MAPK activation cultured cells – phospho-specific antibodies

Important considerations for kinase sample preparation success

Successful preparation of tissue or cells for the subsequent assay of kinase activity depends largely on the three following factors

- Maintain the sample at low temperature during the preparation process i.e. washing, scraping and centrifuging the cells after agonist addition. Everything is done with cold buffers and is kept in the ice, NOT on top of the ice.
- Minimizing actual elapsed sample preparation time, i.e. complete the sample preparation protocol from agonist to centrifuging and separation of the two components as soon as possible. No time to wander off and surf the web. Time from harvesting the cells to homogenization of the sample can be critical to keep the protein active and in it's respective intracellular location.
- Inclusion of protease inhibitors and phosphatase inhibitors in the preparation scheme to retain the structural and functional integrity of the kinase. Active kinases are subject to proteolytic activity and phosphatases are kinetically much faster than most kinases

MAPK minireview

The response of cells to growth factors and other mitogens is mediated by a specific receptors including both tyrosine kinases and heterotrimeric G proteins. Several related intracellular signaling pathways, collectively known as mitogen-activated protein kinase (MAPK) signaling cascades, have been elucidated in the past decade. Transmission of signals via these cascades is usually initiated by activation of a small G protein (e.g., Ras) and followed by sequential stimulation of several sets of cytosolic protein kinases. Four distinct MAPK cascades, ERK (extracellular signal-related protein kinase), JNK (c-Jun NH2-terminal kinase), SPK (stress-related protein kinase), and BMK (big MAPK), have been elucidated to date. These cascades can cooperate in transmitting signals from most extracellular stimuli and can thus determine a cell's fate in response to the ever-changing environment. Several controlling factors have been identified including PKC, Raf-1, MEK, and Ras. A general activation scheme involves the activation of a receptor tyrosine kinase such as the insulin receptor or the epidermal growth factor receptor. Dimerization and or activation of these receptors result in the autophosphorylation of the receptor and provide a docking site for SH2 containing proteins. One such protein isGrb2, which in turn localizes Sos to the plasma membrane. Sos activates Ras the exchange of GDP for GTP. Ras-GTP then binds directly to a serine/threonine kinase, Raf, forming a transient membrane anchoring signal. Raf-1 itself is a protein kinase and will modify MEK, a duel specific protein kinase. Once phosphorylated at two serine residues, MEK becomes active and will in turn phosphorylate ERK1 and ERK2 on a threonine and tyrosine (at a TEY consensus sequence). The major targets of activated ERK1/2 is p90 ribosomal S6 kinase (Rsk) and a phospholipase (PLA2). There are several very specific protein phosphatases that will remove the phosphoryls from ERK 1/2, so it is imperative that processing time is kept to a minimum and phosphatase inhibitors are used to maximise the ERK 1/2 phosphohrylation.

Part 1- Cell preparation/agonist stimulation

1. 2 to 3 days prior to the experiment, subculture one T-25 1:24 as follows
   - Remove old media, rinse and incubate with one ml of trypsin.
   - Add 9 ml of complete high glucose DMEM media and triturate 5-8 times
   - Add an additional 15 ml of complete media and mix
   - Place two ml in each six well dish or in each 25 mm culture dish
2. 12-18 hours prior to the experiment starve the cells
   - Note confluence of the cells they should be 70-80% if more than 90 it is not wise to continue
   - Remove media and rinse with sterile PBS
   - Add 2 ml of 0.1 % serum and 1% free fatty acid BSA, in the media
3. Agonist stimulation (on the day of the experiment)
   - Record the confluence of the cells - are they different than before you serum starved them?
   - Remove low serum media, rinse with PBS (does not have to be sterile) and add 1 ml of serum free media with 0.5% FFA BSA
   - Incubate for one hour in the incubator.
   - At the appropriate times add inhibitors and Place the cells back into the incubator
4. Agonist Activation – Add the indicated activator. The time will depend on the agonist and cell type. 2 to 5 minutes should be fine for the first time. All actions from here on out need to be done precisely and quickly lest the effect of translocation is lost.

Part 2 – Cell Harvesting

5. Remove media and wash 3 times with ice cold PBS. From this point on the cells must be on ice. Be certain all of the PBS has been removed after the last wash. This is critical, residual PBS will dilute the protein.
6. Once the last of the PBS has been removed in a timely fashion, add 0.1 to 0.2 ml of 1X SDS- Page Sample buffer containing sodium orthovanadate and ß glycerol phosphate.
   10 mM ß glycerol phosphate
   0.5 mM Na_3VO_4
7. Scrape the cells, being careful to scrape all of the cells towards the bottom of the dish
8. Lyse cells with 3 x 15 second bursts of sonication or by 5 passes through a 27 gauge needle.
9. Boil the sample for 5 min and freeze if not continuing for the day.
10. Prepare a 10 % SDS-PAGE gel. Load the gel in the following order.

    - The First lane gets the positive control if there is one. For MAPK this usually is 20 µl of cell lysate activated by LPA. If no positive control is used then load the low range pre-stained MW standards in the first lane
    - If using positive blotting controls load the pre-stained standards in the second lane
    - See Drs. Wallert or Provost for the order of the rest.