Hepatic epidermal growth factor-regulated mitogen-activated protein kinase kinase kinase activity in the rat: lack of identity with known forms of Raf and MEKK

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Abstract **Mitogenic signaling involves protein kinases that phosphorylate the mitogen-activated protein kinase (MAPK) activator, MEK. In rats, basal hepatic MEK kinase activity is low in vivo in both adult rats and late gestation fetal rats, and is markedly stimulated by intraperitoneal administration of epidermal growth factor (EGF). The level of stimulated MEK phosphorylating activity is approximately 15 times higher in fetal liver than in adult liver. To identify regulated forms of the two categories of MEK kinase, Raf and MEKK, Western immunoblotting, immunoprecipitation kinase assays and immunodepletion studies were performed. Western immunoblotting confirmed that Raf-1, A-Raf, B-Raf, MEKK1 and MEKK2 were present at similar levels in E19 and adult liver. However, specific immunoprecipitation kinase assays did not detect any kinases that could account for marked EGF sensitivity or the higher level of activity in E19 fetuses. Immunodepletion studies produced a marked reduction in immunoreactive Raf/MEKK content and activity, but a minimal decrease in the ability of chromatography fractions to phosphorylate and activate recombinant MEK-1. Our results indicate that hepatic, EGF-sensitive MEK kinase activity may reside with a previously unidentified and physiologically relevant form of Raf and/or MEKK.**

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Key words: Mitogen; Signal transduction; Protein kinase; Protein phosphorylation; Liver

1. Introduction

Members of the *raf* family of serine/threonine protein kinases are effectors for the mitogenic actions of GTP-bound p21^{ras} and related small GTP binding proteins [1–4]. This observation established the central role of the Raf protein kinase family in mitogenic signal transduction. Recruitment of Raf to the cell membrane by GTP-bound Ras is a required early step in activation of the mitogen-activated protein kinase (MAPK) pathway [4]. In addition to a role in MAPK signaling, the various forms of Raf may be involved in other aspects of cell regulation, including the NF-κB pathway [5,6].

The initial identification of v-*raf*, the transforming gene of murine sarcoma virus 3611 [7], was followed by the identification of three cellular homologues, c-*raf*-1 [8], A-*raf* [9] and B-*raf* [10]. Subsequently, a family of kinases denoted by the term MEK kinase (MEKK1–4) were identified and characterized [11–13]. MEKK1–4 can activate the c-Jun amino-terminal kinase (JNK) pathway, while MEKK3 may be a physiologic regulator for both the JNK and p38 MAPK pathways [14]. MEKK1 and MEKK2 clearly have significant relevance to the regulation of the prototypical MAPKs, ERK1 and ERK2.

Our laboratory has focused on mitogenic signal transduction during late gestation liver development in the rat. Our initial studies on the MAPK signaling pathway showed a low level of constitutive MAPK activity in fetal liver under basal conditions [15,16]. We then attempted to define the stimuli for fetal hepatic MAPK activation, utilizing in situ activation of mitogenic pathways by direct intraperitoneal administration of epidermal growth factor (EGF) to late gestation fetal rats [17]. As part of these studies, the activation of Raf/MEKK was examined. Results showed that MEK phosphorylating activity, measured in FPLC anion exchange (MonoQ) column fractions, was markedly increased in fetal liver within 15 min after the intraperitoneal injection of EGF. EGF-mediated activation was also seen in adult liver, although the post-EGF activity recovered after MonoQ chromatography was 15-fold higher in fetal samples compared to adult samples. The present studies were undertaken with the aim of characterizing the Raf and/or MEKK forms responsible for hepatic EGF-dependent MEK kinase activity in the adult rat and, in particular, the high level of activity in the late gestation fetus.

2. Materials and methods

2.1. Materials

Human EGF was purchased from PeproTech, Inc. (Rocky Hill, NJ). Rabbit polyclonal antibodies directed against the carboxy-terminal regions of human Raf-1, A-Raf and B-Raf were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), as were antibodies against the amino-terminus of human MEKK1 and MEKK2.

Recombinant, histidine-tagged, human ERK-1 and kinase inactive human MEK-1 were prepared as described previously [17]. Recombinant, histidine-tagged wild type MEK-1 was expressed and purified as described by Gardner et al. [18].

2.2. Animals

For fetal studies, timed pregnant Sprague–Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) were delivered by Cesarean section under pentobarbital anesthesia (50 μg/g body weight, intraperitoneal) on day 19 of gestation, term being 21 days. Adult studies were performed using male Sprague–Dawley rats weighing

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Abbreviations: MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; MEKK, MEK kinase; EGF, epidermal growth factor; MBP, myelin basic protein

125–175 g. Activation of hepatic EGF signaling was accomplished by intraperitoneal injection of EGF at a dose of 0.5 µg/g body weight to fetuses in situ or to adult male rats. Injection of an equal volume of vehicle, phosphate-buffered saline, was used as a control.

Liver samples were taken 15 min after saline or EGF injection. Preparation and fractionation of homogenates by MonoQ ion exchange chromatography followed previously described procedures [16].

2.3. Biochemical analyses

For all immunoprecipitations, antibodies were cross-linked to protein A-Sepharose CL-4B using the chemical cross-linking reagent, dimethyl pimelimidate (20 mM in 0.1 M sodium borate, pH 9.0) [16]. Western immunoblotting of immunoprecipitates was performed using standard methods with detection by chemiluminescence [16,17].

Immune complex kinase assays for Raf and MEKK isoforms also employed immunoprecipitation with antibody cross-linked to protein A-Sepharose beads. Immunoprecipitated complexes were used directly in a MEK phosphorylating assay that used recombinant, histidine-tagged, kinase inactive human MEK-1 as substrate. Other assay conditions were identical to those previously employed in our laboratory [17]. Analysis of MEK phosphorylating activity in fractions derived from HPLC MonoQ chromatography was carried out under the same conditions, but without preparatory immunoprecipitation [17]. In both cases, phospho-MEK-1 was detected by polyacrylamide gel electrophoresis and autoradiography followed by densitometric quantification. Results are expressed as arbitrary absorbance units.

For immunodepletion studies, samples containing 2 mg of rat liver homogenate protein were precleared with protein A-Sepharose then incubated at 4°C for two successive 2 h cycles with a mixture of cross-linked antibody beads directed at the five Raf and MEKK forms of interest. The antibody beads were saved after each cycle and treated as above for Western immunoblotting. The supernatant that had been immunodepleted for two cycles was fractionated by MonoQ ion exchange chromatography. Fractions were analyzed either for MEK phosphorylating activity, or for their ability to activate wild type MEK-1. The latter was determined using a coupled assay that measured activity as an increase in myelin basic protein (MBP) phosphorylation by recombinant ERK-1. In brief, 17 µl of each fraction was mixed with 3 µl of the recombinant ERK-1 preparation. Samples were incubated at 30°C for 30 min followed by the addition of 20 µl assay buffer (50 mM β-glycerophosphate pH 7.2, 100 µM Na_3VO_4, 100 nM microcystin, 20 mM $MgCl_2$, 3 mM EGTA, 200 µM $[\gamma\text{-}^{32}P]ATP$ (0.25µC/nmol), and recombinant wild type MEK-1). After a 30 min incubation at 30°C, 16 µg of MBP was added. This was followed by an additional incubation for 15 min at 30°C. The reaction was stopped with 10 µl of 250 mM EDTA. Samples were separated on a 12% polyacrylamide gel to visualize the phosphorylated MBP. Each fraction was also run without the addition of recombinant MEK-1 to control for endogenous MEK-1 activity. The latter proved to be negligible relative to the added MEK-1.

3. Results and discussion

As we have described previously [17], EGF administration in vivo to late gestation fetal (E19) or adult rats produced a maximal level of MEK phosphorylating activity recovered from MonoQ chromatography that was approximately 15-fold higher in fetal liver homogenates than in adult liver homogenates. As a first step in identifying the specific enzymes accounting for EGF-responsive MEK phosphorylating activity, as well as the high level of fetal MEK kinase activity, fetal and adult liver homogenates were analyzed for Raf and MEKK content. We focused on Raf-1, A-Raf and B-Raf, as well as the forms of MEKK that are likely candidates for regulation of ERK-1 and ERK-2, namely MEKK1 and MEKK2. Immunoprecipitation of fetal liver homogenates followed by Western immunoblotting (Fig. 1) detected immunoreactive Raf-1 and A-Raf bands with the expected sizes of approximately 73 and 65 kDa, respectively [8,9]. In both cases, smaller forms ranging from 35 to 47 kDa were observed
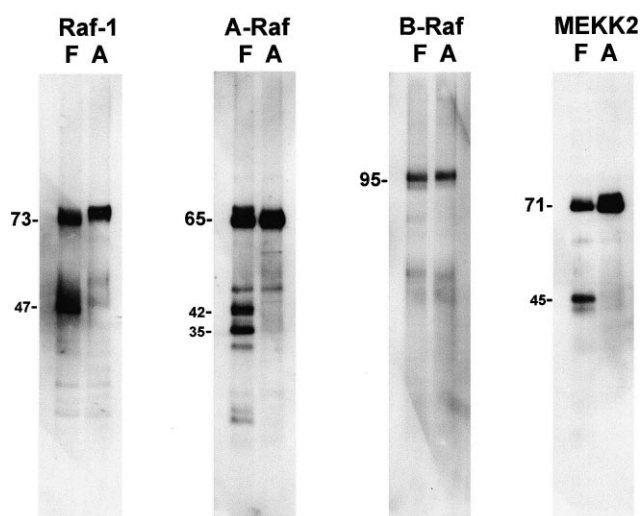

Fig. 1. Western immunoblotting for fetal and adult liver Raf-1, A-Raf, B-Raf and MEKK2. Fetal (F) and adult (A) liver homogenates were subjected to immunoprecipitation followed by Western immunoblotting using antibodies specific for various MEK kinases. Numbers to the left of the autoradiograms represent molecular mass in kDa. Larger numbers designate the apparent size of the expected forms for each MEK kinase.

in fetal samples but not adult samples. The significance of these forms is uncertain.

B-Raf was detected in both fetal and adult liver homogenates at the expected size of 95 kDa [19]. MEKK2 was detected as expected at approximately 70 kDa [12]. Again, a smaller immunoreactive protein of 45 kDa was seen in the fetal but not the adult sample. Very low levels of immunoreactive MEKK1 could be detected at the expected molecular weight of 195 kDa (not shown). This minimal signal was present at a higher level in adult liver than in fetal liver. Of note, the Western immunoblotting results did not demonstrate the presence of a Raf/MEKK in fetal liver that was absent from adult liver, or a form that was present in greater amounts in fetal compared to adult liver.

We next determined the activation states of the aforementioned forms of Raf and MEKK that were detected by Western immunoblotting. Liver homogenates from fetal and adult animals that were injected with saline or EGF prior to death were analyzed for MEK phosphorylating activity. Immunoprecipitation kinase assays (Fig. 2) revealed that B-Raf was the predominant immunoprecipitable active MEK kinase in both fetal and adult liver. However, activity was high under basal conditions and minimally stimulated by EGF. Immunoprecipitation with antibodies directed towards Raf-1, A-Raf, MEKK1 and MEKK2 all resulted in low but detectable MEK phosphorylation. In no case did we identify the expected kinase showing low activity under basal conditions, marked responsiveness for EGF, and EGF-stimulated activity in the fetus that markedly exceeded that seen in the adult.

These results were interpreted as indicating that the aforementioned forms of Raf or MEKK might not account for the MEK phosphorylating activity recovered from MonoQ chromatography. In order to confirm this, an immunodepletion approach was employed. Liver homogenates prepared from EGF-injected fetuses and an EGF-injected adult were subjected to two 2 h cycles of immunoprecipitation using a combination of immobilized antibodies directed towards Raf-1, A-
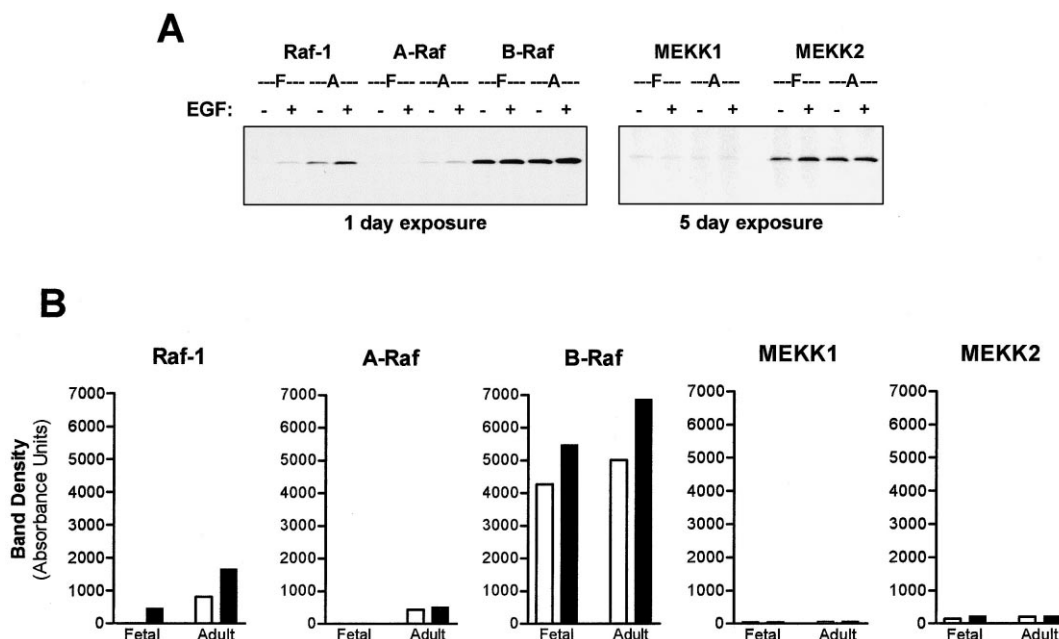
A

Fig. 2. Raf and MEKK activities in fetal and adult liver homogenates. Samples from fetal (F) and adult (A) animals injected with saline (−, open bars) or EGF (+, solid bars) were analyzed by immunoprecipitation kinase assay using antibodies specific for Raf-1, A-Raf, B-Raf, MEKK1 and MEKK2. A: Autoradiograms showing phosphorylated MEK-1 were obtained after 1 day or 5 days exposure of dried gels. B: Bars represent the densitometric quantification of the results shown in A corrected for autoradiography exposure time. Note that all results are shown to scale.

Raf, B-Raf, MEKK1 and MEKK2. Total MEK kinase activity was determined after both immunoprecipitation cycles. Results showed that this immunodepletion protocol resulted in removal of over 90% of the combined, immunoprecipitable MEK phosphorylating activity (Fig. 3, upper panel). In order to confirm the effectiveness of the immunodepletion process, the immunodepleted samples were also analyzed by Western immunoblot. Results (Fig. 3, lower panel) demonstrated that two cycles of immunodepletion were adequate to reduce levels of immunoreactive Raf-1, A-Raf, B-Raf and MEKK2 by more than two thirds.

Control (undepleted) and depleted samples were analyzed for MEK phosphorylating activity using MonoQ chromatography. Results (Fig. 4) showed that immunodepletion resulted in only a minimal reduction (approximately 10%) in the activity recovered following MonoQ chromatography for both the fetal and adult samples.

These results did not rule out the possibility that the in vitro MEK phosphorylation we were measuring was at sites on MEK other than those that are involved in regulating MEK activity. To address this, the experiment was repeated using kinase intact MEK-1 as substrate in combination with recombinant human ERK-1 and MBP. Control (undepleted) and depleted fetal liver homogenates were fractionated by MonoQ chromatography. Samples were analyzed for their ability to activate recombinant MEK-1, as indicated by an increase in MBP phosphorylation by recombinant ERK-1 included in the assay. As a control, each fraction was assayed for MBP phosphorylation after the addition of MEK-1 plus ERK-1 versus the addition of ERK-1 alone. Thus, the reported activity was dependent on the activation of exogenous MEK-1. Results (Fig. 5) showed that total activity across the profile was decreased by approximately 10% in the immunodepleted sample relative to the undepleted sample. This loss of

activity was interpreted as being far less than would be expected if the measured activity were due to any of the Raf or MEKK forms being examined.

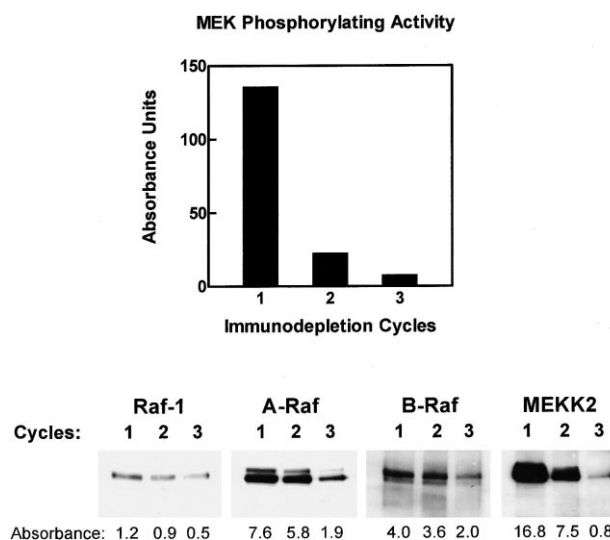An alternative explanation consistent with the above results

Fig. 3. Western immunoblot analysis of immunodepleted fetal liver homogenates. Upper panel: In order to confirm the effectiveness of the immunodepletion protocol, fetal liver homogenates were subjected to three cycles of immunoprecipitation with a mixture of antibody beads directed towards Raf-1, A-Raf, B-Raf, MEKK1 and MEKK2. The sequential immunoprecipitates were analyzed for MEK phosphorylating activity. Lower panel: The immunodepletion protocol was repeated with Raf-1, A-Raf, B-Raf and MEKK2 antibody beads. Resulting immunoprecipitates were analyzed for immunoreactive Rafs and MEKK2. Numbers below each lane correspond to absorbance of the immunoreactive bands as determined by densitometry.
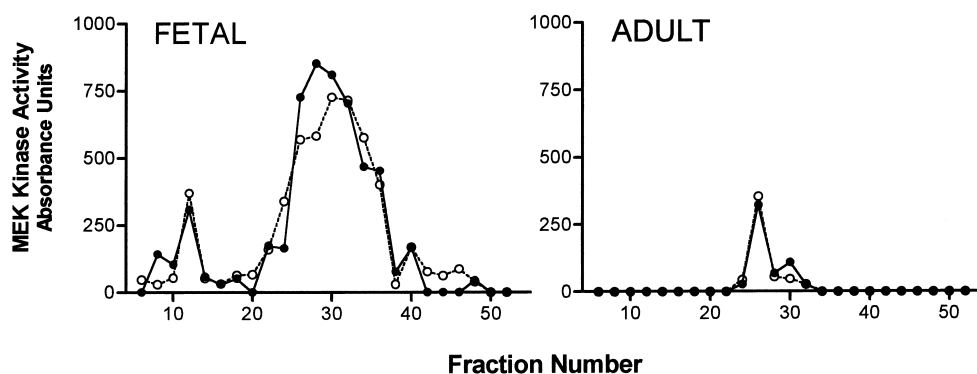
Fig. 4. MonoQ fractionation of MEK phosphorylating activity from fetal and adult liver homogenates analyzed before or after immunodepletion. Samples were subjected to MonoQ chromatography before (●) or after (○) immunodepletion with a combination of five Raf/MEKK antibodies. Column fractions were analyzed for MEK phosphorylating activity.

was that incorporation of regulated Rafs or MEKKs into multiprotein complexes interfered with immunoprecipitation. Given that such complexes would be unlikely to remain intact after ion exchange chromatography, two experiments were performed. In the first, chromatography fractions were subjected to Western immunoblotting for the various forms of Raf and MEKK. Minimal immunoreactivity was observed (data not shown), and in no case did immunoreactive species correspond with MEK kinase activity in the fractions. For the second experiment, MEK kinase activity eluted from MonoQ was pooled and subjected to immunoprecipitation with the panel of antibodies. Results (not shown) demonstrated low but detectable levels of B-Raf activity that were similar for both control and EGF-activated preparations. None of the other antibodies yielded any detectable kinase activity.

Previous studies of MEK kinases in liver have focused on signal transduction in the adult. We observed that the level of maximal hepatic MEK kinase activation following EGF injection to a fetus was 15-fold higher than that seen in adult liver [17]. This led us to hypothesize that known forms of Raf and/or MEKK are present in greater abundance in fetal liver than in adult liver. The observation that fetal liver MEK, like

adult liver MEK, was markedly activated by the in vivo administration of EGF supported the hypothesis that the main form(s) of MEK kinase in fetal liver would be EGF-sensitive.

The present studies lead to the conclusion that none of the currently characterized forms of Raf and MEKK meet our criteria of higher activity in fetal (relative to adult) homogenates and marked activation in response to EGF. The physiologic relevance of the MEK kinase activity recovered after MonoQ fractionation of liver homogenates is indicated by in vivo EGF-mediated activation of MEK-1 and MEK-2 in fetal and adult liver [17]. By using MEK-1 as a substrate for MEK kinase activity measurements, we intended to focus on enzymes that were relevant to EGF signal transduction through the MAPK pathway. The demonstration that residual MEK-phosphorylating activity recovered from MonoQ after immunodepletion could also activate recombinant MEK-1 supports the relevance of our findings to the physiologic regulation of the MAPK signaling pathway in liver.

Our data do not allow conclusions regarding the identity of the EGF-responsive form(s) of MEK kinase in rat liver. Examination of the known Rafs and MEKKs shows marked heterogeneity within the two families. This supports the plausibility of the most obvious interpretation of our findings more plausible; that is, the existence of a key signaling kinase involved in the activation of the hepatic MAP kinase pathway that has not yet been identified.
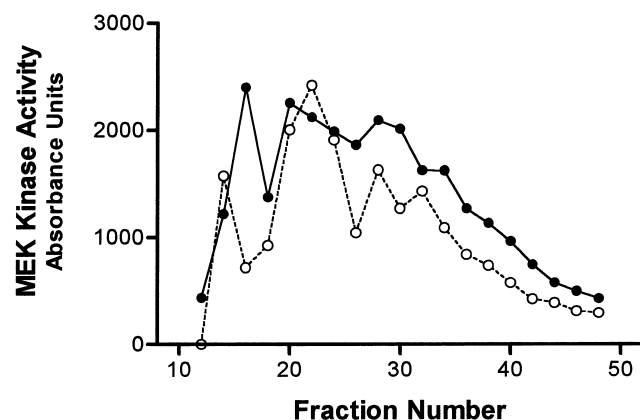
Fig. 5. MonoQ fractionation of MEK kinase activity from a fetal liver homogenate analyzed before or after immunodepletion. Samples were subjected to MonoQ chromatography before (●) or after (○) immunodepletion with combined Raf/MEKK antibodies. Column fractions were analyzed for Raf/MEKK activity by determining the ability of each fraction to stimulate the sequential activation of recombinant MEK-1 and ERK-1. Activity data represent phosphorylation of the ERK-1 substrate, MBP.

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