Uncoupling of Hepatic, Epidermal Growth Factor-mediated Mitogen-activated Protein Kinase Activation in the Fetal Rat*

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Joan M. Boylan and Philip A. Gruppuso‡

From the Department of Pediatrics, Division of Pediatric Endocrinology and Metabolism, Rhode Island Hospital and Brown University, Providence, Rhode Island 02903

Stimulation of cell proliferation by mitogens involves tyrosine phosphorylation of proteins at the cell membrane by receptor tyrosine kinases. This promotes formation of multi-protein complexes that can activate the small G-protein, Ras. Activation of Ras, in turn, leads to sequential activation of the following three serine-threonine kinases: Raf, extracellular signal-regulated kinase kinase (MEK), and members of the family of mitogen-activated protein (MAP) kinases. Prior studies have shown that intraperitoneal injection of epidermal growth factor (EGF) leads to rapid activation of hepatic MAP kinases in adult rats but not in late gestation (E19) fetal rats (Boylan, J. M., and Gruppuso, P. A. (1996) Cell Growth & Differ. 7, 1261–1269). The present studies were undertaken to determine the mechanism for this “uncoupling” of the MAP kinase pathway. E19 fetal rats and adult male rats were injected with EGF (0.5 μg/g body weight, intraperitoneally) or with saline. After 15 min, livers were removed and prepared for kinase analyses. EGF injection led to a rapid and marked activation of hepatic Raf and MEK in both fetal and adult rats, whereas MAP kinase activation was minimal in fetal as opposed to adult rats. Examination of the ontogeny of this dissociation of MAP kinase activation from MEK activation showed gradual acquisition of intact signaling as an adult hepatocyte phenotype was attained during the first 4 postnatal weeks. Over this period, MAP kinase content as determined by Western immunoblotting was constant. Recombination experiments using partially purified fetal and adult rat liver MEK and MAP kinase showed intact MAP kinase activation in vitro, indicating that neither enzyme was irreversibly altered in the fetus. In studies using primary cultures of E19 fetal rat hepatocytes, uncoupling of MAP kinase activation from MEK activation could be induced by incubation of fetal hepatocytes for 24 h with a potent fetal hepatocyte mitogen, transforming growth factor-α. These findings indicate that a novel negative feedback mechanism for MAP kinase regulation may be active in developing rat hepatocytes.

A major signaling pathway through which virtually all known mitogens exert intracellular responses is the mitogen-activated protein (MAP)1 kinase cascade (1, 2). A primary mechanism by which growth factor receptor tyrosine kinases activate MAP kinases involves tyrosine phosphorylation of an adaptor protein, Shc. Shc binds to a second adaptor protein, Grb2, by SH2/3 interactions. This complex incorporates a guanyl nucleotide exchange factor, Sos, which interacts with and activates Ras. The GTP-bound Ras recruits Raf kinase to the cell membrane and initiates a protein kinase cascade that phosphorylates and activates a MAP kinase kinase termed MEK (MAP kinase or extracellular signal-regulated kinase) MEK in turn phosphorylates MAP kinases on both tyrosyl and threonyl sites. Active MAP kinases translocate to the nucleus where they phosphorylate other serine-threonine kinases and transcription factors which can mediate immediate early gene induction.

The growth and development of the fetus during late gestation is an intricate process dependent upon multiple regulatory mechanisms. Changes in hepatic growth tend to parallel effects on fetal somatic growth (3, 4), making liver an appropriate organ for studying fetal growth control. When we embarked on the present studies we expected that the proliferation of fetal rat hepatocytes, both in vivo and in vitro, would require the action of fetal growth factors. However, late gestation (19 day; E19) rat fetal hepatocytes cultured without serum, growth factors, or insulin were found to be highly proliferative (5, 6). By using a variety of approaches, we were able to find no direct evidence for the production of autocrine growth factors. Thus, we went on to employ MAP kinase activity as an indirect indicator of mitogenic signaling. Studies showed that the MAP kinases present in cultured fetal hepatocytes and in fetal liver were minimally active, although MAP kinase could be activated in cultures of primary fetal hepatocytes by at least two mitogens, transforming growth factor-α (TGF-α) and hepatocyte growth factor (7).

Given the abundance of these growth factors in the late gestation fetal rat, we hypothesized that the relatively low activity of hepatic MAP kinases in vivo might be the result of negative feedback inhibition. In our initial studies (8), we demonstrated that intraperitoneal injection of epidermal growth factor (EGF)2 into E19 fetuses or adult rats resulted in the activation of the proximal portion of the EGF signaling pathway in the liver. This was based on the EGF-induced tyrosine phosphorylation of Shc and Shc-Grb2 complex formation. When the downstream activation of MAP kinases was examined, EGF was shown to result in the marked (∼50-fold) activation of

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‡ To whom correspondence should be addressed: Dept. of Pediatrics, Rhode Island Hospital, 593 Eddy St., Providence, RI 02903. Tel.: 401- 444-5504; Fax: 401-444-8845; E-mail: Philip_Gruppuso@brown.edu.

1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase or extracellular signal-regulated kinase; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; MBP, myelin basic protein; E, embryonal day; P, postnatal day; PAGE, polyacrylamide gel electrophoresis; JNK, c-Jun amino-terminal kinases; bFGF, basic fibroblast growth factor.

2 EGF was used for in vivo experiments rather than TGF-α because of its availability in larger quantities. Comparative signal transduction experiments using primary cultures of fetal rat hepatocytes have shown that there are no differences in the ability of these two growth factors to stimulate phosphorylation of Shc or activation of MAP kinase (28).
MAP kinase in the adult liver. However, minimal MAP kinase activation occurred in the fetus (8).

The present studies were performed following our initial observation of uncoupling of the MAP kinase signaling cascade in E19 fetal liver. We have determined when in development uncoupling of the hepatic MAP kinase cascade occurs as well as the site in this cascade where the actual uncoupling of MAP kinase activation takes place. We proceeded to characterize the constituents of the MAP kinase pathway to better understand this physiologic mechanism for down-regulation of mitogenic signaling.

EXPERIMENTAL PROCEDURES

Animals—Pregnant Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, MA), of known gestation (term specified as 21 days), were delivered by cesarean section under pentobarbital anesthetia (50 mg/kg, intraperitoneally). Male Sprague-Dawley rats (150–175 g) were used for adult liver preparation. Rats were injected intraperitoneally with EGF (0.5 μg/g body weight) or phosphate-buffered saline and sacrificed 15 min later.

Hepatocyte Isolation and Primary Culture—Fetal hepatocytes were isolated on day 19 of gestation by collagenase digestion as described previously (9). Cell suspensions were diluted to 3 x 10^7 cells/100-mm Primaria tissue culture plate with supplemented minimum essential medium (6) containing 5% fetal bovine serum. After a 2-h attachment period, the medium was removed and replaced with supplemented minimum essential medium without fetal bovine serum. Media contained 0.1 mg/ml bovine serum albumin to avoid nonspecific binding of growth factors.

Immune Complex Kinase Assay—Rat liver homogenates were prepared as described previously for MAP kinase activity (7). Homogenates corrected for protein (4 mg) were preclarified with Protein A-Sepharose CL-4B (Pharmacia Biotech Inc.). MAP kinase immunoprecipitation was accomplished with a peptide antibody against the rat ERK-1 sequence (anti-MAP kinase R2, Upstate Biotechnology, Lake Placid, NY) that had been covalently coupled to Protein A-Sepharose CL-4B using di-methyl pimelimidate (20 mM in 0.1 M sodium borate, pH 9.0). The immunoprecipitates were washed 4 x in 10 mM Tris, pH 7.6, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 100 μM Na_2VO_4, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 25 μg/ml phenylmethylsulfonyl fluoride, and then 1 x with 25 mM β-glycerophosphate, 50 μM Na_2VO_4, 10 mM MgCl_2, 0.5 mM EGTA, 10 mM NaF, 0.2% Triton X-100, plus the above protease inhibitors. The immunoprecipitated kinase was resuspended in 25 μl of this buffer. MAP kinase assay was performed by adding an equal volume of 2 x reaction mixture containing 50 mM β-glycerophosphate, pH 7.2, 100 μM Na_2VO_4, 20 mM MgCl_2, 1 mM EGTA, 200 μM [γ-32P]ATP (0.05 μCi/ml), and 0.667 mg/ml MBP and incubating for 20 min at 30 °C. The assays were terminated by adding 10 μl of 250 mM EDTA. MBP was separated on a 12% polyacrylamide gel under reducing conditions. This dried gel was exposed to Kodak XAR-5 film at −70 °C in the presence of intensifying screens, and phosphate incorporation into MBP was quantified with a Hoefer model 300S scanning densitometer connected to a Hewlett-Packard model 3390A integrator.

Mono Q Fractionation and Kinase Assays—Rat liver homogenates and hepatocyte lysates were prepared for MAP kinase activity determinations as described previously (7). Where indicated, samples were fractionated using Mono Q fast protein liquid chromatography. MAP kinase activity was determined using myelin basic protein (MBP) as substrate (7).

Adult and fetal rat liver samples were prepared for determination of Raf or MEK activity by homogenizing liver in Raf/MEK homogenization buffer (10 mM Tris, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM Na_2VO_4, 1% Triton X-100, 10 μg/ml leupeptin, and 25 μg/ml phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 100,000 g for 30 min and frozen at −70 °C until use. Thawed samples were diluted 10-fold in Raf/MEK Mono Q buffer A (10 mM β-glycerophosphate, pH 7.2, 0.1 mM Na_2VO_4, 1 mM EGTA, 1 mM dithiothreitol, and 0.1% Triton X-100) and then loaded at 0.5 ml/min onto a Mono Q column. Raf was eluted with a 40-ml gradient from 0 to 100% buffer B (buffer A + 1 M NaCl). Column fractions of 0.5 ml were collected. MEK was eluted with a 40-ml gradient from 0 to 45% buffer B. Fractions of 1 ml were collected.

The Raf activity assay used recombinant, histidine-tagged, kinase inactive MEK-1 as substrate. Expression and purification were performed as described by Gardner et al. (9). Raf was assayed by mixing 20 μl of each fraction with 20 μl of assay buffer (50 mM β-glycerophosphate, pH 7.2, 0.1 mM Na_2VO_4, 20 mM MgCl_2, 1 mM EGTA, 200 μM [γ-32P]ATP (0.25 μCi/ml), with or without 1.5 μg of recombinant kinase-inactive MEK-1. The assay mixture was incubated for 1 h at 30 °C and stopped with 10 μl of 250 mM EDTA. Proteins were separated on a 10% polyacrylamide gel in the presence of dodecyl sulfate. The dried gel was exposed to film and quantitated as above.

The MEK activity assay used histidine-tagged human ERK-1 as substrate. MEK was assayed by mixing 20 μl of each fraction with 20 μl of the above assay buffer containing 50 mM staurosporine, with or without 2 μg of recombinant human ERK-1. The mixture was incubated for 15 min at 30 °C, and then MBP was added to a final concentration of 0.33 μg/ml, followed by incubation for an additional 15 min. The reaction was stopped with 10 μl of 250 mM EDTA, and proteins were separated by SDS-PAGE, and protein phosphorylation was quantitated as above. MEK activity was assessed as both MAP kinase phosphorylation and activation.

In Vitro Activation of Rat Liver MAP Kinase—Livers from fetal and adult rats injected with EGF were homogenized in 50 mM Hepes, pH 7.2, 50 mM NaCl, 1 mM Na_2VO_4, 20 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 25 μg/ml phenylmethylsulfonyl fluoride. After centrifugation at 100,000 x g for 1 h, 250 μl was loaded onto a Superose 6 gel filtration column (Pharmacia), run at 0.5 ml/min with the above buffer. Fifty 0.5-ml fractions were collected to separate active MEK. The reaction was started by mixing 10 μl of each fraction with 5 μl of either control fetal rat liver MAP kinase partially purified by Mono Q chromatography (7), recombinant human ERK-1 (0.4 μg), or MAP kinase Mono Q buffer A as control (7). After incubation for 3 min at 30 °C, 5 μl of 20 mM magnesium acetate plus 800 μM ATP was added. This mixture was incubated for 20 min at 30 °C, after which it was added 80 μl of 50 mM Tris, pH 7.4, 0.125 mM EGTA, 0.125 mM Na_2VO_4, 12.5 mM magnesium acetate, 250 μM [γ-32P]ATP (0.05 μCi/ml), and 0.4 mg/ml MBP. After an additional 10 min incubation, the reaction was stopped with 10 μl of 250 mM EDTA. Proteins were separated on a 10% polyacrylamide gel and analyzed as above.

Partial Purification of Activated MEK—Liver from an adult rat injected with EGF was fractionated on a Mono Q column. For these experiments, Triton X-100 was not added to Mono Q buffers. Fractions containing MEK activity were pooled and then precipitated by adding a volume of 3.6 mM ammonium sulfate (2 ml final concentration). The pellet was resuspended in 25 mM Tris, pH 7.4, 1 mM EDTA, 5% glycerol (w/v), 0.02% Brij 35 (w/v), 25 mM NaF, 0.1% β-mercaptoethanol, and 1 mM benzamidine. The solution was dialyzed overnight using the same buffer plus 50% glycerol.

Western Immunoblotting—For detection of immunoreactive kinases,
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Fig. 2. Ontogeny of MAP kinase activation in developing rat liver. Livers from 21-day fetal rats, and 1-, 4-, 7-, and 28-day postnatal (P) rats were collected 15 min after intraperitoneal injection of 0.5 μg of EGF/g of body weight (solid circles) or from un.injected animals (control, open circles). Homogenates were prepared and fractionated by Mono Q chromatography (7). Individual fractions were assayed for MAP kinase activity (7). Previous studies have established the elution of 42- and 44-kDa MAP kinases in fractions 32–46. 

Fig. 3. Levels of total versus active MAP kinase in developing rat liver. Fractions 32–46 from each chromatogram in Fig. 2 (uninjected (-) and EGF-injected (+) animals) were pooled, subjected to SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with anti-MAP kinase R2. These samples were also immunoblotted with an antibody to the dually phosphorylated (Thr/Tyr) MAP kinase. Numbers to the right of the autoradiograms indicate apparent molecular mass in kilodaltons.

Fig. 4. Translocation of fetal versus adult liver MAP kinase from the cytosol to the nucleus in control (−) and EGF-injected (+) rats. E19 fetal (Fet) and adult (Ad) rats were injected with phosphate-buffered saline (control) or 0.5 μg of EGF/g of body weight. Fifteen minutes later livers were removed and homogenized, and nuclei were separated from cytosol as described previously (11). Cytosol (2.9 mg of protein) and nuclear extracts (0.46 mg of protein) were immunoprecipitated using anti-MAP kinase R2, separated by SDS-PAGE, and Western immunoblotted with the MAP kinase R2 antibody. Nuclear fraction autoradiograms were exposed to film for 30 s, whereas cytosolic fraction autoradiograms were exposed for 2 s. Arrows indicate the positions of the 44- and 42-kDa MAP kinases.

RESULTS

MAP Kinase Activation in Developing Liver—To confirm our previous observations that MAP kinase activation in fetal liver is uncoupled (8), we immunoprecipitated MAP kinase in fetal liver homogenates from E19 fetal and adult rats given an intraperitoneal injection of phosphate-buffered saline (control) or EGF. MAP kinase activity was then measured directly on immunoprecipitated MAP kinase. Results showed that control fetal and adult rats showed minimal MAP kinase activity (Fig. 1). Upon EGF administration, there was a slight increase in fetal MAP kinase activity, whereas adult MAP kinase was seen to increase approximately 15-fold (Fig. 1). Western immunoblotting of parallel immunoprecipitates (Fig. 1, bottom) showed similar MAP kinase content in fetal and adult samples, indicating that the diminished ability of EGF to stimulate MAP kinase activity in the fetal rat was not due to a decrease in MAP kinase content.

The next series of studies were performed to determine the point in development at which hepatic MAP kinases could be stimulated by in vivo EGF injection. MAP kinase activity was measured in liver homogenates from rats before or after intraperitoneal EGF administration to term fetal rats, newborn rats aged 1, 4, and 7 days, and 28-day-old animals (E21, P1, P4, P7, P28; Fig. 2). Minimal EGF-mediated activation of MAP kinases was seen in E21 fetuses. A low level of activation occurred on P1 and P4. The degree of activation increased on P7. By P28, MAP kinase activation was indistinguishable from that seen in adult rats.

To ensure that hepatic MAP kinase content was not changing during this period, Mono Q fractions 32–46 from each column in Fig. 2 were pooled and analyzed by Western immunoblotting (Fig. 3). Results showed that 42- and 44-kDa MAP kinases were present at constant levels from late gestation to the 28th postnatal day. The immunoblot analysis was repeated on the same samples using an antibody that recognizes active, dually phosphorylated (Thr/Tyr) MAP kinase. Results indicated that MAP kinase phosphorylation (Fig. 3) correlated with MAP kinase activation (Fig. 2).

To determine if the absence of hepatic MAP kinase activation in EGF-injected fetal rats coincided with an effect on MAP kinase translocation to the nucleus, nuclei and cytosol were isolated from livers of fetal and adult animals that had been injected with saline (control) or EGF. Western immunoblotting (Fig. 4) revealed that EGF injection of adult animals induced an increase in nuclear MAP kinase content. No such increase in MAP kinase content was detected in fetal nuclear extracts after EGF administration. No decrease in the amount of MAP kinase in the cytosol of the adult rats was observed. This is presumably due to the large pool of MAP kinase found in the cytosolic versus the nuclear fraction masking any detectable decrease.

In Vivo Activation of Raf and MEK—We hypothesized that the attenuation of growth factor-mediated phosphorylation and activation of MAP kinase were due to the failure to activate the
kinases upstream of this enzyme. The activity of Raf (MEK kinase), the proximal kinase in the signaling pathway, was examined to determine indirectly if Ras activation was intact. Liver homogenates were fractionated by Mono Q ion exchange chromatography. Individual fractions were assayed for MEK kinase activity using recombinant, kinase-deficient MEK-1 as substrate. Fetuses injected with EGF showed marked activation of MEK kinase activity, present in multiple peaks (Fig. 5).

EGF-inducible MEK kinase activity was also present in the adult, albeit at a lower level than in the fetus and as a single chromatographic peak (Fig. 5). Although we have not yet characterized the multiple MEK kinase activity peaks present in liver from EGF-injected fetuses, the response to EGF was taken as evidence for intact signaling at this step.

Given these results, similar experiments were performed to study the in vivo activation of MEK. Recombinant human ERK-1 was used as the kinase substrate. Fetal and adult rat liver homogenates fractionated by Mono Q chromatography (Fig. 5) showed similar patterns of EGF-induced MEK activation. In both cases, MEK activity was minimal in the basal (control-injected) samples.

**In Vitro Activation of Fetal MAP Kinase**—The results to this point were interpreted as indicating that the ability of EGF to only minimally activate hepatic MAP kinase in vivo resided at the level of MAP kinase phosphorylation by MEK. The first hypothetical mechanism that was investigated was that MAP kinase and/or MEK were altered in fetal liver such that they did not interact normally. This possibility had not been addressed in the experiments utilizing recombinant kinases as substrates.

Active fetal or adult liver MEKs obtained from EGF-injected animals and fractionated by high pressure liquid chromatography gel filtration chromatography were examined for their ability to activate rat hepatic MAP kinases. Inactive fetal rat liver MAP kinase from an uninjected animal, partially purified by Mono Q chromatography, and recombinant human ERK-1 were used as substrates. Results (Fig. 6) showed that both MAP kinases were potently activated by both fetal and adult rat liver MEK that had been activated in vivo by EGF injection. This was interpreted as indicating that there was no significant alteration in fetal MAP kinases that could account for their resistance to in vivo activation by MEK. Western immunoblotting of the gel filtration fractions that contained MEK activity (fractions 30–37) using antibodies to either MEK-1 or MEK-2 revealed that both proteins were present in approximately equal amounts in the fetus and adult. Immunoblotting with an antibody against the active (Ser-217/221 phosphorylated) MEK-1 and -2 proteins demonstrated immunoreactive proteins with Mr~47,000 in fractions coinciding with MAP kinase kinase activity (Fig. 7). The presence of these immunoreactive proteins was interpreted as supporting activation of MEK-1 and/or MEK-2, although we were not able to discern which of the MEKs was activated due to the similarity of their molecular weights. Nonetheless, these data were interpreted as supporting the conclusion that MEK activation was intact in fetal liver, whereas MAP kinase activation was markedly attenuated.

In a confirmatory experiment, liver from an adult rat injected with EGF was homogenized and fractionated by Mono Q ion exchange chromatography. Fractions showing MEK activ-
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ity were pooled, ammonium sulfate-precipitated, and dialyzed (see “Experimental Procedures”). The resulting protein solution showed MEK activity measured as the ability to activate bacterially expressed human ERK-1. This MEK preparation was used in a recombination experiment. It was added to individual Mono Q chromatography fractions obtained by preparing liver homogenates from normal (uninjected) fetal and adult rats. The predicted elution of inactive MAP kinase was based on previous chromatograms that had been analyzed by Western immunoblotting. The partially purified, active hepatic MEK derived from an EGF-injected adult rat was found to be potent in activating both adult and fetal rat liver MAP kinase (Fig. 8). These results were interpreted as confirming that fetal hepatic MAP kinases are intact as in vitro substrates for MEK following their partial purification.

Uncoupling of MAP Kinase Activation in Fetal Hepatocytes—We had shown previously that 24 h exposure of fetal hepatocytes in primary culture to TGF-α or hepatocyte growth factor resulted in desensitization of MAP kinase to activation by either growth factor (11). We hypothesized that this desensitization might be analogous to the uncoupling seen in vivo. To examine this, E19 fetal hepatocytes were cultured in the absence or presence of TGF-α for 24 h and then stimulated with additional TGF-α for 10 min. Shc immunoprecipitation showed an increase in tyrosine phosphorylation as well as Shc-Grb2 complex formation upon acute TGF-α stimulation. This was not affected by 24 h preincubation with TGF-α (Fig. 9). This was interpreted as indicating that tonic growth factor exposure for 24 h had not resulted in sufficient down-regulation of EGF receptor number or tyrosine kinase activity to cause an attenuation of proximal signaling. Given that this result was consistent with the in vivo findings, we went on to examine downstream signaling.

By using the same experimental design, MAP kinase reactivation following incubation with TGF-α for 24 h was examined. It was found to be only one-third of that seen with no TGF-α preincubation (Fig. 10). In contrast, MEK was activated to the same degree under both conditions. (The final peak of MBP kinase activity that eluted in fraction 23 of Fig. 9 and was higher in the untreated hepatocytes could be accounted for by the endogenous, previously activated MAP kinase.) Similar uncoupling of MAP kinase from active MEK was seen in an experiment where E19 fetal hepatocytes were pretreated for 48 h with TGF-α (data not shown). Western immunoblotting (not shown) indicated that this effect of growth factor preincubation was not due to a decrease in MAP kinase content. These results were interpreted as demonstrating that in vivo uncoupling of the MAP kinase signaling pathway could be reproduced in cultured fetal hepatocytes by tonic growth factor stimulation.

Investigation of a Potential Role for MAP Kinase Phosphatases in MAP Kinase/MEK Uncoupling—Recently cloned dual specificity phosphatases like MKP-1 (MAP kinase phosphatase 1, encoded by the murine gene 3CH134 (12)) or CL100, the human homologue of MKP-1 (13), exhibit dual catalytic activity toward phosphotyrosine and phosphothreonine, thus providing a mechanism for the inactivation of MAP kinase. Based on the above studies, we treated cultured (E19) fetal hepatocytes incubated with or without TGF-α for 24 h with either the tyrosine phosphatase inhibitor, sodium orthovanadate, or the serine-threonine phosphatase inhibitor, okadaic acid. Phosphatase inhibitors were added for 30 min prior to restimulation with TGF-α. In both cases, suppression of MAP kinase activation remained intact, that is MEK activation was unaffected by growth factor preincubation while MAP kinase activation was attenuated in a manner indistinguishable from that shown in Fig. 10.

Parallel in vivo studies were performed as well. We administered the protein tyrosine phosphatase inhibitor mpV(pic), a peroxovanadium compound that is over 100-fold more potent than orthovanadate, to fetal rats by intraperitoneal injection. Subsequent injection of EGF (15 min later) produced no discernible MAP kinase activation (results not shown).

An additional experiment was carried out to obtain indirect evidence that a MAP kinase phosphatase is present in fetal hepatocytes under basal conditions (i.e. without tonic growth factor stimulation). E19 fetal hepatocytes were exposed to TGF-α for 10 min. The TGF-α-containing medium was then replaced with fresh media that did not contain the growth factor. Within 15 min, MAP kinase activity returned to basal

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**Fig. 7.** MEK and phospho-MEK content in fetal and adult rat liver after EGF stimulation. Fractions 30–37 from the fetal and adult gel filtration columns shown in Fig. 6 were separated on a 10% SDS gel, transferred to nitrocellulose, and sequentially immunoblotted with the antibody to dually phosphorylated MEK-1/2, to MEK-1, and to MEK-2. Numbers to the right of the immunoblots represent the position of the 45-kDa marker.

**Fig. 8.** In vitro activation of fetal and adult rat liver MAP kinase by partially purified active MEK. Activated MEK from an EGF-injected adult rat was partially purified (see “Experimental Procedures”). Individual fractions obtained by Mono Q chromatography of control fetal (left) or adult (right) rat liver homogenates (7) were then combined with the partially purified MEK (solid circles) or buffer as control (open circles). MAP kinase activity was measured as MBP phosphorylation. Thus, the resulting chromatograms represent the in vitro activation of MAP kinase contained in the individual column fractions.
levels, consistent with the presence of an active MAP kinase phosphatase. Upon restimulating these cells for 10 min with TGF-α, MAP kinase was reactivated, indicating that inactivation was not due to MAP kinase turnover.

Investigation into the Presence of an Inhibitory MAP Kinase Binding Protein—An alternative hypothesis to explain the dissociation of MAP kinase and MEK activation in fetal liver could involve a protein which binds to MAP kinase, thereby preventing its activation in vivo. To obtain an indicator of the relative size of fetal and adult liver MAP kinases, fetal and adult rat liver homogenates, prepared using conditions aimed at minimizing disruption of protein-protein interactions, were analyzed using a Superose 6 gel filtration column (Pharmacia). Results (not shown) were consistent with the elution of MAP kinases from both fetal and adult rat liver as soluble monomers.

Gel filtration chromatographic analysis was performed on lysates derived from fetal hepatocytes preincubated for 24 h with or without TGF-α to detect the induction of a MAP kinase binding protein. Again, no shift in the apparent size of the 42- and 44-kDa MAP kinases was seen (not shown). An additional approach to detect a MAP kinase binding protein was employed. Cultured E19 hepatocytes were labeled with [35S]methionine for 24 h in the absence or presence of TGF-α. Immunoprecipitation with antibodies toward MAP kinase was preincubated with or without TGF-α for 24 h for 10 min (11). Samples were immunoprecipitated with immobilized Shc antibody followed by sequential Western immunoblotting with antibodies to Shc, phosphotyrosine (PY), and Grb2. These results were replicated in several additional experiments.

![Fig. 9. Shc tyrosine phosphorylation and Shc-Grb2 complex formation in fetal rat hepatocytes.](image)

Cultured fetal rat hepatocytes (E19) were incubated for 24 h under defined conditions in the absence (none) or presence (24 h) of 1.7 nM TGF-α. The cells were then stimulated with the re-addition of TGF-α for 10 min (11). Samples were immunoprecipitated with immobilized Shc antibody followed by sequential Western immunoblotting with antibodies to Shc, phosphotyrosine (PY), and Grb2. These results were replicated in several additional experiments.

![Fig. 10. Induction of the uncoupling of MAP kinase activation from MEK activation in fetal hepatocytes.](image)

E19 fetal hepatocytes were cultured in the absence (closed circles) or presence (open circles) of 1.7 nM TGF-α for 24 h. The cells were then stimulated with the re-addition of TGF-α for 10 min (11). MEK activity was measured as the increase in the activity of MAP kinase (phosphorylation of MBP), and MAP kinase was measured directly using MBP as substrate.

Our prior observation that intraperitoneal injection of late gestation fetal rats in situ with EGF showed only minimal activation of hepatic MAP kinases (8) was initially interpreted as indicating a probable block at the level of Ras activation. This was based on observations from several laboratories indicating that growth factor activation of the MAP kinase pathway can lead to phosphorylation of the Ras guanyl nucleotide exchange protein SOS, thereby down-regulating the MAP kinase pathway (14–19). Such a mechanism might pertain to negative feedback inhibition of hepatic MAP kinase activation in the growth factor-rich fetal environment. The ability of EGF to activate hepatic MAP kinases in vivo was recovered during the early phase of postnatal development, a period when hepatocyte proliferation is slowing and fetal hepatocytes are making the transformation to an adult hepatocyte phenotype. However, the observation that Raf and MEK activation were intact in the absence of MAP kinase activation indicated an alternative regulatory mechanism.

The conclusion that in situ EGF injection of a late gestation fetal rat could produce MEK activation with minimal MAP kinase activation is consistent with the entirety of the experiments we performed. In addition to activity measurements, Western immunoblotting confirmed the presence of MAP kinase but the absence of its phosphorylation in fetal liver. Furthermore, the lack of activation was accompanied by the lack of nuclear translocation. Gonzalez et al. (20) have reported that the majority of the MAP kinase in quiescent cells is in the cytoplasm. Upon activation of the cells with serum, cytoplasmic MAP kinase translocates into the nucleus. Similar translocation could be detected in subcellular fractions from adult, but not fetal, liver. The evidence for intact MEK activation included not only activity measurements but also Western immunoblotting for the active forms of MEK-1 and -2.

The physiologic in vivo uncoupling of MAP kinase activation at the level of MAP kinase phosphorylation by MEK has not been reported previously. However, several reports have suggested similar uncoupling of MAP kinase activation in vitro. By using a mouse myoblast cell line, Campbell et al. (21) showed that following withdrawal from serum and bFGF for 3 h bFGF stimulated MAP kinase kinase activity but that MAP kinase and S6 peptide kinase activities were not detected. When se-
rum and bFGF were withdrawn for 10 h, the activities of MAP kinase kinase, MAP kinase, and S6 peptide kinase were coordinate stimulated by bFGF. Similar results were seen using EGF. Furthermore, it was demonstrated that incubation with the tyrosine phosphatase inhibitor, sodium orthovanadate, restored MAP kinase activation. Microcystin, a serine-threonine phosphatase inhibitor, had little effect.

Samuels et al. (22), using retroviruses encoding a fusion protein consisting of an oncogenic form of human p74raf and the hormone-binding domain of the human estrogen receptor (hrafER), have studied MAP kinase regulation in rat1a cells conditionally transformed by hrafER. In these cells, estradiol (hrafER), have studied MAP kinase regulation in rat1a cells in v-infected macrophages, MEK activation occurs normally in vivo uncoupling of MAP kinase activation from MEK activation, whereas MAP kinase shows no activation correlating with lack of tyrosine phosphorylation of this enzyme (23). Suppression of MAP kinase activation was reversed in these cells by treating with sodium orthovanadate.

We did not observe an effect of phosphatase inhibitors on either the in vivo or in vitro uncoupling of MAP kinase activation from MEK activation. Although these findings do not rule out a fetal hepatic MAP kinase phosphatase as the factor required for uncoupling of MAP kinase activation from MEK activation, they also do not support this hypothesis. A responsible dual function phosphatase would have to be insensitive to vanadate and okadaic acid. In addition, there appears to be a MAP kinase phosphatase expressed in fetal hepatocytes maintained under non-growth factor-stimulated conditions. Thus, induction of a phosphatase would have to modify the effects of one already present. Nonetheless, given that the transcription of the dual specificity phosphatases has been shown to be stimulated by serum and growth factors (12, 24, 25), this remains a viable hypothesis.

Our hypothesis that MAP kinase activation by MEK could be blocked by a MAP kinase binding protein is not without precedent. It has recently been reported that the protein p21WAF1/cip1/ldc1, a DNA damage-inducible cell cycle inhibitor, acts as an inhibitor of the stress-stimulated protein kinases, also called the c-Jun amino-terminal kinases (JNKs). Dickens et al. (26) have described an additional mechanism for attenuating effects mediated by the JNK pathway. These investigators have cloned a cytoplasmic inhibitor of the JNK pathway, JIP-1, that binds specifically to JNK. JIP-1 causes cytoplasmic retention of JNK and inhibition of JNK-related gene expression. Given that the JNK enzymes represent a subfamily of the MAP kinases (27), we considered an analogous mechanism. However, we were unable to obtain evidence for fetal hepatic MAP kinases being present in a form other than soluble monomers. In addition, our data indicated a lack of MAP kinase phosphorylation, an effect not attributed to JIP-1.

In summary, our results demonstrate that hepatic, EGF-induced MAP kinase activation is suppressed in late gestation through the early postnatal period in the developing rat. Western immunoblotting indicated that the absence of MAP kinase activity correlates with the absence of MAP kinase phosphorylation. However, activation of MEK, the kinase which phosphorylates and activates MAP kinase, remains intact upon acute stimulation with EGF. Furthermore, the uncoupling of MAP kinase from the signaling cascade can be induced in vitro by the incubation of fetal hepatocytes for 24 h with growth factors. These findings may define a mechanism for attenuating MAP kinase activation under conditions in which cells are subject to tonic growth factor stimulation.

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Joan M. Boylan and Philip A. Gruppuso

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