and when the P3X cells were also growing well in a T25 flask, the mouse was injected a final time intravenously with 50 μg ERK1 in 200 μl 0.85% saline solution (Harlow and Lane, 1988).

The immunized mouse was sacrificed by CO_2 inhalation. The spleen was then removed using sterile technique. The spleen was mashed with a plunger from a sterile syringe to release the white blood cells. The spleen cells were then washed twice with sterile RPMI 1640 medium containing no fetal calf serum (FCS). The spleen cell suspension was transferred to a 50 ml conical tube, and large clumps were allowed to settle in the tube over a period of 5-10 minutes. The supernatant was saved, and the cells were washed three times with RPMI that contained no FCS. The purpose of this washing was to remove FCS and serum components. The P3X myeloma cells were fed the day before fusion with complete RPMI 1640 medium containing OPI (oxaloacetate, pyruvate, and insulin) and harvested during the log phase of cell growth. To prepare 100 ml of 100X OPI, 1.5 grams of oxaloacetate and 500 mg sodium pyruvate were dissolved in 100 ml of H_2O.

Then, 2000 IU (international units) of bovine insulin were added, and the finished media was filter-sterilized and dispensed into sterile tubes in 2.0 ml aliquots (Harlow and Lane, 1988).

The myeloma cells were washed in 37° C RPMI 1640 medium containing no FCS. The cells were then counted. Spleen cells and myeloma cells were then pelleted together for 5 minutes at 400 x g in the proportions of 2-4 spleen cells: 1 myeloma cell. The cells were fused by the addition of 1 ml of 50 % PEG (polyethylene glycol) at 37° C and at neutral pH to the pellet over a period of 5 minutes. The PEG was then diluted by slow addition of 20 ml of RPMI 1640 medium, without FCS, over a period of 4 minutes with gentle swirling (Harlow and Lane, 1988; Morgan and Darling, 1993).

The cells were then centrifuged for 5 minutes at 250 X g, and the supernatant was removed. Complete medium (30 ml RPMI 1640, 20 ml FCS, 1 ml 50 ml spleen cell supernatant, 1 ml L-glutamine, and 1 ml penicillin/streptomycin) was added to the cell suspension, and the cells were dispersed into a 24-well plate (1 ml/well). After overnight incubation, 1 ml of 1X hypoxanthine-aminopterin-thymidine (HAT) medium (RPMI 1640 containing 20% FCS, and 1X HAT) was added to all the wells. The HAT stock to produce the media was diluted to give the final concentrations of 1

X 10^{-4} M hypoxanthine, 4 X 10^{-7} M aminopterin, and 1.6 X 10^{-5} M thymidine. Every other day for 14 days, 1 ml of HAT was removed, and 1 ml of HAT was added. During the first week of HAT addition, 20% spleen cell supernatant was added to the medium to provide extra growth factors. After 14 days, hypoxanthine-thymidine (HT) medium containing 80% RPMI 1640, 20% FCS, and 1 ml of 1.5 M HT was added to the wells and removed every other day as performed with HAT. After this two-week period of media changing, complete medium (80 ml RPMI 1640, 20 ml FCS, 1 ml P/S, and 1 ml L-glutamine) was added to the wells. Once growth in the wells became confluent, the cells from one well were transferred to a T25 flask with 5 ml of medium. When the cells in the T25 flask were close to confluence, antibodies to ERK1 were identified by performing an ELISA. Aliquots of the hybridomas in the T25 flasks were frozen in a -70° C freezer for future studies (Harlow and Lane, 1988; Morgan and Darling, 1993).

Limiting Dilution Cloning of Hybridoma Cells

The hybridomas were cloned by the rapid method of limiting dilutions (Harlow and Lane, 1988). First, 100 µl of medium was added to all wells of a 96-well plate. Next, 100 µl of hybridoma cell suspension was added to the top left-hand well and mixed by pipetting. Serial dilutions (1 in 2) followed down the left-hand row of the plate (8 wells, 7 dilution steps). Next, 1 in 2 dilutions were performed across the plate using an 8-well multi-pipetter. Macrophages (100 µl) were added to the central 60 wells of the 96-well plate (Harlow and Lane, 1988). The macrophages used were plated out at 2 X 10^4/well. Once a week, 100 µl of medium was removed and replaced with fresh medium. The media to be replaced was RPMI (80 ml RPMI 1640, 20 ml FCS, 1 ml L-glutamine).

ELISAs

The ELISA (Bringman and Aggarwal, 1987) was used to determine antibody titer. Microtiter plates (96-well plates) were coated with 100 ng/well antigen (human recombinant GST/ERK1) dissolved in 50 mM sodium carbonate buffer at pH 9.6. Only as many rows as samples being tested were coated with the antigen. The solutions were then removed from the plates by flicking the plates over the sink and blotting the wells on a

paper towel. A 30-minute incubation with blocking buffer [(1% bovine serum albumin (BSA) in 150 mM NaCl, 50 mM Tris, 2 mM EDTA, pH 7.4 (TBS)] at 22° C followed to block unreacted protein-binding sites on the wells. Next, the wells were washed three times by flooding the wells with PBS-Tween (0.05% Tween-20). The first well was left blank with only 100 µl of sample buffer (TBS with 5 mg/ml BSA and 0.05% Tween-20) added. Now, 1 µl of the positive control (mouse anti-GST/ERK1) plus 100 µl sample buffer were added to a second well. A third well was used as a negative control (non-immune mouse serum) and was set up the same way as the positive control well. Next, 100 µl of sample buffer was added to all the wells which were tested. The first well contained 100 µl of a hybridoma supernatant of interest. Binding was allowed to occur at room temperature for 2 hours (or overnight at 4° C). The wells were then washed as before, and 100 µl goat anti-mouse IgM, IgA, IgG (Gibco BRL) diluted 1:1000 in sample buffer was added to each well. Binding took place for 2 hours at room temperature or at 4° C overnight. The wells were then washed as before. Now, 100 µl of orthophenylenediamine (OPD) solution was added to all the wells (0.1 mg/ml OPD diluted 1:20 in phosphate/citrate buffer containing 1 µl of 30% H_2O_2 (Sigma Chem. Co.)/ml of solution). A 30-

minute incubation at room temperature followed. To stop the reaction, 50 µl of 2.5N H_2SO_4 was added to the wells. The absorbance of each well was read using an ELISA reader (Beckman Instruments, Inc.) set to 490 nm (Bringman and Aggarwal, 1987).

Cleveland Gel Peptide Mapping of ERK1

Cleveland gel (Cleveland et al., 1977) peptide mapping analysis was employed on the ERK1 samples for additional characterization of the protein by proteolytic fractions. The purpose of this digest was to try to compare proteolytic ERK1 fragments from the recombinant GST/ERK1 fusion protein and fragments generated from sea urchin egg cytoskeleton fractions containing ERK. Before performing the Cleveland gel, an SDS-PAGE mini-gel was run. A mixture of 40 µl ERK1, 40 µl thrombin, and 8 µl cation solution (100 mM Ca^{2+} , 100 Mn^{2+} , and 100 mM Mg^{2+} brought up to 1 ml stock in distilled water) was allowed to digest for 4 hours at 37° C. Afterwards, to the tube containing the digestion mixture, 30 µl 4X SDS sample buffer was added. The mixture was boiled for 2 minutes. Next, 4 lanes were loaded, each of which contained 30 µl sample. The samples were electrophoresed at 100 volts until the dye front reached the end of the stacking gel (2.7 ml water, 0.75 ml bis-acrylamide, 1 ml stacking buffer at

pH 6.8 with SDS, 30 µl ammonium persulfate, and 11 µl TEMED). Then, the voltage was increased to 200 volts as the dye front from the samples met the resolving gel (3.2 ml glycerol, 12 ml of pH 8.8 resolving buffer, 6.4 ml distilled water, 28.4 ml bis-acrylamide, 160 µl ammonium persulfate, and 48 µl TEMED). Once the dye front came within 1 cm of the bottom of the resolving gel, the voltage was turned off to stop protein migration. The gel was then stained in Coomassie blue for 5-10 minutes and then destained in Coomassie blue destain (350 ml methanol, 100 ml glacial acetic acid, 550 ml distilled water) for three 5-minute washes. The excised bands were then mixed with Gel Slice Buffer I (0.125 M Tris, 20% glycerol, 0.1 g SDS, and 1 ml β-mercaptoethanol brought up to 100 ml in distilled water) for 30 minutes. Diluted *Staphylococcus aureus* V8-protease (Sigma Chem. Co.) at a concentration of 50 ng was added to Gel Slice Buffer III (0.125 M Tris, 10% glycerol, pH 6.8 brought up to 100 ml in distilled water) and mixed with bromophenol blue.

Four lanes on a 1 mm wide gel (as opposed to a 0.5 mm gel as ran above) were established. One lane contained 5 µl prestained standards (Sigma Chem. Co.). Another lane contained 10 µl V8 protease, the excised ERK1 band, and 10 µl Gel Slice Buffer II. A third lane contained Gel Slice buffer II (10 µl) and 10 µl V8 protease. A fourth lane contained 10 µl

unfertilized sea urchin cytoskeleton mixed with 3 µl 4X SDS sample buffer. The gel was electrophoresed at 100 volts until the dye front approached the stacking gel. Then, the gel was turned off for 30 minutes to allow for V8 protease digestion. After 30 minutes, the gel was electrophoresed through the resolving gel at 200 volts until the dye front reached approximately 1 cm from the bottom of the gel. The gel was then stained with Coomassie blue overnight and then destained with Coomassie destain (3 X 5 minute washes). The gels were then scanned for digitized analysis. High molecular weight standards (Sigma Chem. Co.) were used in this study to determine approximate molecular weights of samples used on gels and blots.

Western Blot Analysis

Western blot analysis (Towbin et al., 1979) was performed as follows. First, a polyvinylidene fluoride (PVDF) membrane was prepared by prewetting in methanol and then in water. Proteins were transferred electrophoretically from the gel onto a PVDF membrane by electrophoresing for 30 minutes at 24 volts in a Genie apparatus (Idea Scientific). Transfer occurred in transfer buffer (57.6 g glycine, 12.1 g tris, 800 ml methanol, 3200 ml distilled water). The membrane was now blocked in blocking buffer (5 g 5% non-fat dry milk in 100 ml TBS-Tween) for 1 hour at room

temperature on a rocking table or overnight in the refrigerator. After blocking the membrane, primary antibody (8 ml TBS, 2 ml blocking buffer, 20 µl or 3.3 µl primary antibody) was added to the membrane and allowed to incubate for 1 hour with the respective membranes. Mouse antiserum to the human recombinant GST/ERK1 fusion protein was one source of primary antibody used, and anti-ERK1 (Transduction, Inc.) was another source of primary antibody used. Next, the unbound primary antibody was washed from the membranes by performing 3 X 5 minute washes with TBS-Tween (2 ml Tween-20 in 1,000 ml TBS). Now, the membranes were incubated with secondary antibody. A 1:3000 dilution of BioRad's goat-anti-mouse IgG conjugated to horseradish peroxidase was added to secondary antibody solution for all the blots in this study (8 ml TBS, 2 ml blocking buffer, 20 µl or 3.3 µl secondary antibody), and the membranes were incubated for 1 hour at room temperature. Afterwards, 3 X 5 minute washes with TBS (58.44 g 0.5 M NaCl, 4.84 g 20 mM tris base, brought up to 2.0 liters of distilled water with pH adjusted to 7.5 with HCl) followed. The antibodies bound to the membrane were now detected by chemiluminescence. First, the blot was submerged with a mixture containing 5 ml luminol (Pierce Chem. Co.) and 5 ml peroxide (Pierce Chem. Co.) for 5 minutes while swirling in a container.

X-ray film (Kodak) was gently placed over the blot in a darkroom for 5 to 10 seconds. The film was developed with GBX film developer (Kodak).

IV. Results

The pGEX-ERK1 plasmid was subjected to an ECoR1 restriction enzyme digest (Figure 2). Lane A contained the λ molecular weight standard. Lane B contained pGEX-2T, the unrecombinant commercial plasmid with no insert in it. Lane C contained the cut pGEX-ERK1 plasmid, and lane D contained the uncut plasmid containing ERK1. Lane C had bands appearing in the linearized plasmid as well as in the ERK1 region, and only one band was apparent in lane B which represented the plasmid alone. The uncut plasmid (lane D) should only show one band, pGEX-ERK1. However, the presence of the uppermost band may represent DNA supercoiling changing the migration of ERK1 on the DNA gel.

The recombinant GST/ERK1 fusion protein was derived from an induced pGEX-ERK1-containing culture by affinity chromatography. The 25 fractions eluted from the glutathione column were run on an SDS-PAGE mini-gel (Figure 3). The gel showed greatest protein concentration, as indicated by the intense staining of bands in the ~60 kDa GST/ERK1 molecular weight region, in lanes shown. These lanes corresponded to fractions 4-6 from the glutathione column.

Peak fractions were quantified by the Bradford protein assay method (Figure 4). Fractions containing recombinant GST/ERK1 protein, fractions

4-6, had significant amounts of protein. The fractions were pooled, and the total recombinant GST/ERK1 amount was then determined. The pooled concentration was 5.27 mg/ml in a total of 1.5 mls. The total amount of antigen obtained was 7.905 mg.

Figure 5 shows a thrombin digest of the recombinant protein containing GST and ERK1. Thrombin cleaves the bond between serine and proline in Figure 2. The recombinant protein (10 μl) was added to a microcentrifuge tube with 10 μl thrombin (10 units/ml), and 2 μl 100 mM cation (Ca^{2+} , Mn^{2+} , Mg^{2+}) solution. The tube was incubated for 4 hours at 37° C before adding 1/3 volume 4X SDS and loading onto an SDS-PAGE gel (Laemmli, 1970). Lane A contained undigested recombinant ERK1, and lane B contained thrombin-digested recombinant ERK1. The thrombin-digested recombinant ERK1 lane showed bands in the molecular weight regions for undigested recombinant protein (~50-60 kDa), for ERK1 (~42-43 kDa), and for GST (~26 kDa), whereas the undigested lane showed only bands (~50-60 kDa) corresponding to the molecular weight of the recombinant fusion protein and its breakdown product since ERK1 and GST were not separated from each other here.

To purify ERK1 and GST, fragments were subjected to specific precipitation of GST by glutathione-agarose beads. Figure 6 shows the

results of this separation. Lane A contained the GST pellet sample, and lane B contained the ERK1 supernatant. Interestingly, lane A showed bands corresponding to the recombinant GST/ERK1 protein and its breakdown product (~50-60 kDa), as well as to ERK1 (~43 kDa) and GST (~26 kDa) on the gel. Lane B showed a distinct band in the GST/ERK1 molecular weight range, also.

Figure 7 further supports the data of Figure 6. Lane A contained the GST pellet. Lane B contained ERK1 supernatant, and lane C contained undigested ERK1. In lane A, a distinct band appeared in the recombinant GST/ERK1 protein molecular weight range (~60 kDa), in the ERK1 range (~43 kDa), and also in the GST range (~26 kDa). The ERK1 supernatant lane showed a band in the recombinant GST/ERK1 molecular region only. Also, since lane C was untreated with thrombin, a distinct band in both the ERK1 and the GST molecular weight regions did not appear. However, bands appeared in the molecular weight region for the recombinant GST/ERK1 protein and its breakdown product (~50-60 kDa) in this lane.

To better characterize the structure of ERK1, peptide mapping was performed. Figure 8 shows results from a Cleveland peptide map. V8-protease digestion of purified ERK1 from the recombinant GST/ERK1 protein resulted in a series of distinct bands. In particular, V8-protease is a

glutamic acid endopeptidase that cleaves at the carboxyl group of this amino acid in a peptide bond (Cleveland, 1977). The V8-protease concentration was 50 ng/10 µl. A Western blot was performed on the proteolytic ERK1 fragments (data not shown) to see if there was any binding of antibody to a potential epitope in the fragments, but no bands were detected by commercial anti-ERK1 antibody. This may have been because the digest rendered such little protein.

To confirm if the mouse was producing antibodies to the recombinant GST/ERK1 fusion protein, an ELISA was performed as previously described. Table 1 shows the data from the test. Since O.D. readings for the immunized mouse antiserum were consistently over 0.5 up to the 1/12800 dilution, the mouse appeared to be producing the GST/ERK1 antibody (Row A, Table 1). The commercial antibody was also used (Row B, Table 1), and the titers were not nearly as strong as for the mouse since the commercial antibody was diluted 1:100 in TBS before performing the serial dilutions with the ELISA.

Mouse antiserum against GST/ERK1 was tested against the recombinant GST/ERK1 fusion protein and against a 90-minute post-fertilization cytoskeleton fraction from sea urchin by Western blot analysis (Figure 9). Lane A contained the GST/ERK1 fusion protein. Lane B

contained the sea urchin cytoskeleton fraction. Lane A showed a very significant amount of binding on the blot in the molecular weight range of the GST/ERK1 protein (~60 kDa). The large bands in the ~60 kDa range indicated strong binding of the antibody to the fusion protein with a large amount of breakdown from the fusion protein. Lane B showed significant amounts of binding in the ~50-60 kDa region, as well, which is consistent with the region where ERK1 may be present on a gel.

A Western blot using the immunized mouse anti-GST/ERK1 serum and commercial anti-ERK1 antibodies was performed to compare the specificity of binding of these antibody sources to thrombin-digested ERK1 (Figure 10). Lane A (1:500 dilution) contained mouse antiserum against GST/ERK1. Lane B contained normal mouse serum (1:500 dilution), and lane C contained commercial anti-ERK1 (Transduction, Inc.) at a 1:500 dilution. Lane A and lane C showed binding patterns in the ~60 kDa molecular weight region which is where the recombinant GST/ERK1 fusion protein runs on the gel. These two lanes also showed breakdown bands from the ~60 kDa region which also appear to be binding ERK1. Lane C shows some nonspecific binding, as well.

A Western blot using the mouse GST/ERK1 antiserum (1:5000 dilution) versus sea urchin egg cytoskeleton fractions and versus purified

ERK1 from the GST/ERK1 fusion protein was performed to compare binding specificity with purified ERK1 in the protein samples (Figure 11). Lane A represented the 90-minute post-fertilization cytoskeleton fraction, and lane B represented the 95-minute fraction. Interestingly, strong binding on the blot in these lanes occurred at the ~60 kDa molecular weight region which would correspond to where GST/ERK1 would run on a gel. The purified ERK1 sample in lane C appeared to show a strong signal with the antibody in the ~60 kDa region, also, whereas ERK1 is a ~42-43 kDa protein.

Since the mouse GST/ERK1 antiserum was binding the ERK1 at the region on the gel where the recombinant GST/ERK1 fusion protein was expected to run on the gel (~60 kDa), it was necessary to determine specifically what component of the GST/ERK1 fusion protein the mouse antiserum (1:5000 dilution) was binding. A Western blot was performed (Figure 12) on purified components of the GST/ERK1 protein to help reveal the answer. Lane A represented the lane where thrombin-digested ERK1 was loaded on the gel. Lane B represented the lane where GST was loaded on the gel. Both lanes show doublets in the ~50-60 kDa region when compared to molecular weight standards (Sigma Chem. Co.). The ~60 kDa region corresponds to the molecular weight region for GST/ERK1. Perhaps,

the thrombin digest was not totally effective, and some undigested recombinant protein was left in both lanes that were blotted. Since no banding occurred in the ERK1 molecular weight region in either lane, therefore, one possible explanation could be that GST/ERK1 has one epitope and that this epitope was cleaved from the ERK1 sample and remained intact in the undigested GST/ERK1 molecular weight region. Thus, antibody may have been bound to the ERK1 epitope portion of GST/ERK1. Neither lane had banding in the ~26 kDa region which would indicate the presence of GST.

A Western blot using commercial anti-ERK1 (Transduction, Inc.) antibody (1:5000 dilution) was also performed against the components of the GST/ERK1 fusion protein (Figure 13). The purpose of this blot was to compare specificity of binding results with those results obtained from Figure 12. Lane A represented the lane where GST was run on the gel. Lane B represented the lane where ERK1 was run on the gel. Again, both lanes showed doublets in the ~50-60 kDa region. Therefore, the antibody appeared to be binding ERK1, since the commercial antibody was specific for ERK1, but in the GST/ERK1 region.

Since binding on Western blots was evident to what was believed to be the ERK1 portion of the recombinant GST/ERK1 fusion protein, I grew

P3X myeloma cell/spleen cell hybridomas which would multiply indefinitely in culture and which would produce the desired antibody to ERK1 for future studies. The hybridomas could then be subjected to limiting dilution cloning to produce monoclonal antibodies to a single epitope on ERK1. Tables 2 and 3 show data for the first ELISA testing the hybridoma production of antibody. Table 2 shows the data testing ERK1 as an antigen, and Table 3 shows data for the plate coated with GST/ERK1. The readings for the GST/ERK1 plate were much higher in general than for the ERK1 plate. Table 4 shows data for the second ELISA for the hybridoma cells producing antibody against GST/ERK1. The highest absorbance readings on the table were A2, A3, A6, C12, D1, D23, D25, and D26. The absorbances of these wells were determined high enough to perform cloning. Table 5 shows ELISA data for these eight hybridoma supernatants grown for an additional week before cloning.

The purpose of limiting dilution cloning was to produce single clones of the specific antibody to an ERK1 epitope for future studies. Figures 14-17 show the results of the limiting dilution clones of four positive P3X/spleen hybridoma supernatant samples from Table 5. The data show where single clones arise on the 96-well plates. None of the cloned cells producing antibody were cloned a subsequent time. The cloned antibodies and cell lines

were not tested at this time, and they were frozen for future use.

Table 1. Results from the ELISA showing production of mouse antibody to GST/ERK1.

	control	*1/100*	*1/200*	*1/400*	*1/800*	*1/1600*	*1/3200*	*1/6400*	*1/12800*	*1/25600*	*1/51200*	*1/102400*	*1/204800*
A	N/A	0.711	0.778	0.944	1.047	0.701	1.005	0.615	0.665	0.447	0.321	0.317	
B	N/A	0.751	0.255	0.245	0.182	0.236	0.259	0.243	0.185	0.222	0.196	0.197	0.122
C	0.231												
D	0.117												

Row A is immunized mouse antiserum
Row B is anti-ERK1 (Transduction Labs, Inc.)
Row C is non-immunized serum at 1/100 dilution
Row D is blank (no serum)

Table 2. Results from the first ELISA of hybridomas producing antibody to purified ERK1.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
A	0.245	0.191	0.229	0.215	0.224	0.156	0.173	0.190	0.159	0.165
B	0.445									
C	0.138									
D	0.262									

Row A is hybridoma supernatant samples
Row B is immunized mouse antiserum at 1/100 dilution
Row C is blank (no serum)
Row D is non-immunized serum at 1/100 dilution

Table 3. Results from the first ELISA of hybridomas producing antibodies to GST/ERK1.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
A	0.344	0.408	0.386	0.418	0.352	0.382	0.273	0.279	0.376	0.439
B	0.797									
C	0.140									
D	0.268									

Row A is hybridoma supernantant samples
Row B is immunized mouse antiserum at 1/100 dilution
Row C is blank (no serum)
Row D is non-immunized serum at 1/100 dilution

Table 4. Second ELISA experiment for hybridomas producing antibodies to GST/ERK1.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
A	.233	***0.272***	***0.320***	0.198	0.214	***0.262***	0.217	0.217	0.216	0.229
B	.483	.179	.179							
	11	12	13	14	15	16	17	18	19	20
C	0.185	***0.285***	0.203	0.189	0.201	0.210	0.218	0.189	0.213	0.258
	21	22	23	24	25	26	27			
D	***0.272***	0.234	***0.323***	0.264	***0.268***	***0.267***	0.228			

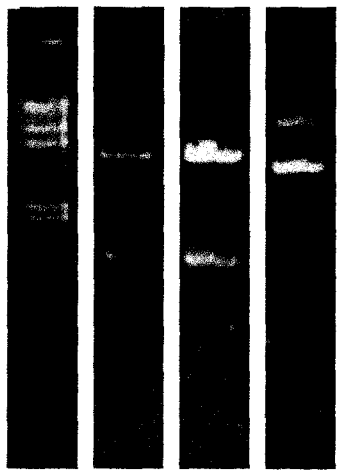
Row A represents hybridoma supernatant samples 1-10
B1 is immunized mouse antiserum at 1/100 dilution
B2 is blank (no serum)
B3 is non-immunized serum at 1/100 dilution
Row C represents hybridoma supernatant samples 11-20
Row D represents hybridoma supernatant samples 21-30
* bold/italics equals the particular samples subjected to limiting dilution cloning

Table 5. ELISA experiment on positive wells from Table 4.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
A	.259	.247	.261	.275	.273	.251	.290	.274
B	.290	.126	.110					

A1-A4 represent A2, A3, C12, and D23 supernatants respectively (the wells that were chosen to clone) from Table 4
A5-A8 represent A6, D1, D25, and D26 supernatants respectively from Table 4
B1 is immunized mouse antiserum at 1/100 dilution
B2 is blank (no serum)
B3 is non-immunized serum at 1/100 dilution

Figure 2. DNA restriction digest of pGEX-ERK1 using ECoR1. Lane A represents the λ molecular weight standard. Lane B represents GST (unrecombinant commercial plasmid). Lane C represents the recombinant ERK1 plasmid cut with ECoR1. Lane D represents the uncut recombinant plasmid. The second band in lane D may represent supercoiling of DNA. All lanes contain 20 μl DNA.



A. B. C. D.

Figure 3. Affinity chromatography results. Lanes A, B, and C (corresponding to fractions 4-6 from the protein column) show the recombinant GST/ERK1 fusion protein. The arrow indicates where the recombinant protein is being eluted from the glutathione column. Each lane contains 10 μl sample.

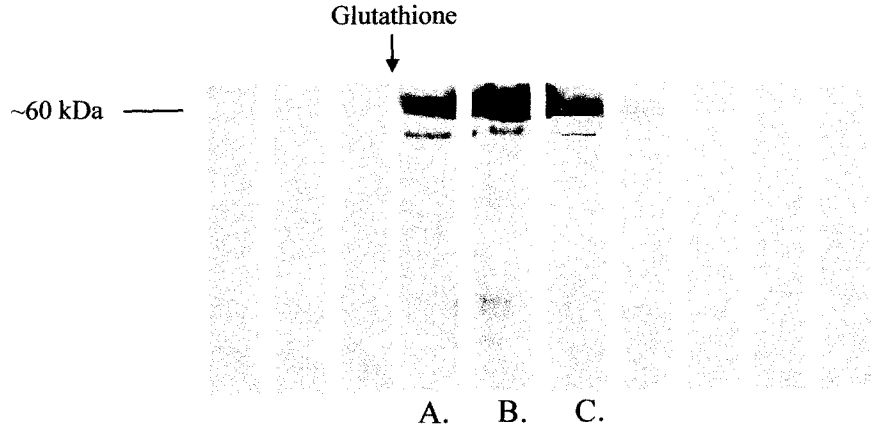

Glutathione
~60 kDa
A.
B.
C.

Figure 4. GST/ERK1 protein assay data showing concentration versus fraction.

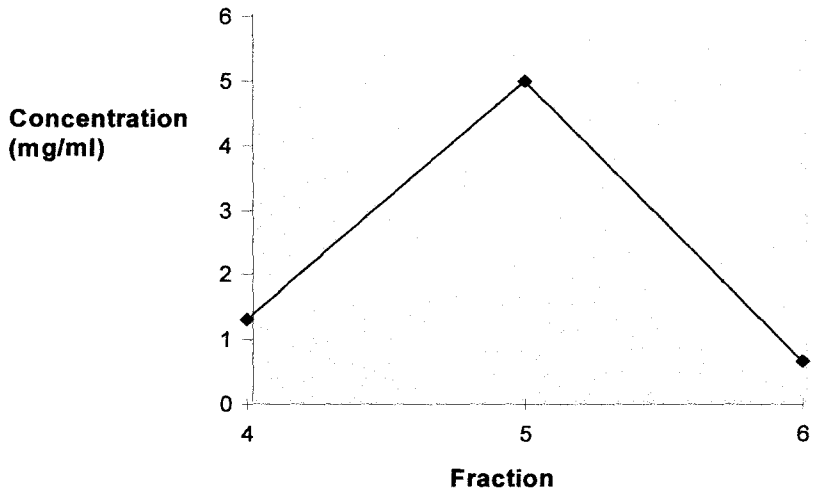

Concentration
(mg/ml)
6
5
4
3
2
1
0
4
5
6
Fraction

Figure 5. Thrombin digest. Lane A shows the undigested GST/ERK1 recombinant protein. Lane B shows the thrombin-digested recombinant protein. Each lane contains ~1 μg sample.

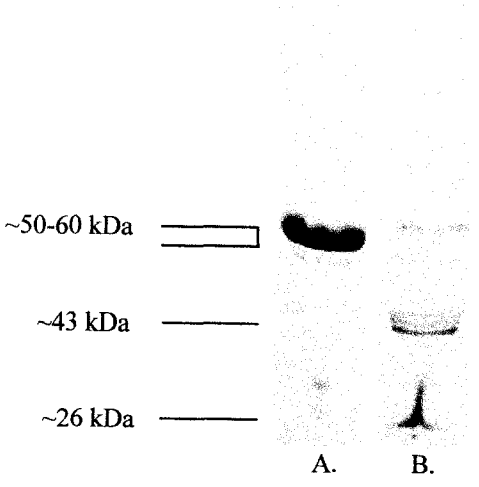

~50-60 kDa
~43 kDa
~26 kDa
A.
B.

Figure 6. Purified GST pellet (Lane A) and purified ERK1 supernatant (Lane B). Each lane contains 1 μg sample.

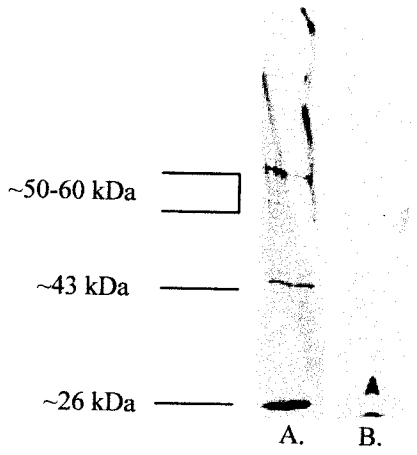

~50-60 kDa
~43 kDa
~26 kDa
A.
B.

Figure 7. Second experiment run on the purified components of the recombinant protein. Lane A is the GST pellet. Lane B is the ERK1 supernatant. Lane C is the undigested recombinant protein containing GST and ERK1. Each lane contains ~1 μg sample.

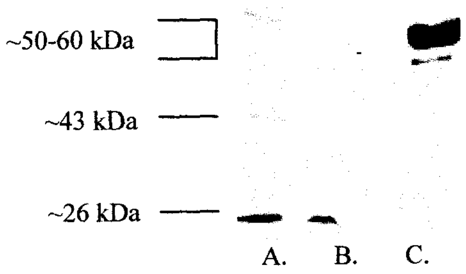

~50-60 kDa
~43 kDa
~26 kDa
A.
B.
C.

Figure 8. Peptide mapping gel of purified human ERK1.

Figure 9. Western blot using mouse antiserum against the human recombinant GST/ERK1 fusion protein (Lane A) and against 90-minute sea urchin egg extract (Lane B). Each lane contains ~1 μg sample.

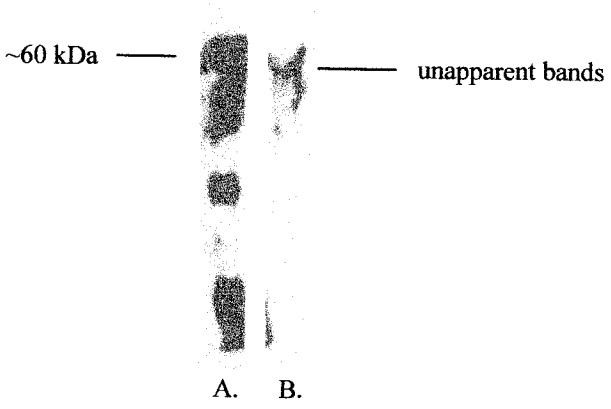

~60 kDa
unapparent bands
A.
B.

Figure 10. Western blot showing mouse anti-GST/ERK1 (Lane A) and non-immunized mouse (Lane B) serum to thrombin-digested ERK1. Lane C shows commercial anti-ERK1 antibody to thrombin-digested ERK1. Each lane contains ~1 μg sample.

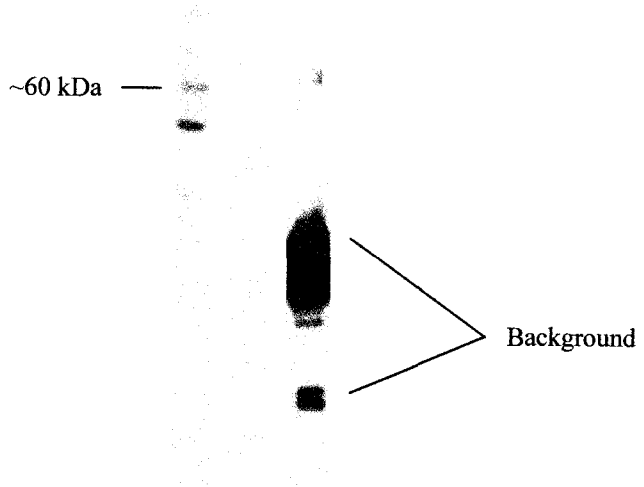

~60 kDa
Background
A. B. C.

Figure 11. Western blot of the mouse anti-GST/ERK1 versus sea urchin egg cytoskeleton fractions and thrombin-digested ERK1 from the recombinant GST/ERK1 fusion protein. Lane A represents a 90-minute post-fertilization sea urchin cytoskeleton fraction. Lane B represents a 95-minute fraction. Lane C represents purified ERK1 from the human GST/ERK1 recombinant protein. Lane D represents purified ERK1 spillover onto the next lane. Each lane contains ~1 μg sample.

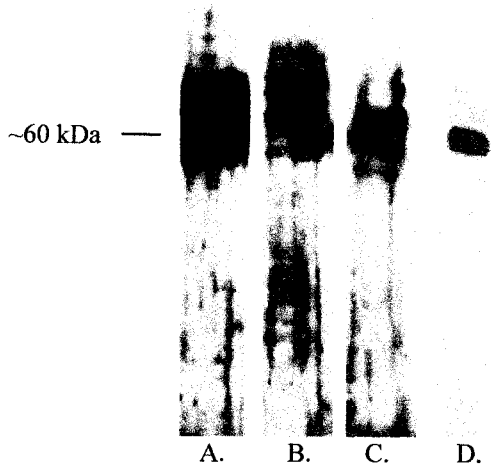
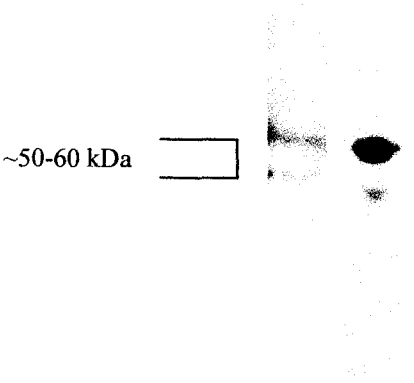

~60 kDa
A.
B.
C.
D.

Figure 12. Western blot using mouse GST/ERK1 antiserum. Lane A is the ERK1 supernatant lane. Lane B is the GST pellet lane. Each lane contains ~1 μg sample.

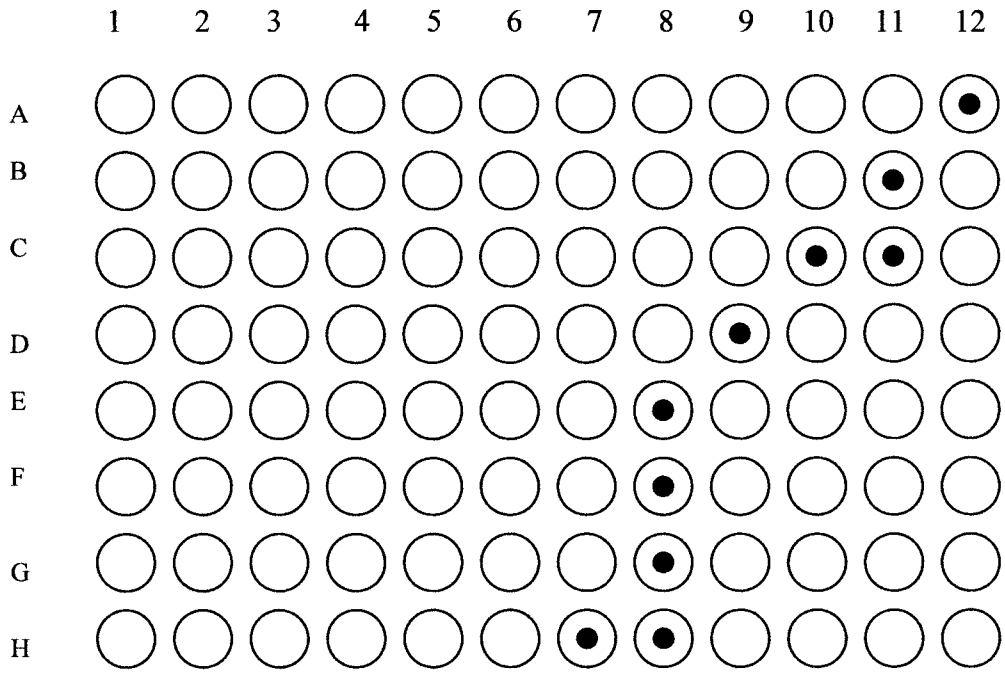


A. B.

Figure 13. Western blot using commercial anti-ERK1 antibody. Lane A is the GST pellet lane. Lane B is the ERK1 supernatant lane. Each lane contains ~1 μg sample.

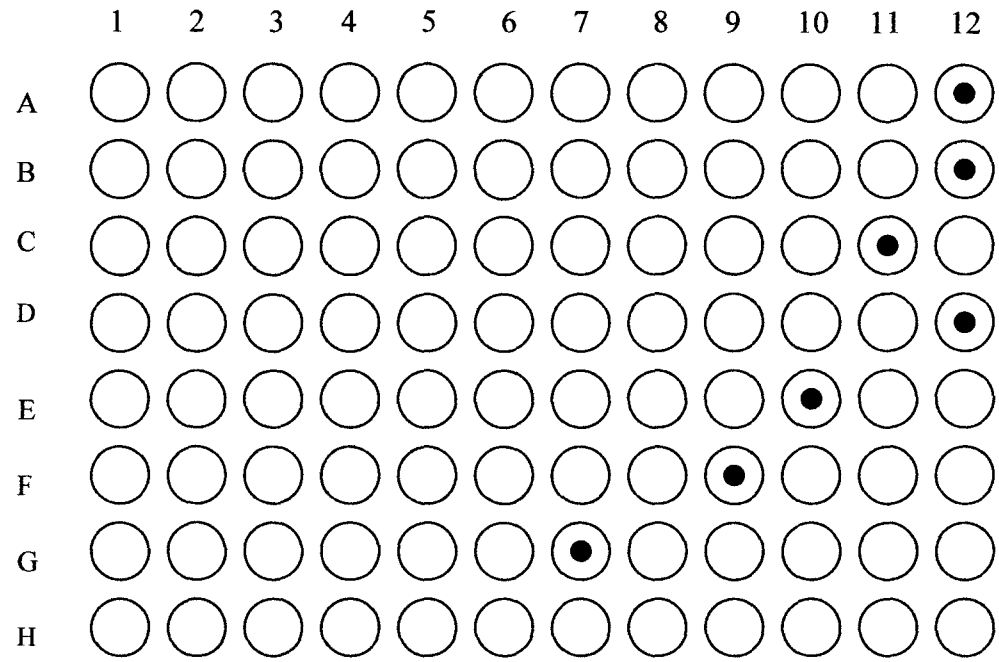
A. B.

Figure 14. Characterization of single clones by testing hybridoma supernatant sample A2 from Table 4.



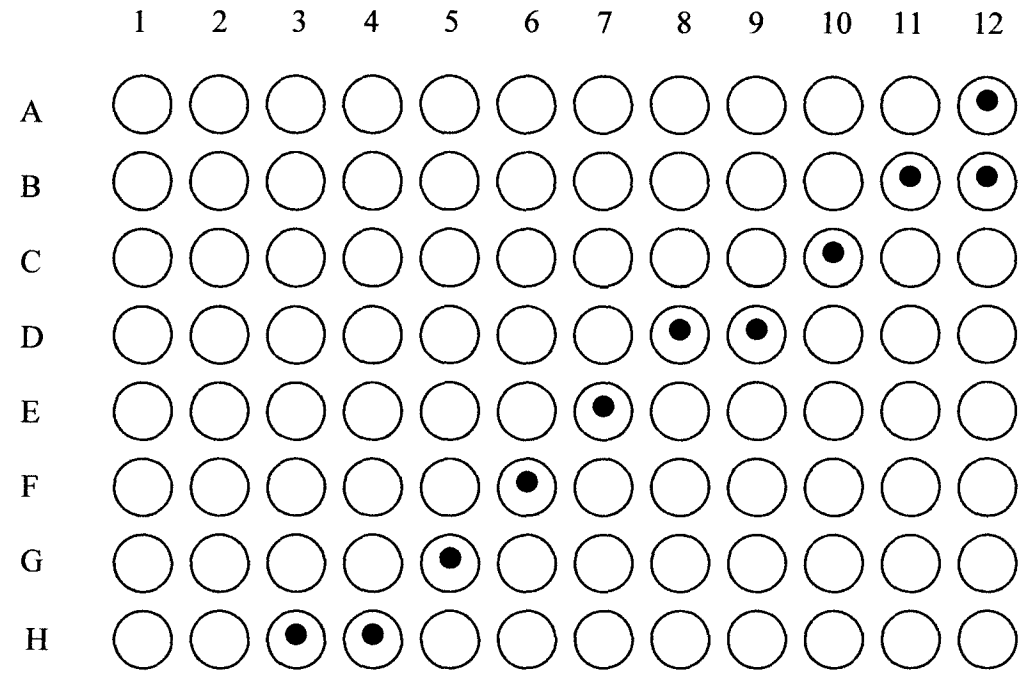
* filled circles within big circles indicate wells with single clones.

Figure 15. Characterization of single clones by testing hybridoma supernatant sample A3 from Table 4.



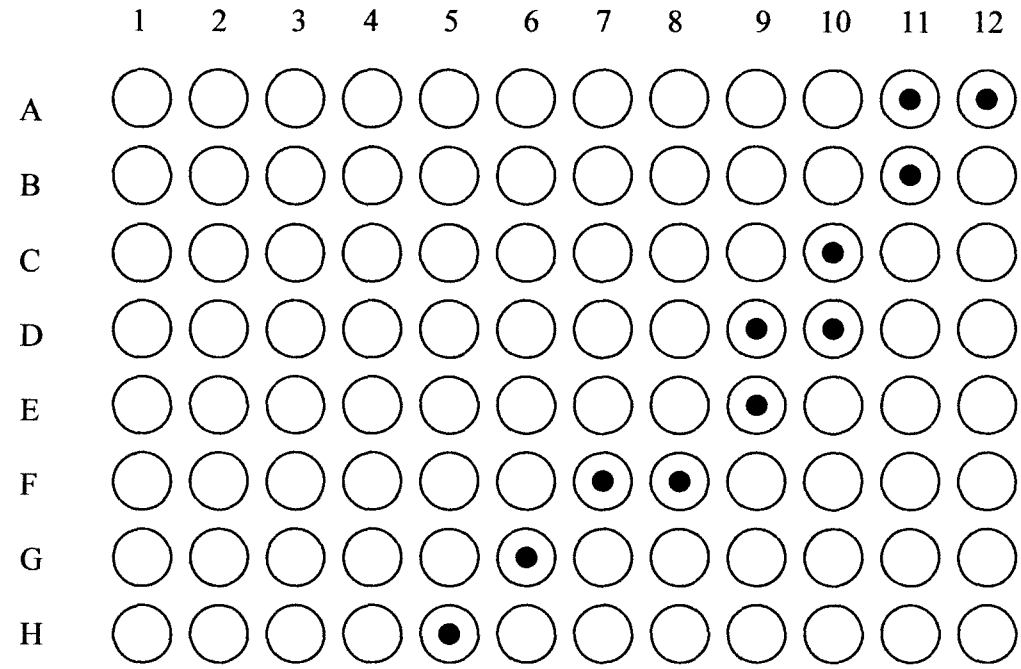
* filled circles within big circles indicate wells with single clones.

Figure 16. Characterization of single clones by testing hybridoma supernatant sample C12 from Table 4.



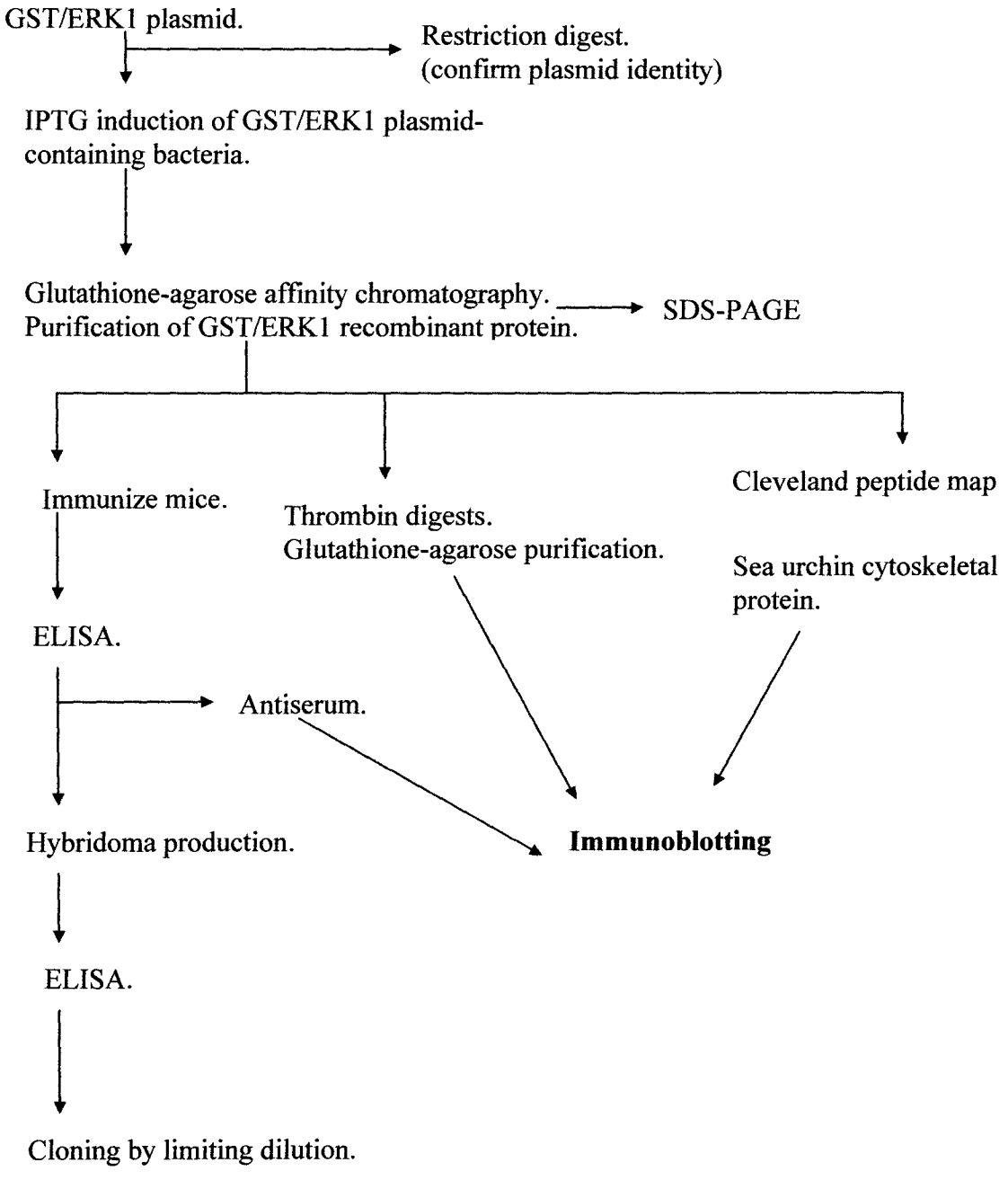
* filled circles within big circles indicate wells with single clones.

Figure 17. Characterization of single clones by testing hybridoma supernatant sample D23 from Table 4.



* filled circles within big circles indicate wells with single clones.

Figure 18. Summary of study.


GST/ERK1 plasmid.
Restriction digest.
(confirm plasmid identity)
IPTG induction of GST/ERK1 plasmid-containing bacteria.
Glutathione-agarose affinity chromatography.
Purification of GST/ERK1 recombinant protein.
SDS-PAGE
Immunize mice.
Thrombin digests.
Glutathione-agarose purification.
Cleveland peptide map
Sea urchin cytoskeletal protein.
ELISA.
Antiserum.
Hybridoma production.
Immunoblotting
ELISA.
Cloning by limiting dilution.

V. Discussion

ERKs are essential proteins to cell division. A vast array of extracellular proliferation- and differentiation-inducing signals turn on these kinases, and ERKs are essential to signalling cascades. Upon activation via phosphorylation on both a threonine and a tyrosine, ERKs become involved in such cellular processes as cell growth, cell division, and cell differentiation. The goal of my study was to compare sea urchin ERK1 and ERK1 from a human recombinant GST/ERK1 fusion protein.

Data from my Western blots suggest that the mouse serum against the recombinant GST/ERK1 fusion protein was binding to the ERK1 portion of the recombinant protein (Figure 12) since no band to the GST region was detected. The bands seen on this blot were in the ~50-60 kDa molecular weight region. The ~60 kDa region is where GST/ERK1 runs on a gel. One would expect a ~42-43 kDa band, which is where ERK1 should run on the gel. The reason for no ~42-43 kDa band was that the thrombin digest for this blot may not have been strictly specific, and although some, if not most, ERK1 was cleaved from GST, perhaps the essential epitope was a small sequence that was retained in the recombinant protein upon cleavage but destroyed by digestion. Even if there were more than one epitope, there is a chance that all these epitopes could have been destroyed by digestion.

Antibody binding to the recombinant protein occurred in the ERK1 sample (lane A) and in the GST sample (lane B) to indicate that undigested recombinant protein was found in both samples. Another reason for this result may be that digestion of GST/ERK1 may have resulted in doublet patterns seen in Figure 12, but I had no way of detecting whether ERK1 alone and not both ERK1 and the undigested recombinant protein were present in this ~60 kDa region since, theoretically, antibody to ERK1 or to the recombinant GST/ERK1 protein could bind an ERK1 component in either case. Regardless of which presumption could be held true, GST was not bound by antibody on this blot.

A Western blot using commercial anti-ERK1 (Transduction, Inc.) was performed to show where specifically ERK1 was located on the gel in Figure 12. The contents of this blot were GST (lane A) and ERK1 (lane B). Results here were practically identical to those for Figure 12 using the mouse antiserum. Again, there appeared to be undigested recombinant protein in the GST (lane A) and ERK1 (lane B) samples, and the antibody binding was to the ~50-60 kDa region for both samples indicating that not only was there undigested recombinant protein in either sample, but there was also binding to the ERK1 portion of the recombinant protein. No binding of GST occurred in this blot also.

The results from the Western blot in Figure 11 were critical to this study. The results here showed that the 90- and 95-minute sea urchin cytoskeleton fractions were bound by the mouse anti-GST/ERK1 antibody at the same location on the blot as the purified ERK1 sample on the blot. This region of binding was in the ~60 kDa molecular weight region. Since the sea urchin extract has no GST/ERK1 fusion protein endogenous to it and since no banding occurred in the ERK1 molecular weight region, one possibility for binding to the ~50-60 kDa region is that an ~49-50 kDa ERK may be endogenous to sea urchins. Walker et al. (in press) discovered a potential higher molecular weight ERK1 in sea urchins. Further studies would be necessary to prove or disprove this notion. Perhaps, also, the reason that binding occurred in the GST/ERK1 molecular weight region is that cross-reactivity was occurring in binding. Thus, there appears to be similarity in protein structure between sea urchin egg extracts and the human recombinant GST/ERK1 fusion protein in this region which may indicate the presence of ERK1 in the ~50-60 kDa region in both the human and sea urchin examples. The sea urchin fractions may have had a similar enough epitope(s) to the human recombinant ERK1 in the GST/ERK1 region which allowed for binding.

Data from the ELISAs were used in comparison with the Western

blots. The first ELISA (Table 1) was performed on wells coated with the GST/ERK1 recombinant protein to see if the mouse antibody titer to this protein was high enough to begin growing hybridomas. Absorbance reading were relatively high (most > 0.5) for almost all wells in the 1:2 serial dilutions. The commercial anti-ERK1 antibody (Transduction, Inc.) produced lower readings than did the mouse antiserum. Perhaps, since the commercial antibody was diluted 1:100 before even being applied to the plates, this may have caused for lower absorbances in the serially diluted wells.

ELISAs were performed on hybridoma supernatants periodically to determine when titers of anti-GST/ERK1 were high enough to begin cloning. The first ELISAs using the hybridomas (Tables 2 and 3) as the source of primary antibody produced key findings. For example, the wells coated with just ERK1 produced much lower absorbance readings in general than did the wells containing the GST/ERK1 fusion protein. These results complemented the Western blot analysis in that probably the thrombin digest was an incomplete digestion and that this digestion cleaved the epitope from the purified ERK1. This epitope was possibly retained with the GST/ERK1 fusion protein.

The second ELISA with the hybridoma antibody (Table 4) produced

lower readings than did the first ELISA with the hybridomas. Perhaps, the hybridomas were dying and therefore produced lower titers of antibody. Regardless, the four wells producing the highest titers were used for cloning.

Future work can stem from this research. One study in particular could be to analyze if the sea urchin extract has an ERK1 component in the ~50-60 kDa region that bound the commercial ERK1 antibody and the mouse GST/ERK1 antiserum in this study. Substrate binding assays with either MAP-2 or myelin basic protein could give more information on whether this ~50-60 kDa protein is an ERK. Also, perhaps, performing this experiment over but with the use of a GST/ERK2 protein instead of a GST/ERK1 protein could be useful for comparison to see if blots on thrombin-digested ERK2 give similar binding patterns as did ERK1. Perhaps, a better way to digest ERK1 from GST than by thrombin digestion could be found. With a more purifed ERK1, one could redo this experiment using ERK1 rather than the entire recombinant GST/ERK1 fusion protein for immunizations and could then generate monoclonal antibodies to a specific site on ERK1. The monoclonal antibodies could then be tested against sea urchin egg extracts to see if this antigenic sequence is also found in sea urchin extracts.

VI. References

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February 14, 1995

Dr. Diana Fagan
Department of Biological Sciences
U N I V E R S I T Y

Dear Dr. Fagan:

Upon recommendation of the Animal Care and Use Committee, the annual update of your ongoing protocol 92-016 has been approved with the expiration date as requested on your application.

You must adhere to procedures described in your approved request; any modifications must first be authorized by the Animal Care and Use Committee.

Sincerely,

Peter J. Kasvinsky
Dean of Graduate Studies

kb

Enclosure

c: Dr. Leipheimer, Chair, IACUC
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December 19, 1996

Dr. Diana Fagan
Department of Biological Sciences
UNIVERSITY

Dear Dr. Fagan:

Upon recommendation of the Animal Care and Use Committee, your proposals entitled "Use of Animals in Research: Immunization, Collection of Cells and Cell Culture," (Protocol #97-2); and "Natural Killer Cell Hybridoma Preparation," (Protocol #97-3) have been approved.

You must adhere to procedures described in your approved request; any modification must first be authorized by the Animal Care and Use Committee.

Sincerely,

Peter J. Kasvinsky
Dean of Graduate Studies

cc

c: Dr. Paul Peterson, Chair,
Biological Sciences
IACUC Committee