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Mitogen-Activated Protein Kinase Signaling in Drug-Resistant Neuroblastoma Cells

Raymond R. Mattingly

Abstract

Widespread inherent or acquired resistance to cytotoxic drugs is a major limitation to chemotherapy. There are many mechanisms that contribute to such resistance. In neuroblastomas there is evidence that acquired drug resistance may be associated with altered response to growth factor signals. The ubiquitous mitogen-activated protein kinase (MAPk) cascade, which transmits growth factor signals from the cell membrane to the nucleus, provides a principal mechanism for regulation of cell cycle progression and proliferation. We have shown that there is a relationship between acquired drug resistance in human neuroblastoma cells to doxorubicin, a topoisomerase-2 inhibitor, and to MDL-28842, an inhibitor of S-adenosylhomocysteine hydrolase, and reductions in the activation and nuclear translocation of MAPk.

Key Words: Neuroblastoma; MAP kinase; ERK; epidermal growth factor; confocal immunofluorescence; nuclear localization; doxorubicin.

1. Introduction

The ubiquitous mitogen-activated protein kinase (MAPk) cascade, which transmits growth factor signals from the cell membrane to the nucleus ***(1)***, provides a principal mechanism for regulation of cell-cycle progression and proliferation ***(2)***. Constitutive and inappropriate activation of MAPk may be a critical component in some human tumors ***(3,4)***, and thus pharmacological intervention in the MAPk cascade has been identified as a promising new approach to cancer

From: *Methods in Molecular Biology, vol. 218: Cancer Cell Signaling: Methods and Protocols*
Edited by: D. M. Terrian © Humana Press Inc., Totowa, NJ

therapy ***(5)***. Widespread inherent or acquired resistance to cytotoxic drugs is a major limitation to chemotherapy ***(6)***. We have shown that there is a relationship between acquired drug resistance in human neuroblastoma cells to doxorubicin, a topoisomerase-2 inhibitor ***(7)***, and to MDL-28842, an inhibitor of *S*-adenosyl-homocysteine hydrolase ***(8)***, and the activation and nuclear translocation of MAPk ***(9)***.

The activation of MAPk is dependent on its dual phosphorylation at both Threonine-183 and Tyrosine-185 residues ***(10)***. Commercial availability of antibodies that recognize this dually phosphorylated and active form of MAPk with very great selectivity over the dephosphorylated or monophosphorylated forms provides straightforward assay protocols for the activation of MAPk by both Western blotting and immunofluorescence. Confocal immunofluorescence detection allows confirmation that the activated MAPk has been translocated to the nucleus.

2. Materials

2.1. Cell Culture and Lysis

1. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, HyClone, Ogden, UT).
2. Doxorubicin (Sigma, St. Louis, MO) is dissolved in tissue-culture water at 10 m*M*, stored in aliquots at −80ºC, and then added to tissue culture dishes as required.
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 m*M*) from Gibco/BRL.
4. Epidermal growth factor (EGF, Gibco) is dissolved at 1 mg/mL in DMEM and stored in single use aliquots at −80ºC. Working solutions are prepared by dilution in 100 µg/mL BSA.
5. Phorbol 12-myristate 13-acetate (PMA, Sigma) is dissolved at 2 m*M* in dimethyl sulfoxide (DMSO) and stored in single use aliquots at −80ºC.
6. Modified Laemmli ***(11)*** buffer for cell lysis: 75 m*M* Tris-HCl, pH 6.8, 1.5% (w/v) sodium dodecyl sulfate (SDS), 7.5 % (w/v) glycerol, 200 m*M* β-mercaptoethanol, 0.03% (w/v) bromophenol blue, 0.003% (w/v) pyronin-Y. Store in aliquots at −20ºC. (*See* **Note 1**).
7. Teflon cell scrapers (Fisher).

2.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer (4X): 1.5 *M* Tris-HCl, pH 8.7, 0.4% SDS. Store at room temperature.
2. Stacking buffer (4X): 0.5 *M* Tris-HCl, pH 6.8, 0.4% SDS. Store at room temperature.
3. Thirty percent acrylamide/bis solution (37.5:1 with 2.6% C) (this is a neurotoxin when unpolymerized and so care should be taken not to receive exposure) and N,N,N,N'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad, Hercules, CA) (*see* **Note 2**).

4. Ammonium persulfate: prepare 10% solution in water and immediately freeze in single use (200 µL) aliquots at –20ºC.
5. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow to separate. Use the top layer. Store at room temperature.
6. Running buffer (5X): 125 m*M* Tris, 960 m*M* glycine, 0.5% (w/v) SDS. Store at room temperature.
7. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad, Hercules, CA).

2.3. Western Blotting for Active MAPk

1. Setup buffer: 25 m*M* Tris (do not adjust pH), 190 m*M* glycine, 20% (v/v) methanol.
2. Transfer buffer: Setup buffer plus 0.05% (w/v) SDS. Store in the transfer apparatus at room temperature (with cooling during use, *see* **Note 3**).
3. Supported nitrocellulose membrane from Millipore, Bedford, MA, and 3MM Chr chromatography paper from Whatman, Maidstone, UK.
4. Tris-buffered saline with Tween (TBS-T): Prepare 10X stock with 1.37 *M* NaCl, 27 m*M* KCl, 250 m*M* Tris-HCl, pH 7.4, 1% Tween-20. Dilute 100 mL with 900-mL water for use.
5. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
6. Primary antibody dilution buffer: TBS-T supplemented with 2% (w/v) fraction V bovine serum albumen (BSA).
7. Antidually phosphorylated MAPk ***(12)*** (available from Sigma, *see* **Note 4**).
8. Secondary antibody: Antimouse IgG conjugated to horse radish peroxidase (Santa Cruz, Santa Cruz, CA).
9. Enhanced chemiluminescent (ECL) reagents from Kirkegaard and Perry (Gaithersburg, MD) and Bio-Max ML film (Kodak, Rochester, NY) (*see* **Note 5**).

2.4. Stripping and Reprobing Blots for Total MAPk

1. Stripping buffer: 62.5 m*M* Tris-HCl, pH 6.8, 2% (w/v) SDS. Store at room temperature. Warm to working temperature of 70ºC and add 100 m*M* β-mercaptoethanol.
2. Wash buffer: 0.1% (w/v) BSA in TBS-T.
3. Primary antibody: Anti-ERK (Transduction, Lexington, KY).

2.5. Confocal Immunofluorescence for Active and Total MAPk

1. Microscope cover slips (22 × 40 × 0.15 mm) from Fisher, Pittsburgh, PA, and Lab-Tek two-well glass chamber slides from Nalge Nunc, Naperville, IL.
2. Phosphate buffered saline (PBS): Prepare 10X stock with 1.37 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, 18 m*M* KH_2PO_4 (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by dilution of one part with nine parts water.
3. Paraformaldehyde (Fisher): Prepare a 4% (w/v) solution in PBS fresh for each experiment. The solution may need to be carefully heated (use a stirring hot-plate in a fume hood) to dissolve, and then cool to room temperature for use.

4. Quench solution: 50 m*M* NH_4Cl in PBS.
5. Permeabilization solution: 0.5% (v/v) Triton X-100 in PBS.
6. Antibody dilution buffer: 3% (w/v) BSA in PBS.
7. Secondary antibody: Antimouse IgG conjugated to Cy3 (Jackson, West Grove, PA).
8. Nuclear stain: 300 n*M* DAPI (4,6-diamidino-2-phenylindole) in water.
9. Mounting medium: Antifade (Molecular Probes, Eugene, OR).

3. Methods

The active, dually phosphorylated form of MAPk is inherently labile owing to protein phosphatase activities within the cell. To obtain reliable and reproducible results, therefore, it is important to terminate the samples rapidly and effectively at the end of the treatment protocol. The antibody to the active, dually phosphorylated form of MAPk is sufficiently sensitive to allow detection in relatively small amounts of whole cell lysates, allowing the samples to be prepared by addition of boiling cell lysis buffer. Confirmation of equal recovery of the samples through the procedure is provided at the end by reprobing the blots for the total amount of MAPk that is present (phosphorylated or not). Treatment with PMA provides a positive control for the activation of MAPk in many cell types ***(13)***.

Ultimately it is important to determine whether the active MAPk is being translocated into the nucleus where many of its critical substrates reside ***(14)***. This can be accomplished through confocal indirect immunofluorescence. Confocal localization of total MAPk confirms whether a significant fraction of cellular MAPk is relocalized to the nucleus following stimulation. It also indicates whether appearance of active MAPk in the nucleus is more likely to be owing to phosphorylation of MAPk in the cytosol and its subsequent translocation to the nucleus, or whether there may be preexisting inactive MAPk in the nucleus that may be phosphorylated and activated *in situ*. The former paradigm apparently operates in SKNSH cells (*see* data figures later).

3.1. Preparation of Samples for Assay of Active MAPk by Western Blotting

1. The wild-type and drug-resistant SKNSH human neuroblastoma cells are passaged when approaching confluence with trypsin/EDTA to provide new maintenance cultures on 100-mm tissue dishes and experimental cultures on 35-mm dishes. One 35-mm dish is required for each experimental data point. A 1:40 split of the wild-type cells and a 1:20 split of the resistant cells will provide experimental cultures that are approaching confluence after 48 h. At this point the cultures are rinsed twice with DMEM (without serum) and incubated for a further 24 h in DMEM (without serum) (*see* **Note 6**).
2. All the materials required for the treatment and termination protocol are made ready: the agonists at appropriate stock concentration for 1:1000 dilution into the

cultures; a labeled microcentrifuge tube for each sample with a hole poked in the cap using a 26-gauge syringe needle; a vacuum aspirator; a hot-block at 100ºC; cell lysis buffer heated to 100ºC.
3. The cultures are treated with agonists according to the protocol and the medium then removed by aspiration. Immediately, 100 µL of boiling cell lysis buffer is added and the material scraped into the appropriate labeled tube (*see* **Note 7**).
4. The tubes are closed and then boiled for a further 10 min. After cooling to room temperature, they are ready for separation by SDS-PAGE.

3.2. SDS-PAGE

1. These instructions assume the use of a Hoeffer SE-400 or SE-600 gel system. They are easily adaptable to other formats, including minigels. It is critical that the glass plates for the gels are scrubbed clean with a rinsable detergent after use (e.g., Alconox, Alconox, New York, NY) and rinsed extensively with distilled water. They can be kept clean until use in a plastic rack in 30% nitric acid (use caution when removing). They will just need rinsing (distilled water then 95% ethanol) to remove the acid and air-dry.
2. Prepare a 1.5-mm thick, 10% gel by mixing 7.5 mL of 4X separating buffer, with 10-mL acrylamide/bis solution, 12.5 mL water, 100 µL ammonium persulfate solution, and 20 µL TEMED. Pour the gel, leaving space for a stacking gel, and overlay with water-saturated isobutanol. The gel should polymerize in about 30 min.
3. Pour off the isobutanol and rinse the top of the gel twice with water.
4. Prepare the stacking gel by mixing 2.5 mL of 4X stacking buffer with 1.3 mL acrylamide/*bis* solution, 6.1 mL water, 50 µL ammonium persulfate solution, and 10 µL TEMED. Use about 0.5 mL of this to quickly rinse the top of the gel and then pour the stack and insert the comb. The stacking gel should polymerize within 30 min.
5. Prepare the running buffer by diluting 100 mL of the 4X running buffer with 400 mL of water in a measuring cylinder. Cover with Para-Film and invert to mix.
6. Once the stacking gel has set, carefully remove the comb and use a 3-mL syringe fitted with a 22-gauge needle to wash the wells with running buffer.
7. Add the running buffer to the upper and lower chambers of the gel unit and load the 50 µL of each sample in a well. Include one well for prestained molecular weight markers.
8. Complete the assembly of the gel unit and connect to a power supply. The gel can be run either overnight at 50 V or, if cooling is available for the gel unit, then during the day (about 5 h) at 20 mA through the stacking gel and 30–40 mA through the separating gel. The dye fronts (blue and pink) can be run off the gel if desired, but if the pink dye (pyronin-Y) is retained then it will be transferred to the nitrocellulose membrane and identify the positions of the lanes.

3.3. Western Blotting for Active MAPk

1. The samples that have been separated by SDS-page are transferred to supported nitrocellulose membranes electrophoretically. These directions assume the use of a

Hoeffer transfer tank system. A tray of setup buffer is prepared that is large enough to lay out a transfer cassette with its pieces of foam and with two sheets of 3MM paper submerged on one side. A sheet of the nitrocellulose cut just larger than the size of the separating gel is laid on the surface of a separate tray of distilled water to allow the membrane to wet by capillary action. The membrane is then submerged in the setup buffer on top of the 3MM paper.

2. The gel unit is disconnected from the power supply and disassembled. The stacking gel is removed and discarded and one corner cut from the separating gel to allow its orientation to be tracked. The separating gel is then laid on top of the nitrocellulose membrane.
3. Two further sheets of 3MM paper are wetted in the setup buffer and carefully laid on top of the gel, ensuring that no bubbles are trapped in the resulting sandwich. The second wet foam sheet is laid on top and the transfer cassette closed.
4. The cassette is placed into the transfer tank such that the nitrocellulose membrane is between the gel and the anode. It is vitally important to ensure this orientation or the proteins will be lost from the gel into the buffer rather than transferred to the nitrocellulose.
5. The refrigerated/circulating water bath is switched on to maintain a temperature between 10–15ºC and a magnetic stir-bar in the tank activated.
6. The lid is put on the tank and the power supply activated. Transfers can be accomplished at either 30 V overnight or 70 V for 2 h.
7. Once the transfer is complete the cassette is taken out of the tank and carefully disassembled, with the top sponge and sheets of 3MM paper removed. The gel is left in place on top of the nitrocellulose and these are laid on a glass plate so that the shape of the gel (including the cut corner for orientation) can be cut into the membrane using a razor blade. The gel and excess nitrocellulose can then be discarded. The coloured molecular weight markers (and pyronin-Y lane markers, if the latter were retained on the gel during the PAGE run) should be clearly visible on the membrane.
8. The nitrocellulose is then incubated in 50 mL blocking buffer for 1 h at room temperature on a rocking platform.
9. The blocking buffer is discarded and the membrane quickly rinsed prior to addition of a 1:2000 dilution of the antidually phosphorylated MAPk antibody in TBS-T/2% BSA for 1 h at room temperature on a rocking platform.
10. The primary antibody is then removed (*see* **Note 8**) and the membrane washed three times for 5 min each with 50 mL TBS-T.
11. The secondary antibody is freshly prepared for each experiment as 1:20,000-fold dilution in blocking buffer and added to the membrane for 30 min at room temperature on a rocking platform.
12. The secondary antibody is discarded and the membrane washed six times for 10 min each with TBS-T.
13. During the final wash, 2 mL aliquots of each portion of the ECL reagent are warmed separately to room temperature and the remaining steps are done in a dark room under safe light conditions. Once the final wash is removed from the blot, the ECL

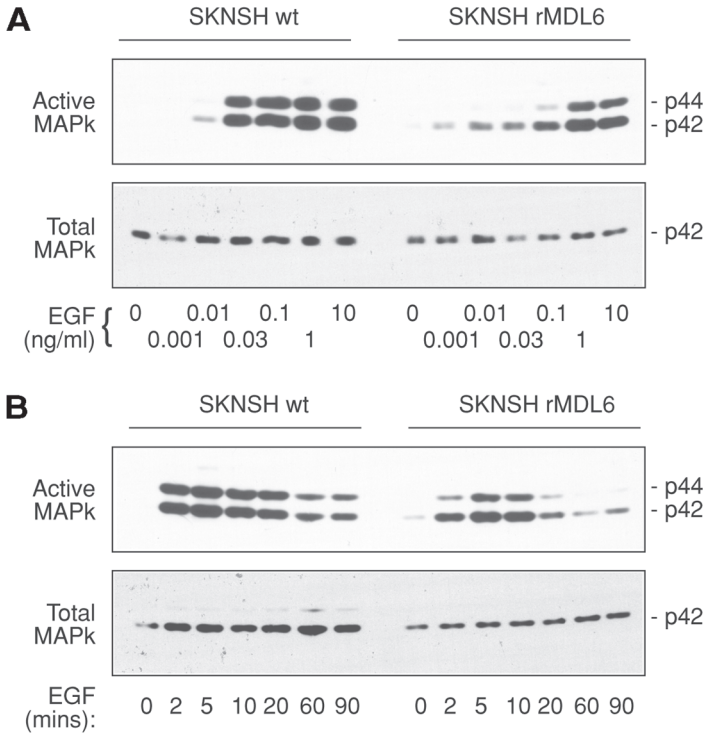

Fig. 1. Concentration-response and kinetics of the activation of MAPk by EGF in SKNSH rMDL6 cells. (**A**) Wild-type and SKNSH rMDL6 cells were treated for 5 min with the indicated concentrations of EGF and processed for assay of active and total MAPk by western blotting. (**B**) Wild-type and SKNSH rMDL6 cells were treated for the indicated time with 10 ng/mL EGF and processed for assay of active and total MAPk by western blotting. Activation of MAPk by EGF is clearly less potent and more transient in rMDL6 cells than in wild-type SKNSH cells. EGF, even up to 175 ng/mL, was incapable of activation of MAPk in rDOX6 cells (data not shown). (Reproduced from **ref. *9*** with permission from Elsevier Science.)

reagents are mixed together and then immediately added to the blot, which is then rotated by hand for 1 min to ensure even coverage.

14. The blot is removed from the ECL reagents, blotted with Kim-Wipes, and then placed between the leaves of an acetate sheet protector that has been cut to the size of an X-ray film cassette (*see* **Note 9**).
15. The acetate containing the membrane is then placed in an X-ray film cassette with film for a suitable exposure time, typically a few minutes. An example of the results produced is shown in **Fig. 1**.

3.4. Stripping and Reprobing Blots for Total MAPk

1. Once a satisfactory exposure for the result of the active MAPk has been obtained, the membrane is then stripped of that signal and then reprobed with an antibody that recognizes MAPk irrespective of whether it is phosphorylated. This provides a loading control that confirms equal recovery of the samples through the procedure.
2. Stripping buffer (50 mL per blot—*see* **Note 10**) is warmed to 70ºC and the β-mercaptoethanol and blot added. The blot is incubated for 30 min with occasional agitation.
3. Once the blot is stripped it is extensively washed in washing buffer (three times 150 mL, each wash for 10 min), and then blocked again in blocking buffer.
4. The membrane is then ready to be reprobed with anti-total MAPk (1:5000 in TBS-T/2% BSA) with washes, secondary antibody, and ECL detection as above. An example result is shown in **Fig. 1**.

3.5. Confocal Immunofluorescence for Active and Total MAPk

1. Neuroblastoma cells are passaged as described above (***see*** **Subheading 3.1.**), except that the experimental samples are either 60-mm dishes with sterile cover-slips or two-well chamber slides. If cover-slips are to be used, they must first be sterilized by holding with tweezers, addition of 95% ethanol, and passing through the flame of a Bunsen burner (take extra care to keep the flame away from the ethanol bottle and not to allow the burning ethanol to drip onto a flammable surface), and then placed in the culture dishes to cool.
2. The cells should be rinsed and changed to serum-free DMEM when below subconfluence (typically, 24–48 h of culture time) so that individual cells are clearly visible in the immunofluorescence.
3. The cells are treated with agonists according to the protocol, and then rinsed rapidly twice with ice-cold PBS.
4. Paraformaldehyde solution is then added for 10 min at room temperature to fix the cells.
5. The paraformaldehyde is discarded (into a hazardous waste container) and the samples washed twice for 5 min each with PBS.
6. Residual formaldehyde is quenched by incubation in NH_4Cl for 10 min at room temperature, followed by a further two washes with PBS.
7. The cells are permeabilized by incubation in PBS/Triton X-100 for 5 min at room temperature, and then rinsed twice more with PBS.
8. The samples are blocked by incubation in antibody dilution buffer for either 2 h at room temperature or at 4ºC overnight. If chamber slides are used, then the upper plastic housing should be removed, leaving the gasket on the slide.
9. The blocking solution is removed and replaced with the antiactive MAPk antibody (1:200) or antitotal MAPk (1:50) in antibody dilution buffer for 1 h at room temperature (*see* **Note 11**).
10. The primary antibody is removed and the sample washed three times for 5 min each with PBS. The sample is then put under aluminium foil and the room lights dimmed for subsequent steps.

11. The secondary antibody is prepared at 1:250 in antibody dilution buffer and added to the samples for 30 min at room temperature (*see* **Note 11**).
12. The secondary antibody is discarded and DAPI is added for 10 min at room temperature to stain the DNA and identify the nuclei.
13. The samples are washed five times for 10 min each with PBS and then aspirated dry from one corner.
14. The samples are then ready to be mounted. If they are on a cover-slip then the slip is carefully inverted into a drop of mounting medium on a microscope slide. If on a chamber slide, then the gasket is carefully removed (a razor blade may be required) and then mounting medium and a cover-slip added. In either case, nail varnish is used to seal the sample (*see* **Note 12**). The sample can be viewed immediately that the varnish is dry, or be stored in the dark at 4ºC for up to a month.
15. The slides are viewed under phase contrast microscopy (to locate the cells and identify the focal plane) and under confocal microscopy. Excitation at 543 nm induces the Cy3 fluorescence (red emission) for the MAPk, while excitation at 364 nm induces DAPI fluorescence (blue emission). Software can be used to overlay the phase contrast and fluorescence images. Examples of the signals for active MAPK and total MAPk are shown in **Figs. 2** and **3**.

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. This standard is referred to as "water" in this text.
2. TEMED is best stored at room temperature in a desiccator. Buy small bottles as it may decline in quality (gels will take longer to polymerize) after opening.
3. Transfer buffer can be used for up to five transfers within 1 wk so long as the voltage is maintained constant for each successive run (the current will increase each time). Adequate cooling to keep the buffer no warmer than room temperature by use of a refrigerated/circulating bath is essential to prevent heat-induced damage to the apparatus and the experiment.
4. We have found this antibody to be excellent for both Western blotting and immunofluorescence. Numerous competitive reagents are available from other commercial sources.
5. Quantification of data may be desired and this can be done by scanning densitometry of the films, providing that care is taken to ensure that the signal has not saturated. Alternatively, the chemiluminescent signal can be captured digitally with an instrument such as a FujiFilm LAS-1000 plus.
6. This protocol can be adapted for many other cell culture systems. For cells that grow in suspension, such as Jurkat T cells, the cells can easily be counted after the serum starvation and then aliquotted for the treatment protocol ***(13)***.
7. The cell material will be very viscous at this stage owing to release of DNA and thus hard to pipet. It is easiest to use the pipet tip to transfer the sample to the tube by dragging and pushing rather than by drawing the sample up and down. The viscosity

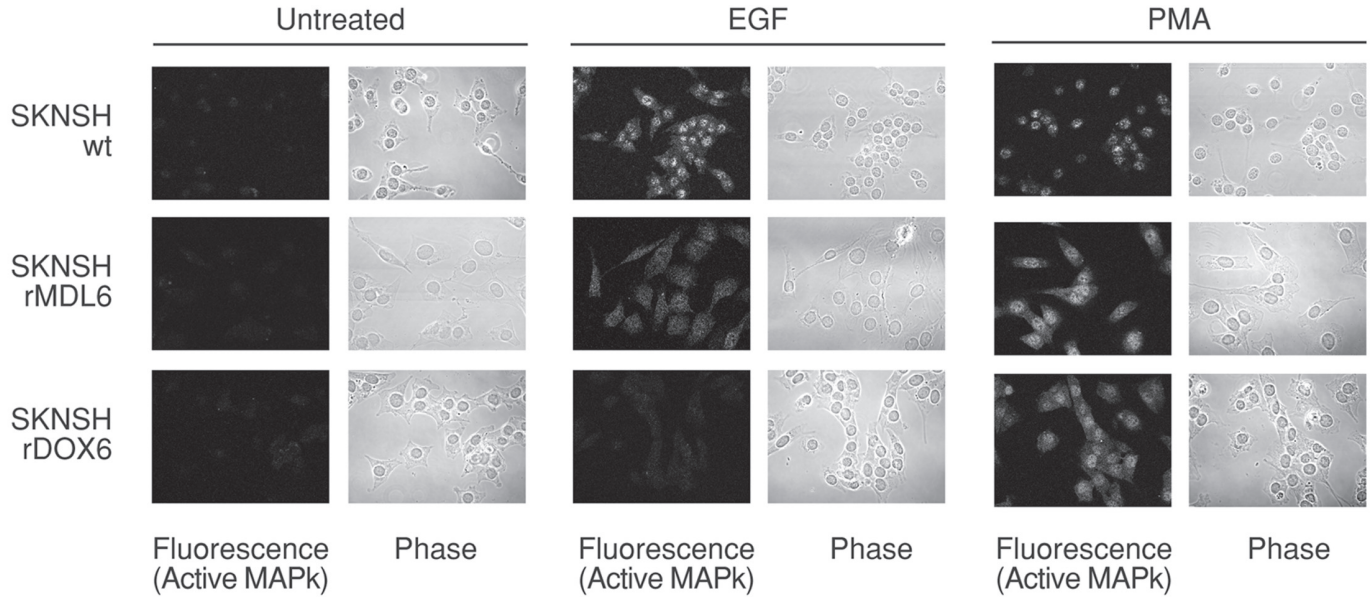

Fig. 2. Accumulation of active MAPk in the nuclei of control and stimulated SKNSH cells and drug-resistant variants. Wild-type SKNSH and the rMDL6 and rDOX6 variants were treated for 5 min with 10 ng/mL EGF or 200 n*M* PMA and processed for immunofluorescence of active MAPk. Confocal images were obtained on a Zeiss LSM310 microscope using a 63X oil-immersion lens as described ***(15)***. Phase contrast pictures of the same field of cells at the same magnification are also shown. The apparent nuclear localization that is evident following agonist stimulation of the wild-type cells was confirmed by colocalization with the fluorescence from DAPI, a DNA marker (data not shown). EGF is evidently better able to induce nuclear accumulation of active MAPk in wild-type SKNSH cells than it is in the resistant lines. PMA stimulates some active MAPk accumulation in the resistant lines, although, again it appears less effective than in the wild-type SKNSH cells. (Reproduced from **ref. *9*** with permission from Elsevier Science.)

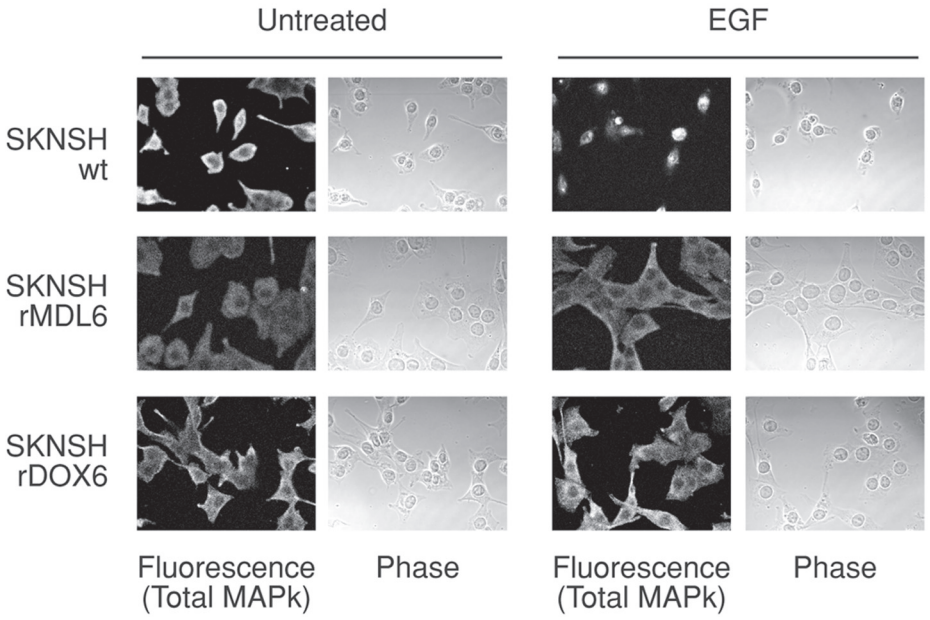

Fig. 3. EGF stimulation of nuclear translocation of MAPk in SKNSH cells. Wild-type SKNSH and the rMDL6 and rDOX6 variants were treated for 10 min with 10 ng/mL EGF and processed for confocal immunofluorescence of total MAPk. Phase contrast pictures of the same field of cells at the same magnification are also shown. The apparent nuclear localization that is evident following agonist stimulation of the wild-type cells was confirmed by colocalization with the fluorescence from DAPI, a DNA marker (data not shown). These results support a model whereby EGF induces the translocation of active MAPk from the cytosol to the nucleus of wild-type SKNSH cells, but is much less effective in the resistant lines. (Reproduced from **ref. *9*** with permission from Elsevier Science.)

of the material will decrease during subsequent boiling, allowing accurate loading of the gel.

8. The primary antibody can be saved for subsequent experiments by addition of 0.02% final concentration sodium azide (conveniently done by dilution from a 10% stock solution; exercise caution since azide is highly toxic) and storage at 4ºC. These primary antibodies have been used for up to 20 blots over several months, with the only adjustment required being increasing length of exposure to film at the ECL step.
9. Backgrounds in this protocol are normally so clean that exact alignment of the subsequent film with the nitrocellulose can be difficult. We, therefore, apply a square of luminescent tape (Sigma) to the edge of the acetate sheet to provide an alignment

mark for the film and membrane and thus allow identification of the signals with the lanes.

10. This procedure generates a significant, unpleasant smell in the laboratory. We, therefore, routinely wait until several membranes are ready to be stripped and process them in a group to minimize the time required. Containers with tight-fitting lids are an advantage. The use of a supported nitrocellulose membrane, rather than pure nitrocellulose, facilitates the increased manipulation required for stripping and reprobing.
11. For economy, only 100–150 µL of diluted antibody per sample needs to be used at this step. In the chamber slides, this volume will be retained by the gasket around the sample. If using cover-slips, then use an aspirator to dry the dish around the slip thoroughly and establish a good meniscus on top of the cover-slip with this volume of antibody dilution buffer before replacing it with the primary antibody.
12. Air bubbles are undesirable in the mounting medium, and slow, careful application of the top layer minimizes their appearance. A bright color of varnish is easier to apply accurately in the dark, with two thin coats being preferable.

Acknowledgments

The author would like to thank Professor Bernard L. Mirkin for development of the drug-resistant models of human neuroblastoma cells and for his advice and encouragement and Jesse Moya for technical assistance. This work was supported by RO1 CA81150.

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