Methods in Signal Transduction

Summary

The transmission of extracellular signals into intracellular targets is mediated by a network of interacting proteins that regulate a large number of cellular processes. One of the best understood signaling mechanisms is the one initiated by growth factors which transmit their signals through specific receptors. Binding of growth factors to their receptors, initiates intracellular signaling pathways which culminate in the activation of regulatory molecules in the cytoplasm or in the nucleus and initiate proliferation.

In this lab course we will learn advanced methods in determining growth factor signaling. We will transfect tissue culture cells with growth factor receptors, determine levels of expression, and characterize its enzymatic activities (tyrosine phosphorylation, activation of Ras). Then we will turn into the intracellular stimulated signaling and study how to determine the activity of various signaling serine/threonine kinases such as PKC, MAPKK (MEK), MAPKs (ERK JNK p38MAPK) and PI3K-dependent signaling. The methods that will be studied include: Transient transfection; Ras assay; serine/threonine as well as tyrosine kinase assays; protein purification (affinity and anion exchange chromatography) and finally immunological methods such as immunoprecipitation and Western blotting.

For more information see the web site:

http://www.weizmann.ac.il/Biological_Regulation/NewFiles/rony/mainpage.html

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1) Introduction

The ability of cells to respond rapidly to varying conditions and to extracellular agents is crucial for their adaptation to an ever-changing environment. Hormones, growth factors, cytokines, stress conditions, contact with solid surfaces, and other extracellular stimuli, each induces various cellular processes, including synthesis of new proteins, which will determine the cell's fate in response to a particular stimulus. The mechanisms by which extracellular agents transmit messages via specific receptors to evoke intracellular processes are known as "signal transduction" pathways (Fig. 1). A few of these pathways are relatively simple, such as the one triggered by the reproductive hormone estrogen, in which a small ligand diffuses all the way to the cell nucleus, where it binds a receptor protein, and regulates transcription. However, most extracellular triggering agents are not as small and diffusible as the estrogen, and therefore require facilitation mechanisms to transmit their signals. In many cases, such complex signaling mechanisms involve a network of interacting molecules.

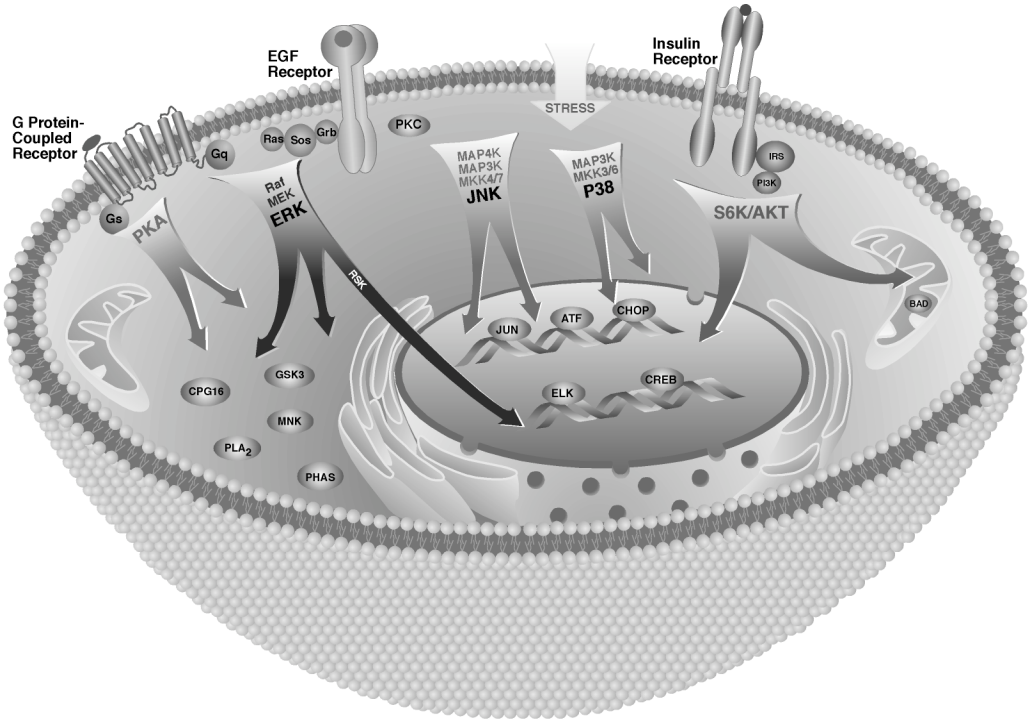

Fig. 1 - Intracellular signaling cascades

A common mechanism of signal transduction in many cellular processes is the sequential activation of kinases ((protein kinase cascades (9)). During the past decade, several related intracellular signaling cascades have been elucidated, which are collectively known as mitogen-activated protein kinase (MAPK) signaling cascades (3,7,9,10). Each of these signaling cascades seems to consist of up to five tiers of protein kinases which sequentially activate each other by phosphorylation. The activation of each of these cascades seems to be initiated by either a small GTP-binding protein or an adapter protein, which transmits the signal to protein kinases at the

MAPK kinase kinase (MAP3K) level of the cascades. In some of the cascades, the activation involves additional upstream kinases at the level of MAP3K kinase (MAP4K), which phosphorylate and activate kinases at the MAP3K level. The signal is then transmitted down the cascade by enzymes at the following tiers, which are referred to as MAPK kinase (MAPKK), MAPK, and MAPK-activated protein kinases (MAPKAPKs). The existence of four to five tiers in each of the MAPK cascades is probably essential for signal amplification, specificity, and tight regulation of the transmitted signals. The four distinct MAPK cascades that are currently known were named according to their MAPK components [the ERK, JNK, SPK (p38MAPK), and BMK (ERK5) cascades]. These MAPK cascades cooperate to transmit signals to their intracellular targets, and thus, to initiate processes such as proliferation, differentiation, development, apoptosis and response to stress.

Apart from the MAPK cascades, additional kinase cascades are activated in response to mitogenic stimulation. These include the NIK-IKK1/2 (5,8) PI3K-AKT (4), Rho-dependent (6), ERK3 kinase-ERK3 (2) and PKA-phosphorylase kinase (1) pathways, which are involved in transmission of many extracellular signals. These pathways do not belong to the family of MAPK cascades, because of the different nature of their components. All the kinase pathways mentioned (MAPK and non-MAPK cascades) are influenced by distinct extracellular agents, leading to activation of these pathways through an elaborate signaling network, and culminating in a characteristic outcome for each stimulation.

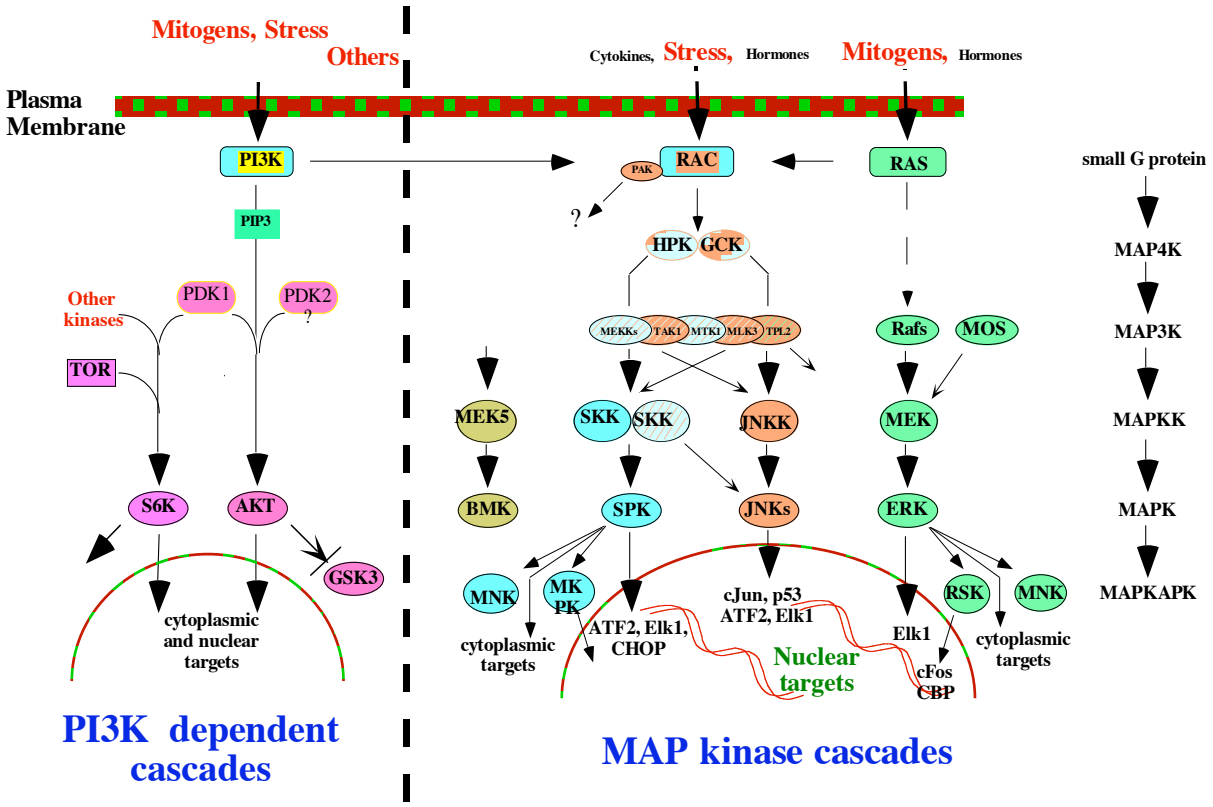

Fig. 2 - Detailed demonstration of the MAPK and PI3K-dependent cascades

2) Transient Transfections

2A. DNA Transfection Using DE-Dextran

MATERIALS

DMEM/FCS = DMEM + 10% FCS + glutamine + antibiotics.

Soln. A = 5 μg Plasmid DNA (GFP in pcDNA1), 500μl of DE-Dextran (0.2 mg/ml in $PBS^{=}$). Make by adding 10 μl of DE-Dextran (1 mg/ml in PBS) to 500 μl of $PBS^{=}$. Prepare fresh for each transfection.

Soln. B = DMEM containing 100 μM chloroquine (10 mg/ml in $PBS^{=}$)
Make by adding 5 μl of 0.1M chloroquine diphosphate (51.6 mg/ml in PBS) to 5 ml DMEM. Prepare fresh for each transfection.

Soln. C = 10% DMSO in $PBS^{=}$.

PROCEDURE

Day 1 - Split cells 24 hours before transfection to reach 50-70% confluency on the next day.

Day 2- * START HERE

1) Wash cells (x2) with $PBS^{=}$ (37^{o}C).

2) Add 0.5 ml of Soln. A to cells (6 cm plate) and swirl gently.

3) Incubate 30 minutes at 37^{o}C (swirl gently every 5 to 10 min).

4) Add 5 ml of Soln. B.

5) Incubate 2.5-3.5 hrs at 37^{o}C and remove medium by aspiration.

6) Aspirate all the medium.

7) Add 2 ml of Soln. C for 1.5 min, and wash twice with PBS.

8) Aspirate and add DMEM/FCS.

2B. DNA Transfection Using $CaPO_4$

MATERIALS

2xBES - 50 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na_2HPO_4; pH 6.96.

0.25 M $CaCl_2$ - 1.35 g of $CaCl_2 \cdot 6H_2O$ in 20 ml DDW.

PROCEDURE

Day 1 - Split cells 24 hours before transfection (a 60 mm plate to 4 plates).

Day 2- * START HERE

1) Mix 5 μg of plasmid DNA with 0.25 ml of 0.25 M $CaCl_2$.

2) Add 0.25 ml of 2xBES to the mixture of step 1 (add dropwise while vortexing the mixture) 3) Incubate the above mixture for 10 minutes at room temperature

4) Add the mixture to the dish, swirling gently to mix well.

5) Incubate 15-24 hrs in incubator set for 3% CO_2

6) Aspirate, wash x2 with DMEM and continue growing under normal conditions.

2C. DNA Transfection Using Polyethylenimine (PEI)

MATERIALS:

Soln A: Mix 5 □g DNA with 125 □l NaCl (150 mM) prepared in polypropylen tubes.

Soln B: Mix 25 □l of PEI (10 mM) with 100 □l of NaCl (150 mM). Prepare both solutions fresh for each transfection.

PROCEDURES:

Day 1 - Split cells (6 cm plates) 24 hours before transfection.

Day 2- * START HERE

1. Add Soln B to Soln A dropwise while vortexing. Incubate the mixture at RT for 15 mins.
2. Add 2.0 ml of serum free medium and mix gently.
3. Wash cells once with DMEM.
4. Carefully cover the cells with the mixture. Incubate for 2.0 hrs.
5. Wash the cells twice with DMEM.
6. Add normal mediun with FCS.

2D. Electroporation

PROCEDURE

1) Harvest cells by trypsinization.
2) Wash twice with DMEM (no serum).
3) Bring to final concentration of 1.5 x10^6/ml and put into a 0.8 ml quevette.
4) Add 10 μg plasmid DNA and place on ice for 10 min.
5) Set "Gene Pulser" on: 300 V, 250 μFD and Resistance = ∞.
6) Place quevette in its holder and push two red buttons, check appearance of small bubbles.
7) Plate the electroporated cells with DMEM/FCS

2E. Lipofectamine

MATERIALS

Solution A: 5 μg DNA, 200 μl Opti-mem (or F12) prepared in polypropylen tubes. Prepare fresh for each transfection.

Solution B: 15 μl lipofectamine, 200 μl Opti-mem (or F12) prepared in polypropylen tubes. Prepare fresh for each transfection.

PROCEDURE

Day 1 - Split cells 24 hrs before transfection (a 60 mm plate to 4 plates).

Day 2- • START HERE

1) Mix solns A+B in a polypropylen tube and leave 30 min at room temperature
2) Add to the mixture 2 ml of Opti-mem (or F12) and mix gently.
3) Wash cells in $PBS^=$, carefully aspirate all the remaining $PBS^=$.
4) Add the lipofectamine mix (from step 2) to the plate.
5) Place at 37^{o}C incubator for 4-5 hrs .
6) Aspirate the mix from the plates, add DMEM/FCS and continue growing in the incubator ON.

2F. β-Galactosidase Staining

MATERIALS

soln C : 1% gluteraldehyde in PBS

soln D : 3.3 mM $K_3(Fe(CN)_6)$, 3.3mM $K_4(Fe(CN)_6)$, 2mg/ml X-Gal and 1 mM $MgCl_2$ in PBS.

PROCEDURE

1) Wash the plate twice with PBS
2) Add 3 ml of soln C to the plate to fix the cells
3) Incubate for 15 min at room temperature.
4) Wash the plate twice with PBS
5) Add 3 ml of soln D to the plate and cover with an aluminum foil.
6) Incubate the plate for 2 - 6 hrs at 37°C
7) Check the results in the microscope without a filter.

3) General Techniques

3A. SDS-PAGE and Western Blotting

SDS-polyacrylamide gel electrophoresis (SDS- PAGE)

MATERIALS

Mini-gel apparatus

Acrylamide (30%):bisacrylamide (0.8%) solution (in water).

Lower (separating) Buffer:1.5 M Tris-HCl, 0.4% SDS, pH 8.8

Upper (stacking) Buffer: 0.5 M Tris-HCl, 0.4% SDS, pH 6.8

TEMED (tetramethylethelendiamine)

10% ammonium persulfate (APS, freshly prepared in water)

Sample buffer (4X concentrated): 200 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 0.2% Bromophenol-blue, 100mM DTT.

Running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3

PROCEDURES

1. Assemble glass plates and spacers.
2. Prepare 10% polyacrylamide separating gel (10 ml): 3.3 ml acrylamide stock solution
   2.5 ml of Lower Buffer
   4.2 ml of water
3. Mix well and add 150 μl APS. Mix and add 15 μl TEMED.
4. Mix the solution and cast the gel.
5. Overlay separating gel with water. Allow gel to polymerize (about 10-15 min).
6. Prepare 5 ml of 3% polyacrylamide stacking gel: 750 μl acrylamide stock solution; 1.25 ml of Upper Buffer; 3.0 ml of water

7. Mix and add 100 μl APS. Mix well and add 10 μl TEMED.
8. Mix the solution and cast the gel.
9. Insert comb and allow to polymerize (5-10 min).
10. Prepare samples: Mix 100 μg protein extract with 1:3 of 4X concentrated sample buffer. Boil the samples for 5 min. and spin down the samples.
11. Assemble gel in apparatus, add running buffer to the upper chamber and watch for leaks.
12. Add buffer to the lower chamber.
13. Load samples.
14. Run gel: Connect the wire leads to the power supply. Turn the power supply on and run the gel at 150 V (constant voltage) for about 75 min.
15. End run: When the bromophenol-blue dye reaches the bottom, turn off the power supply.

Western Blotting (protein blotting)

MATERIALS

Transfer buffer: 15 mM Tris, 120 mM glycine, approximate pH 8.8

Transfer apparatus, Nitrocellulose membrane and two pieces of 3 mm Whatman papers

PROCEDURES

1. Prewet the nitrocellulose membrane in transfer buffer.
2. Once the dye front of the gel has reached the end, take the gel out from between the glass plates.
3. Cut off the stacking gel.
4. Open the transfer sandwich and get rid of air bubbles within the scotch-bright pads.
5. Put a wet scotch-bright pad on the black side of the transfer sandwich and add a 3 mm paper on top of it .
6. Put the SDS-gel on the 3 mm paper, and put the wet nitrocellulose membrane on the gel, discard bubbles
7. Put the other 3 mm paper on the nitrocellulose membrane.
8. Add another wet scotch-bright pad on top of the transfer sandwich, and close the transfer sandwich. Take care not to trap air bubbles between the gel and the other components.
9. Place the transfer sandwich into the buffer-filled transfer apparatus. Nitrocellulose membrane should face the anode side (black to black).
10. Close the transfer apparatus, connect it to the power supply (200 mA constant current, 90 min) The voltage drops as the transfer progresses due to an increase in the buffer conductivity.
11. At the end of the transfer, remove the nitrocellulose membrane from the transfer sandwich. Rinse the transferred nitrocellulose in transfer buffer to remove any adhering polyacrylamide.

staining and quenching the nitrocellulose membrane

MATERIALS

Ponceau S solution: 0.2% (w/v) Ponceau S, in 5% acetic acid (v/v; Sigma p7170]

Washing buffer: 20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST)

Blocking solution: 2% (w/v) Bovine serum albumin (BSA) in washing buffer.

PROCEDURES

1. Immerse blot in Ponceau reagent for 30 sec.
2. Wash the blot in water to obtain transient pattern.
3. Mark major or relevant bands with pen, since pattern is lost if the blot is quenched.
4. Incubate the blot in washing buffer for 5 min to get rid of the dye.
5. Incubate the blot in 2% BSA solution for 30 min at room temperature for blocking.

❦ Incubation of the nitrocellulose membrane with antibodies

MATERIALS

Washing buffer - TBSTx1.

1^{st} antibody solution diluted in a washing buffer.

PROCEDURES

1. Once quenched, incubate the blot with the 1^{st} antibody solution for overnight at 4^{o}C. (incubation for 30 min at 37^{o}C or 1 hr at room temperature are also OK).
2. After overnight incubation, wash the blot at least 3 times for 10 min each with washing buffer.

❦ Incubation of nitrocellulose membrane with HRP-conjugated second antibody

MATERIALS

2^{nd} antibody solution diluted in a washing buffer

PROCEDURES

1. Once washed, incubate the blot with the 2^{nd} antibody solution for 1 hr at room temperature.
2. Wash the blot at least 3 times for 10 min each with washing buffer.

❦ Histochemical detection of labeled proteins

MATERIALS

Substrate solutions:

A: 5 mM 100 mM tris 8.5 + 22 μM 1 mM p-coumarin +50 μM Luminol,

B: 3.3 μl 50% H_2O_2

Piece of 3 mm Whatman paper, Saran wrap and Fluorescent marker

PROCEDURE

1. Mix solutions A , add solution B and mix again
2. Incubate the blot with the substrate solution for 2 min.
2. Dry the blot by 3 mm Whatman paper.
3. Put the fluorescent marker on the right top of the blot.
4. Wrap the blot with saran.
5. Expose the blot to X-ray film in dark room for 30 sec - 5 min and develop the films.

3B. Preparation of Cell Extracts

Preparation of cell extracts with a detergent

MATERIALS

Buffer A: 50 mM β-glycerophosphate (GP), pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate.

Buffer H (homogenization buffer): 50 mM β-GP, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM Na3VO4, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A

PBS=

Cell Scraper

Refrigerated centrifuge

20% NP40/Triton-X-100

VOOH: A: 40 μl of 100mM of Na_3VO_4 +160 μl DDW

B: 8 μ of 1M H_2O_2+ 800 μl PBS=

C: Mix A+B (total of 1 ml) and incubate 10 min at room temperature

D: Add to the cells 25 μl/1 ml meduim (final con.: 0.1mM Na_3VO_4 and 0.2 mM H_2O_2)

PROCEDURE

1. Grow cells [six tissue culture plates per construct, i.e, wild-type and mutant] in appropriate medium + 10% FCS to subconfluency in a tissue culture incubator (37°C, 5% CO_2).

2. Serum starve cells for 18 hours in DMEM:F12 + 0.1% FCS (5 ml/ 10cm plate)

   During serum starvation, place the plates in a tissue culture incubator (37°C, 5% CO_2). Make sure that the plates remain flat and that the medium covers all the plate equally. The aim of this starvation is to make the cells quiescence, which, under these conditions, can be achieved within 14-24 hours. Starvation for too long, or any change in temperature or pH, may be stressful to the cells, and thereby, induce activation of one or more signaling pathways. Although this protocol describes EGF stimulation of CHO cells, this procedure, with minor changes, can be used for mosr stimulated cells.

3. Stimulate cells with various stimuli for 2-45 minutes, with inhibitors for 20 min, or with VOOH for 20 minutes(positive control). The control plates will be left untreated.

   Usually, the stimulus is given first to the plates with the longest incubations, then, at appropriate intervals, to the plates with the next longest incubation periods. It is useful to use a time chart, so that the stimuli will be given at appropriate times and the cells will be harvested within a short period of time (5-10 min).

4. At the appropriate time interval, remove the medium from the plates and rinse them twice with ice-cold PBS (5 ml each) and once with ice-cold Buffer A.

   Since the arrest and slowing down of biological processes is desired at this stage, the plates can be placed on ice. The washing and harvesting of each plate should take 0.5-1.5 min, therefore seven plates should be harvested within 5-10 min.

5. Add 300 μl Buffer H+detergent, scrape cells with a rubber policeman and transfer to pre-cooled appropriately labeled eppendorf tubes, leave on ice for 10 min.

6. Spin at 14,000 rpm for 10 minutes at 4°C, to pellet nuclei.

7. Transfer supernatant to new, labeled, precooled eppendorf tubes and determine protein concentrations.

The protein concentration of each sample should be determined so that identical amounts of proteins from the different samples can be compared, and thus the relative amount of protein kinases in each sample determined accurately. If samples are compared based on cell number, differences of up to 20% in the amount of protein may result. Such differences may cause even larger ones in the following steps when phosphorylation is assessed immunologically.

Preparation of cell extracts using sonication

MATERIALS

Buffer A: 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium orthovanadate

Buffer H (homogenization buffer): 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A

PBS=

Cell Scraper

Mini-sonicator

Refrigerated centrifuge

PROCEDURE

1. Grow one plate each (10 cm) of cells to subconfluency and serum starve for 18 hrs
2. Stimulate cells with various stimuli as directed by the course team.
3. Remove medium and rinsed twice with ice cold PBS and once with ice-cold Buffer A.
4. Add 350 μl of ice-cold Buffer H to each plate, tilt the plate gently (preferably on ice), and scrape the cells into the buffer using a plastic (or rubber) policeman. Using pre-cooled pipette tips, transfer the cells and buffer to prelabeled, pre-cooled 1.5 ml eppendorf tubes.

Special consideration should be given to the composition of buffer H. We recommend using b-glycerophosphate which serves both as a buffer and as a general phosphatase inhibitor, rather than TRIS or HEPES. Sodium orthovanadate is used to inhibit tyrosine phosphatases and the mixture of pepstatin-A, aprotinin, leupeptin, and benzamidine are used to inhibit proteinases. We found that this buffer, when cold, blocks most of the phosphatase and proteinase activities in cell extracts.

5. Disrupt the cells by sonication (two 7 seconds 50W pulses per 0.5 ml sample) on ice.

Over the years, several methods of protein extraction from cells have been successfully used in the study of protein kinases. In this protocol, which utilizes sonication, proteins are extracted from the cytosolic and nuclear fractions, but not from the membrane fraction, and therefore can be considered as "Cytosolic extract". Cellular extraction with non-ionic detergents extracts proteins from the membranal, cytosolic and some nuclear fractions of the cell. Extraction with RIPA buffer or by freezing-thawing can also be used for some kinases.

6. Centrifuge at 14,000 x g for 15 min at 4ºC. The supernatant contains the cytosolic extracts to be examined. All subsequent steps are performed at 4ºC.

Determination of protein concentration

MATERIALS

96-well plate

Bradford solution (Pierce) - ready to use

BSA

lysis buffer

microplate reader

PROCEDURE

1. Dilute 10 μl of each of the supernatants of the cell extracts samples in 190 μl DDW (a final dilution of 1:20) in labeled tubes.

   Usually, dilutions of at least 1:20 or larger are necessary to ensure the samples will be in the linear range of the protein determination assay. For some coomassie blue reagents with extended ranges, this dilution is not always necessary.

2. Place 10 μl of each of the protein standards (0, 10, 25, 50, 75, 100, 150 and 200 μg/ml BSA in lysis buffer) into at least 2 wells of a flat-bottom 96 well microplate (in duplicates).

3. Place 10 μl of each of the diluted samples into the same microplate (in duplicates). Add 200 μl of the Bradford reagent to all wells.
4. Place the microplate in a microplate reader and determine the optical density of the samples at 595 nM.
5. Plot the optical densities of the protein standards against the concentration and calculate by linear regression (y=ax+b) the slope (a) and constant (b). Calculate the protein concentrations of the samples by the formula: Protein conc. (mg/ml) = (a $*$ sample OD - b) $*$ dilution factor.

4) Growth Factor Stimulated Tyr-Phosphorylation

4A. Introduction

Polypeptides such as growth factors and hormones are crucial components of the regulatory system that coordinates development of multicellular organisms. Many of these factors mediate their pleiotropic action upon binding to, and activating cell surface receptors with an intrinsic protein tyrosine kinase activity that share a similar molecular topology.

Receptor tyrosine kinases catalyze the phosphorylation of exogenous substrate as well as tyrosine residues within their own polypeptide chains. These phosphorylations activate cellular proteins and leads to potentiation of various cellular responses.

The EGF receptor (EGFR) is a receptor tyrosine kinase. Following EGF binding to the extracellular domain of EGFR, the receptor kinase is activated to autophosphorylate its own intracellular domain as well as other cellular proteins. These phosphorylations leads to EGF-induced growth promoting effects.

4B. Use of Antibodies

MATERIALS

Washing buffer (TBST).

1st antibody solution: monoclonal phosphotyrosine antibody (PY-99 from "Transduction Lab.") diluted 1:1000 in washing buffer contains 0.1% (w/v) BSA and 0.05% sodium azide. Other antibodies should be used according to the manufacture recommendation.

PROCEDURES

1. Once quenched, incubate the blot with the 1st antibody solution for overnight at 4°C. (incubation of 30 min. at 37°C or 1 hour at room temperature have also been found to be useful).
2. After overnight incubation, wash the blot at least 3 times for 10 min. each with washing buffer.

4C. *In vitro* Tyr-kinase Assay of EGF-Receptor

Introduction

The EGFR is a transmembrane glycoprotein. In order to determine the tyrosine kinase activity of the (EGFR) preparation an *in vitro* kinase assay is performed using a synthetic peptide contains Tyr and Glu residues (poly Glu-Tyr), as a substrate.

Protein kinase assay

MATERIALS

EGF Receptor preparation

Reaction mixture (RM): 10 mM $MgCl_2$, 4 mM $MnCl_2$, 50 mM Hepes pH 7.5,
50 μM ATP and, 1 μl γ-^{32}P ATP.

Poly glu-tyr 5 mg/ml

PROCEDURES

1. Incubate 40 μl EGFR prep, 20 μl Poly glu-tyr and 40 μl RM, for 15 min. at room temperature.
2. Subject 80 μl of each sample to pre-marked (with pencil only) 4 cm^2 paper.
3. Wash the papers 3 times for 30 min. with 10% TCA solution.
4. Wash the papers over night with 10% TCA solution.
5. Wash the papers with 100% acetone and dry the papers.
6. Detect the incorporation of γ-^{32}P to poly Glu-Tyr.
7. Determine the specific activity of each Tyr-Kinase preparation.

5) Ras Assay

REFERENCES

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2. Franke, B., Akkerman, J.W., Bos, J.L. (1996) *EMBO J.* 16: 252-259.
3. WolthuisR.M., Zwartkruis, F., Moen, T.C., Bos, J.N. (1998) *Curr. Biol.* 8: 471-474.

5A. Introduction

The Ras proto-oncogene is a small GTPase, that functions as a molecular switch in many signal transduction processes. Ras is mostly bound to GDP in resting cells. Upon stimulation with growth factors, guanine nucleotide exchange factors (GEFs) for Ras are translocated to the plasma membrane, where they exchange GDP for GTP to Ras. Binding of GTP to Ras induces a conformational change that allow Ras to bind to its effector molecules. The most well known effectors for Ras are the Raf-family kinases, PI-3 kinase and GEFs for the small GTPase Ral.

The classical approach to measure Ras activity is based on labeling of cells with ^{32}P, immunoprecipitation of Ras with a monoclonal antibody, and analyze of the nucleotides that bound to Ras by thin layer chromatography. More recently, an easier approach has been developed, that does not require cell labeling. This assay is based on the differential affinity of effector molecules for the GTP-bound Ras as compared to GDP-bound Ras (ref 1 above). In this assay, a fusion protein consisting of Glutathion-S-Transferase (GST) and the Ras-binding domain (RBD) of Raf is first precoupled to glutathion-agarose beads and then incubated with a cell lysate. Subsequently, the beads are washed and the GTP-bound Ras isolated and quantitated by immunoblotting. This method allows rapid analysis of many different samples. Similar approaches using different effectors have now been developed for several other GTPases like Rap1 and Ral (ref 2,3 above).

MATERIALS

PBS++ : PBS containing: 0.5 mM DTT, 1 μg/ml aprotinin**,** 1 μg/ml leupeptin, 0.1 mg/ml trypsin inhibitor, 250 μM PMSF, 1 mM benzamidine, and 1mM sodium orthovanadate

Reduced Glutathione Buffer: 50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione, 1 μg/ml aprotinin, 1 μg/ml leupeptin**,** 0.1 mg/ml trypsin inhibitor, 250 μM PMSF, 1 mM benzamidine 1mM sodium orthovanadate

Ral-buffer: 50 mM Tris-HCl, pH 7.5, 10% glycerol, 200 mM NaCl, 2.5 mM $MgCl_2$, 1% NP-40 1 μg/ml aprotinin, 1 μg/ml leupeptin,, 250 μM PMSF, 10 mM NaF 1mM sodium orthovanadate

HNTG Buffer: 20 mM Hepes pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, 10% glycerol

PROCEDURE:

5B. Preparation of Fusion Proteins (not done during the course)

1. GST-fusion proteins can be grown either in E. Coli JM109, DH5 or in more specialized, protease deficient strains (e.g., BL21).

2. Bacteria are cultured overnight in 50 ml 2YT medium + ampiciline (100μg/ml) at 37°C.
3. The next day dilute the culture 1:50 by addition to pre-warmed 1000 ml 2YT + ampiciline and grow at 37°C with shaking until OD600=0.5.
4. 1M IPTG (iospropylthiogalactoside) is added to a final concentration of 0.5 mM and the culture is grown for another 3 hours.
5. Bacteria are spun down 15 minutes at 6000xg (Sorvall GS3 rotor 6000 rpm) at 4°C, the bacterial pellet can be frozen at -80°C.
6. Resuspend the pellet on ice with ice-cold 50 ml PBS^{++}.
7. Disrupt by sonication 5 times for 30 seconds on ice with sonicator.
8. Add 20% Triton X-100 to a final concentration of 1%, and mix for 30 minutes at 4°C.
9. Spin the lysate at 12,000 x g (Sorval SS-34 rotor 11000 rpm) at 4°C for 15 minutes at 4°C and collect the supernatant, keep a sample for gel detection.
10 Add 1 ml of 50% (v/v) prewashed glutathion beads to the sup and incubate at 4^{O}C for 1 hour.
11. Wash the beads 3 times with 10X bead volume cold PBS and keep sample for gel detection.
12. Add 0.5 ml reduced glutathion buffer.
13. Elute at room temperature for ten minutes.
14. Spin the beads down and transfer the supernatant to a new eppendorf tube.
15. Repeat elution three more times and keep the supernatants separately.
16. Determine protein concentration for each of the supernatants and pool the most concentrated ones, alternatively analyse on SDS PAGE gel by staining.
17. Dialyse the samples overnight at 4°C against PBS^{++} and for another three hours the next day.
18. Aliquot and freeze at -80°C in.
19. Check purity of GST-fusion protein by SDS-PAGE and Coomassie Brilliant blue staining.

5C. GTPase-Fish Assay

1. Each group will receive six 10 cm plates of cells, cells should be at 70% confluency
2. Serum-starve the cells for 24 hours, with DMEM:F12 medium.
3. Wash Glutathion agarose beads three times in 1 ml Ral-buffer.
4. GST-fusion proteins are thawed on ice and coupled to glutathion agarose beads for at least 1 hour at 4°C (10 μg GST-RBD protein with 14 μl of 50% slurry of beads /10 cm culture plate).

START HERE

5. Treat plates with various stimuli for 5, 10 and 30 minutes. At the end of incubation transfer plates to ice and immediately wash with 6 ml ice cold PBS. Repeat wash two more times.
6. Remove all of the PBS by placing the dishes briefly at a 45° angle.
7. Lyse the cells with 300 μl Ral buffer on ice.
8. Scrape cells with a rubber policeman and transfer to pre-cooled eppendorf tubes labeled appropriately.
9. Vortex briefly and gently and incubate for 10 minutes on ice.
10. Spin down nuclei and cellular debris in microfuge at 14,000 rpm for 10 minutes at 4°C.

11. In the meantime, wash the pre-couple glutathion beads twice with 1 ml $PBS^{=}$ and once with 1 ml Ral buffer and aliquot it into precooled labeled tubes. This can be done best with cut 200 μl tip. Widening the opening of the tip makes it easier to pipette identical amounts of beads into each tube.
12. Transfer the 300 μl of each lysate to the corresponding tube with precoupled beads. Keep an aliquot of 60 μl from the lysate and combine it with 4x sample buffer, which later can be used as a positive control for immunoblotting and to check if the lysates contain identical amount of protein, and for the activity of ERK, etc.
13. Incubate for 45 minutes on a rotary mixer at 4°C.
14. Wash the beads four times with 1 ml ice cold HNTG buffer. Aspirate all of the HNTG buffer once with 1 ml Buffer A and add 30 μl of 1x sample buffer.
15. Vortex briefly, spin down and incubate at 95°C for three minutes. Hereafter the samples can be stored at -20°C or directly analyzed by SDS-PAGE (15% gel). and transfer to PVDF membrane.
16. Western blotting with anti Ras antibody as previously described.

6) IP Assay of Protein Kinases

6A. Introduction to PKB/c-Akt

The serine/threonine kinase PKB, also known as c-Akt or RAC-kinase, consists of a catalytic domain and a PH domain. The PH domain is a protein module that can bind lipids and is found in a variety of signaling molecules with varying function (kinases, lipases, exchange factors etc.). The PH domain of PKB binds predominantly to 3'-phosphorylated lipids. However, lipid binding alone does not regulate kinase activity, phosphorylation of PKB, at threonine 308 and serine 473, is essential for its activation. The upstream kinase that phosphorylates threonine 308 has recently been identified and named phosphatidylinositol dependent kinase (PDK1), since it only phosphorylates PKB in the presence of PI-3P lipids. The PDK1 activity is constitutive, indicating that an increase in PI-3P lipids at the membrane results in PDK1 activation and PKB translocation, whereby the latter is activated by PDK1.

The PKB in vivo substrates have not been identified yet. Plausible targets include the BAD protein, involved in apoptosis regulation; the kinase GSK-3, involved in regulation of glycogen storage; and regulation of protein translation. A consensus PKB phosphorylation site has been identified as being RXRYYS/TZ, where X is any amino acid, Y is a small amino acid except glycine and Z preferably a hydrophobic amino acid. It should be noted that at least in vitro this consensus sequence may also serve as a substrate for other kinases such as $p70^{S6kinase}$ and $p90^{rsk}$.

6B. *In vitro* Kinase Activity Assay (PKB/P38MAPK/ERK).

MATERIALS

Histone 2B (Boehringer)/ MBP (sigma)

Protein A-sepaharose

PBS=

anti-PKB C-terminus Ab

rotating wheel

γ^{32}P-ATP

4x sample buffer

Heating plate

15% SDS-PAGE

Proper shields for radioactive work

Thermomixer or water bath at 30°C

0.5M LiCl in 0.1M Tris pH 8.0

RMx3: (reaction mixture at 3-fold final concentration), 30 mM $MgCl_2$, 1.5 mM DTT, 75 mM β-glycerophosphate, pH 7.3, 0.15 mM sodium orthovanadate, 6 μM PKI peptide, 3.75 mM EGTA, 0.3 mM ATP, 30 μM calmidazolium, 2.5 mg/ml bovine serum albumin (BSA).

Buffer A: 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1.0 mM EDTA, 1.0 mM (DTT), 0.1 mM sodium orthovanadate

RIPA Buffer: 137 mM NaCl, 20 mM Tris, pH 7.4, 10% (v/v) glycerol, 1% (v/v) Triton X-100 0.5% (w/v) Deoxycholate, 0.1% (w/v) SDS, 2.0 mM EDTA, 1.0 mM Phenylmethylsulphonyl flouride (PMSF, add fresh), 20 μM leupeptin

Sample Buffer (X4) for SDS-PAGE

PROCEDURE

❦ Immunoprecipitation

1. Place Protein A-Sepharose beads (~150 μl) in a 1.5 ml plastic test tube, add 1 ml of PBS, and let the beads swell for 10 min at room temperature.

   Although Protein A-conjugated Sepharose is recommended for this method, other commercially available Protein A-conjugated resins, such as agarose, HiTrap, or others, may be used. Protein G-coupled resins are sometimes required to immunoprecipitate certain types of antibodies. If resins are in ready-to-use solutions, they will not require this swelling step.

2. Wash the swollen beads three times with 1 ml PBS. This washing is achieved by centrifuging (1 min, 14,000xg, room temperature) the suspended beads in a microfuge and discarding the supernatant.
3. Add 15 μl of the antibodies to be conjugated, to 120 μl of the swollen packed beads and 365 μl of PBS. Rotate the mixture (1 hour, room temperature) on an end to end rotator to allow the antibodies to bind to the Protein A (this step can be done at 4°C for 16 hours).

   Usually, anti-C terminal antibodies are used for the determination of kinase activity because their binding to the kinase does not interfere with its kinase activity. Ideally, the volumes listed here should be sufficient for 10 reactions, but because of the density of the beads, will probably only be sufficient for eight reactions.

4. Wash resuspend in buffer and then centrifuge (1 min, 14,000xg, 4ºC)] the beads once with 1 ml ice-cold PBS and then three times with 1 ml ice-cold Buffer A. Resuspend the washed beads in an equal volume of ice-cold buffer A (~250 μl for ~250 μl of beads).

   Either use the antibody-conjugated beads immediately, or store at 4ºC until used. It is best to use the conjugated beads within three days of preparation.

5. Add 30 μl of the antibody-conjugated bead suspension (15 μl net) to 300 μl sample of cytosolic extract containing 50-500 μg total protein (in Buffer H) in precooled 1.5 ml plastic test tubes. There will be as many tubes as there are samples to be tested. Rotate end to end for 2 hours at 4˚C.

   Several immunoprecipitation methods have been developed. These methods usually vary in the order in which the antibodies and protein A are added to the cell extracts. In the protocol described here, the antibodies are conjugated to Protein A beads, and only then added to the cytosolic extracts. This procedure minimizes the time the samples are incubated with the antibodies, and thereby, minimizes exposure of the desired kinases to phosphatases and proteinases in the extracts. Furthermore, this procedure ensures that only antibodies recognized by Protein A will be used for the immunoprecipitation. Antibodies that are not recognized by Protein A can bind to the desired antigen, but will not be precipitated when Protein A beads are added, and therefore, will reduce the efficiency of immunoprecipitation.

6. Centrifuge (1 min, 14,000xg at 4˚C) the incubation mixture. Remove and discard the incubation supernatant from the antibody-conjugated beads. Wash the beads once with 1 ml ice-cold RIPA buffer, twice with ice-cold 0.5M LiCl, and twice with 1 ml ice-cold Buffer A.

   These stringent washes are important, because they will remove most "sticky" protein kinases that might non-specifically interact with the Protein A beads.

Phosphorylation reaction

7. After the last washing step in 6, completely remove Buffer A from the conjugated beads.

   Complete removal of the Buffer A can be achieved by removing Buffer A from the beads as above and then centrifuging again and gently removing the residual buffer above the beads.

8. Resuspend the pellets of the beads in 15 μl of double distilled water.

   At this stage, prepare your working bench for working with small amount of radioactivity.

9. Add 10 μl of RMx3 (with 100 μM γ^{32}p-ATP) to each tube.

   The most important components of the reaction mixture are the Mg^{+2} and [γ^{32}P]-ATP which are essential for the phosphorylation reaction. We recommend the use of 100 µM ATP with ~4000 cpm/pmol of the labeled ATP, which provides an extended linear range and reproducible results. When the enzymatic activity of the kinases is very low, which makes detection of phosphorylation difficult, concentration of cold ATP should be reduced to 10-20 µM and the amount of radioactive material elevated. Addition of labeled ATP alone is not recommended because this will result in a nanomolar concentration of ATP, which is much below the Km for ATP and may lead to non-specific phosphorylation. The β-glycerophosphate in the reaction mixture serves as a buffer, but can also inhibit residual phosphatases that may have non-specifically bound to the beads. The BSA serves as a carrier protein but when purity is required, it can be eliminated. The EGTA chelate Ca^{2+}, which may interfere with some kinase activities, DTT keeps the proteins reduced and sodium orthovanadate inhibits tyrosine phosphatases.

10. Start the phosphorylation reaction by adding 5 μl of the phosphorylation substrate (Histone/MBP, 2mg/ml) to the tube and placing the mixture in a termomixer at 30°C.

    Although Histones or MBP are probably not physiological substrates for any MAPK or PKB these are good general substrates for many kinases in vitro. Substrates should be well phosphorylated by the desired kinases to allow accurate detection of their phosphorylation.

11. Incubate 20 min at 30˚C with either constant or frequent shaking.

    If a thermomixer is not available, a water bath or other heating device can be used.

12. Stop the phosphorylation reactions by adding 10 μl sample buffer x4 to each tube.
13. Boil for 5 min, centrifuge for 1 min at 14,000xg, and load the resulting supernatants on a 15% SDS-PAGE as described bellow.

    Since determination of enzymatic activity is not always accurate, when enzymes (in this case protein kinases) are bound to beads, the kinase(s) of interest can be released from the beads at step 6 by adding using excess immunizing peptide. The phosphorylation reaction can thus be performed without the interference of the beads. Then the activity can be measured as described below by a "paper assay".

7) In-gel Kinase Assay

❦ REFERENCES

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❦ INTRODUCTION

The in-gel kinase assay may be used when the identity of a kinase is not known or there are no specific antibodies available for it. This assay involves co-polymerizing a substrate with SDS and polyacrylamide and electrophoresis of protein extract/s on the resulting SDS-polyacrylamide gel. Following several rounds of denaturation and renaturation, radioactive ATP is added and a kinase reaction is performed by the kinase separated in the gel on the substrate which was polymerized in the gel. The phosphorylated bands are visualized by X-ray film or by a phosphoimager and the molecular weight of the protein kinase can be approximated and novel protein kinases identified. However, not all protein kinases can be renatured under the aforementioned conditions and the linear range of this assay is usually limited.

MATERIALS

Wash Buffer: 20% isopropanol, 50mM HEPES, pH 7.6

Buffer A **:** 50mM HEPES, pH 7.6, 5mM □-mercaptoethanol (fresh)

Buffer A+0.05% Tween-20 (at 4°C)

6M urea in Buffer A: 72 g Urea in 200 ml Buffer A

Kinase Buffer: 20mM HEPES, pH7.6, 20mM $MgCl_2$

TCA-PPi 5% trichloroacetic acid, 1% NaPPi

PROCEDURE

1. Preparation of SDS-PAGE containing MBP:

lower gel:	H_2O	-	1.2 ml
	MBP (2mg/ml)	-	2.0 ml
	4x Lower Buffer	-	2.0 ml
	30% Acrylamide:bis	-	2.7 ml
	10% APS	-	150 μl
	TEMED	-	10 μl
			8.0 ml
Upper gel:	H_2O	-	3.15 ml
	4x Upper Buffer	-	1.25 ml
	30% Acrylamide:bis-	0.5 ml	
	10% APS	-	100 μl
	TEMED	-	10 μl
			5.0 ml

2. Apply your protein samples (50μg) to a 10% polyacrylamide-SDS gel which had been polymerized in the presence of 0.5 mg/ml myelin basic protein (MBP, bovine brain, Sigma). **Do not boil samples before loading.**
3. Following electrophoresis (do not exceed 100 volts), after the electrophoresis the gel can be kept over night at 4°C.), wash gel twice for 30 min with 100 ml 20% isopropanol-50mM HEPES (pH 7.6), twice for 30 min each with buffer A, twice for 15 min with 100 ml 6M urea in Buffer A (all washes in room temerature).
4. Place gel in the cold room (4°C), perform stepwise renaturation:
   Step 1 (3M urea solution): take out 50 ml of the 6M urea in Buffer A on the gel and add 50 ml of buffer A+0.05% Tween-20 and wash for 15 min;
   Step 2 (1.5M urea solution): take out 50 ml of the 3M urea in Buffer A and add 50 ml of buffer A+0.05% Tween-20 and wash for 15 min;
   Step 3 (0.75M urea solution): take out 50 ml of the 1.5M urea in Buffer A and add 50 ml of buffer A+0.05% Tween-20 and wash for 15 min.
   Finally, wash gel three times for 15 min each with 100 ml buffer A+0.05% Tween 20. Leave the gel overnight at the cold room.
5. Incubate the gel in 30ml kinase buffer at 30°C for 30 min. change the medium to 20 ml of kinase buffer containing 2mM DTT, (40μl) 20μM ATP (40 μl) and 100μci [γ^{32}P]-ATP. Incubate at 30°C for 2 hrs.
6. Wash gel carefully four times for 15 min with 50 ml TCA-PPi at room temperature. If needed continue overnight.
7. Place gel on a wet Whatman paper, cover with saran wrap, dry and expose to X-ray film.

8) Mini-Columns Assays

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MATERIALS

Spin columns (Bio-Rad)
DE-52 (Whatman)
P81 (Whatman)
myelin basic protein (MBP)
150 mM phosphoric acid
Buffer A: 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium orthovanadate
Buffer H (homogenization buffer): 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A
RM x3: (reaction mixture at 3-fold final concentration): 30 mM $MgCl_2$, 1.5 mM DTT, 75 mM β-glycerophosphate, pH 7.3, 0.15 mM sodium orthovanadate, 6 μM PKI peptide, 3.75 mM EGTA, 0.3 mM ATP, 30 μM calmidazolium, 2.5 mg/ml bovine serum albumin (BSA).

PROCEDURE

Mini-columns

1. DE-52 resin (Whatman) is prepared in Buffer A (check pH and conductivity) and kept as 1:1 ratio of beads to Buffer.
2. Put 0.8 ml of this resin into a small column (the net amount of beads should be 0.4 ml).
3. Wash with ice-cold Buffer A three times (1 ml). Work in the cold room.
4. Load cytosolic extracts (0.5 ml) on the mini-columns
5. Wash with 0.75 ml of Buffer A. The flow-through and wash are collected in separate tubes.
6. Wash again with 1 ml of Buffer A and discard.
7. Elute kinases with 0.75 ml Buffer A containing 0.05M NaCl and collect into a prechilled eppendorf tube. Wash again with 1 ml of the above buffer and discard flow through.
8. Repeat step 7 with Buffer A containing 0.10, 0.20, 0.30 and 0.40M NaCl.

Determination of kinase activity

Kinase activity is determined by phosphate incorporation into substrates such as MBP in the presence of [γ^{32}P]-ATP and magnesium.

1. Incubate aliquots (12.5 μl) of the NaCl fractions with 4.2 μl MBP (2 mg/ml) and 8.3 μl RM containing 100 μM [γ-^{32}P] ATP (1-2 cpm/fmol) for 15 min at 30°C. 4.2 μl H2O instead of MBP are used as a control.
2. Spot 20 μl onto phosphocellulose paper squares
3. Wash three times with 150 mM phosphoric acid and dry.
4. Phosphate incorporation is measured by the addition of 0.5 ml scintillation cocktail (Ecolume, ICN) in a scintillation tube.
5. One unit of MAPK is defined as the amount of enzyme that catalyzes 1 pmol/min of MBP phosphorylation in the above reaction. Under these conditions, MAPK activity is linear up to 100 pmol/min/ml, more concentrated fractions should be diluted in Buffer A.

9) Protein Kinase C (PKC) Activity

REFERENCE

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9A. Preparation of Samples

MATERIALS

Buffer A Tris buffer (20 mM) pH 7.5 containing 2 mM EDTA; 0.5 mM EGTA; 2 mM DTT; 2 mM PMSF, 25 μg/ml aprotinin; 10 μg/ml leupeptin; 10 μg/ml pepstatin and 0.3% Triton-X-100.

Buffer B (same as A except that it contains 30 mM NaCl ; 0.1% Triton X 100 but no protease mix).

Buffer C (same as A except that it contains 200 mM NaCl; 0.1% Triton X 100 but no protease mix).

PROCEDURE

1. Extract proteins with BufferA +0.5% Triton X-100
2. Pipete 2 ml of DEAE-containing slurry into a microfuge tube.
3. Centrifuge at max speed for 2 min and discard upper fluid.
4. Add 1 ml cell supernat (pre-DEAE) and incubate for 15 min at 4°C (continuous shaking).
5. Centrifuge at max speed for 2 min and discard supernat.
6. Add 1 ml of *Buffer B,* mix briefly, and centrifuge at max speed for 2 min.
7. Discard supernat and repeat step 5 , three more times.
8. Add 1 ml of *Buffer C* mix briefly, and incubate for 5 min at 4°C (occasional shaking).
9. Centrifuge at max speed for 1 min.
10. Collect supernat and store at 4°C (PKC enzyme preparation).

11. Determine protein content (Bradford) on pre DEAE and post.DEAE fractions.

9B. PKC Activity Assay (Histone Phosphorylation).

MATERIALS

Assay buffer: Tris 20 mM (pH 7.5, includes EDTA/EGTA mix as above).

*Mixture composition (for 25 assays): 50 mM $MgCl_2$ (375 µl); 15 mM $CaCl_2$ (375 µl); 0.6 mM ATP (124 µl); 200 mM Tris pH 7.5 (360 µl); 200 mM PMSF (15 µl); 2 mg/ml Histone type II (300 µl); H_20 (250 µl); µ-^{32}P-ATP (1µl; sa 3000 Ci/µM).

Activator mix preparation (for 25 assays): Add in a glass tube the following lipids: PS (100 µg); PC (248 µg); (DAG 32 µg) and evaporate organic solvent to dryness under nitrogen. Add assay buffer (250 µl) containing 0.1% triton X100 and sonicate solution for 20 min.

PROCEDURE

1. Prepare a Mix +/- activator. 60 µl of Mixture,, 10 µl of activator/buffer and 30 µl of sample (~15 µg) Assay is started by the addition of the Mixture, and rapid mix.
2. Incubate for exactly 4 min at 30°C.
3. At the end of the incubation, take 80 µl aliquot of the reaction mix and spot on P81 paper strip.
4. Wash paper strips using a beaker and 150 mM phosphoric acid Make sure to discard the wash solution in the appropriate radioactive container
5. Dry paper strip in the hood .
6. Transfer strip paper into a counting vial and add 4 ml scintillation mixture. Count for 1 min.
7. Take a direct count of 48 µl mixture assay (for total ATP added per assay tube).

10) JUN Kinase Assay

REFERENCES

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev* **7,** 2135-2148.

MATERIALS

10X Binding Buffer (Kept frozen in aliquots)

	10X	Final	Stock	For 10 ml
Hepes pH 7.7	220 mM	22 mM	1 M	2.2 ml
NaCl	1.5 M	150 mM	5 M	3.0 ml
$MgCl_2$	20 mM	2 mM	1 M	0.2 ml
EDTA	1 mM	0.1 mM	0.5 M	20 µl
Triton X-100	0.75 %	0.075 %	20 %	0.375 ml

-Glyc. Phos.	200 mM	20 mM	1 M	2 ml
Na ortho vanadate	1 mM	0.1 mM	0.1 M	100 µl
DDW		(up to 10 ml)		2.105 ml

Protease Inhibitors (Prepared fresh before use)

	Final	Stock	For 1 sample	For 20 samples
PMSF	1 : 100	100 mM	3.0 µl	60 µl
Leupeptin	1 : 1000	10 mg/ml	0.06 µl	1.2 µl
Aprotinin	1 : 1000	10 mg/ml	0.6 µl	12 µl
Pepstatin	1 : 500	1 mg/ml	0.6 µl	12 µl
DDW			0.74 µl	14.8 µl
			5 µl	100 µl

Kinase Buffer (Keep frozen, add fresh DTT)

	Final	Stock	For 20 ml
Hepes pH 7.7	20 mM	1 M	0.4 ml
DTT	2 mM	1 M	40 µl
MgCl2	20 mM	1 M	0.4 ml
-Glyc. Phos.	40 mM	1 M	0.8 ml
Na ortho vanadate	0.1 mM	0.1 M	20 µl
DDW			18.34 ml

HB1B Buffer (Keep at 4˚C)

	Final	Stock	400 ml
Hepes pH 7.7	20 mM	1 M	8 ml
NaCl	50 mM	5 M	4 ml
EDTA	0.1 mM	0.5 M	80 µl
MgCl2	25 mM	1 M	10 ml
Triton X-100	0.05 %	20 %	1 ml
DDW			up to 400 ml

****ATP mix X10** (Prepare fresh) For 20 samples
Cold ATP (0.2 mM) 60 l + g ATP (10 mCi/ml) 4 l. Final : Cold 20 mM, Hot : 2 mCi/sample.

PROCEDURE

1. Harvest cells in Buffer H, sonicate and spin as described.
2. Add in eppendorf tubes :

Fraction	150 µl
10X Binding Buffer	30 µl
Protease inhibitors	5 µl
DDW	95 µl
GST-JUN	20 µl
Total volume	300 µl

3. Incubate 1 hr. shaking on vortex at 4˚C (For binding the JNK to GST-JUN).
4. Spin briefly and take out sup.
5. Wash 3 times with 1 ml of HB1B, spin and take out sup. Fix the thermomixer at 30˚C.
6. Wash once with 1 ml of kinase buffer, spin and take out all the sup.
7. Add to the beads 30 µl of kinase buffer and place the tubes in the thermomixer.
8. Add 3 µl of **ATP mix X10 containing hot and cold ATP.

9. Close the tubes and incubate 20 min. shaking.
10. To stop the reaction add 11 μl of Sample Buffer x4.
11. Heat the samples 5 min. at 95˚C. Run on 10% Acrylamide gel. At this stage the samples may be kept frozen.

11) References

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