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Comparative Effects of Hepatocyte Growth Factor and Epidermal Growth Factor on Motility, Morphology, Mitogenesis, and Signal Transduction of Primary Rat Hepatocytes

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Abstract Hepatocyte growth factor (HGF) and epidermal growth factor (EGF) are major hepatocyte mitogens, but HGF, also known as scatter factor (SF), has also been shown as a potent motogen for epithelial and endothelial cells. The mechanisms by which HGF is a stronger motogen compared to other mitogens are not understood. Here we report a comparative study of the effect of the two growth factors on cultured primary rat hepatocytes regarding their differential effects on morphology, mitogenicity, and motility as well as the phosphorylation of cytoskeletal-associated proteins. Using three different motility assays, both HGF and EGF increased the motility of hepatocytes, but HGF consistently elicited a significantly greater motility response than EGF. Additionally, HGF induced a more flattened, highly spread morphology compared to EGF. To examine if HGF and EGF phosphorylated different cytoskeletal elements as signal transduction targets in view of the observed variation in morphology and motility, primary cultures of ^{32}P-loaded rat hepatocytes were stimulated by either HGF or EGF for up to 60 min. Both mitogens rapidly stimulated four isoforms of MAP kinase with similar kinetics and also rapidly facilitated the phosphorylation of cytoskeletal-associated F-actin. Two cytoskeletal-associated proteins, however, were observed to undergo rapid phosphorylation by HGF and not EGF during the time points described. One protein of 28 kDa was observed to become phosphorylated fivefold over controls, while the EGF-stimulated cells showed only a slight increase in the phosphorylation of this protein. Another protein with an apparent mwt of 42 kDa was phosphorylated 20-fold at 1 min and remained phosphorylated over 50-fold over control up to the 60 min time point. This protein was observed to become phosphorylated by EGF only after 10 min, and to a lesser extent (20-fold). Taken together, the data suggest that HGF and EGF stimulate divergent as well as redundant signal transduction pathways in the hepatocyte cytoskeleton, and this may result in unique HGF- or EGF-specific motility, morphology, and mitogenicity in hepatocytes.

Key words: two-dimensional gel electrophoresis, actin phosphorylation, MAP kinase, cytoskeletal protein phosphorylation, scatter factor

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a unique peptide mitogen that is capable of eliciting motogenicity and/or mitogenicity on a number of target cell types [Bhargava et al., 1993; Michalopoulos and Zarnegar, 1992; Matsumoto et al., 1991; Tajima et al., 1992; Weidner et al., 1990; Stoker and Gherardi, 1991]. It has also recently been shown to be angiogenic on endothelial cells [Bussolino et al., 1992; Grant et al., 1993]. Epidermal growth factor (EGF), on the other hand, does not appear to possess such diverse properties and acts primarily as a mitogen on its target cells [Stoker and Gherardi, 1991]. Both growth factors have been implicated as important mitogens in liver regeneration following chemical or mechanical injury, but each appear to manifest their stimulatory effects differently [reviewed by Michalopoulos, 1990; Fausto, 1991; Bucher, 1991]. After partial hepatectomy or hepatotoxin administration (e.g., CCl_4), the level of circulating HGF in the sera increases 13–17-fold within 2 hr [Lindroos et al., 1991], while EGF shows little (1.5–2-fold) to no increase in the circulation [Johansson and Andersson, 1990; Noguchi et al., 1991; Fausto, 1991]. Both growth factors,

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however, are complete mitogens for hepatocytes in vitro. HGF and EGF give comparable mitogenic stimulation at maximal dosage, but on a molar basis HGF is consistently at least 10 times more potent than EGF [Zarnegar and Michalopoulos, 1989; this report]. The mitogenic effects of HGF and EGF have also been shown to be additive [Nakamura et al., 1984; Zarnegar and Michalopoulos, 1989]. The spatial and temporal differences exhibited between the two growth factors in liver regeneration in vivo, as well as their additive effects on hepatocytes in vitro, strongly suggest that they accomplish their proliferative and motogenic effects via different signal transduction mechanisms.

It is now widely accepted that many peptide growth factors elicit rapid intracellular responses on their target cells via transmembrane receptors. Many growth factor receptors are tyrosine kinases that autophosphorylate as well as phosphorylate and activate other enzymes [reviewed by Ullrich and Schlessinger, 1990]. A complicated cascade of events ensues that ultimately leads to DNA replication and mitosis on the target cell. The receptors for each of the growth factors have been shown to be members of the transmembrane growth factor receptor tyrosine kinase family. The proto-oncogene c-*met* has been identified as the HGF/SF receptor [Naldini et al., 1991a,b; Bottaro et al., 1991; Park et al., 1987] and the EGF receptor has been studied in exquisite detail on a number of cell types [Hunter and Cooper, 1981; reviewed by Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992]. The expression of both HGF [Higuchi and Nakamura, 1991] and EGF receptors [Johansson and Andersson, 1990; Earp and O'Keefe, 1981] is downregulated during liver regeneration following partial hepatectomy.

Recently, elegant work by Cantley and colleagues [Cantley et al., 1991; Songyang et al., 1993] has demonstrated that the src-homology region (SH2) of activated c-met and the EGF receptor bind different downstream signal transduction elements. This strongly suggests that each growth factor, via its receptor, might stimulate unique targets temporally distal to the membrane. Many of the components involved in the downstream signal transduction cascade subsequent to ligand binding have membrane-associated, cytosolic, or nuclear locations. A wealth of information is now unfolding about these signal transduction pathways for HGF and EGF [Cantley et al., 1991; Songyang et al., 1993]. There is increasing evidence, however, that cytoskeletal elements may also be the target of growth factor signal transduction pathways [Kellie et al., 1991; Luna, 1991; Luna and Hitt, 1992; Payrastre et al., 1991; Rosen et al., 1990a; Hoffman, 1991], although the mechanism is poorly understood. This should not be totally unexpected since the machinery that drives morphological changes, locomotion, and mitosis coincident with stimulation of cell growth and motility is cytoskeletal in nature.

This report compares the morphogenic, motogenic, and mitogenic properties as well as novel cytoskeletal signal transduction events in cultured rat hepatocytes following stimulation with HGF or EGF in an attempt to decipher the mechanisms governing the pathways leading to morphogenic changes as well as motogenesis or mitogenesis that may accompany liver regeneration. HGF has only been suggested as a possible motogen for hepatocytes in vitro, and direct comparisons between HGF and EGF regarding this phenomenon have not been reported. We show that HGF and EGF elicit unique morphological, motogenic, and mitotic responses. We also show that many cytoskeletally associated proteins, including actin and an isoform of MAP kinase, are signal transduction targets via rapid phosphorylation in less than 1 min after stimulation with either HGF or EGF. Furthermore, at least two cytoskeletal-associated proteins, p28 and p42, are phosphorylated rapidly by stimulation with HGF but not EGF. These data suggest that HGF and EGF do not simply follow redundant signal transduction pathways in the rat hepatocyte cytoskeleton and the phosphorylation of p28 and p42 may represent an important bifurcation signal(s) that commits a cell to a specific response.

METHODS AND MATERIALS

Reagents

All chemicals were purchased from Sigma (St. Louis, MO) unless indicated otherwise. Human recombinant HGF was the very generous gift of Genentech (S. San Francisco, CA). It was determined to be pure by running as a single band under nonreducing conditions at 64 kDa, or four bands (single chain ~97 kDa, heavy chain ~62 kDa, and light chain ~34/32 kDa) upon reduction after radioiodination [Mars et al., 1993]. This is similar to what others have reported for human and rabbit plasma HGF [Zarnegar and Michalopoulos, 1989; Zarnegar et al., 1990]. Mu-

rine submaxillary gland EGF was purchased from Collaborative Research (Waltham, MA).

Cell Isolation and Culture

Hepatocytes from 100–150 gm male Fischer 344 rats were obtained freshly isolated by two-stage collagenase perfusion technique [Zarnegar and Michalopoulos, 1989]. Typically 2–3.5 × 10^8 viable cells were obtained per liver. Cells were plated at various densities onto collagen type I (Vitrogen-100, Celtrix, Palo Alto, CA)–coated tissue culture plates (15 μg/cm^2, allowed to dry under UV light in a sterile hood overnight prior to use) in serum-free Modified Eagle's Medium supplemented with essential amino acids, pyruvate, and gentimycin (MEM) (all from Gibco). Cells were plated at low density (10^4 cells/cm^2), high density (3 × 10^4 cells/cm^2), or near confluent density (5–6 × 10^4 cells/cm^2) for specific assays where indicated. Cells were allowed to attach for 2–4 hr after which unadherent cells were removed and cultures were refed with MEM. Cells were serum starved 24 hr before being stimulated with growth factors.

Mitogenicity Bioassays

Cells seeded at low density or near confluent density were examined for DNA synthesis by ^{3}H-thymidine uptake as described by Zarnegar and Michalopoulos [1989]. DNA synthesis was determined at 24 and 48 hr at both densities.

Motogenicity Assays

Microcarrier bead assays. Microcarrier bead motility assays were performed essentially as described by Rosen et al. [1990a], but with some modifications. Cells were seeded onto Cytodex 3 collagen-coated microcarrier beads in serum-free MEM at a concentration of ~4–5 × 10^4 cells/cm^2 of bead surface. For most efficient bead–cell adhesion, the cell–bead suspension was placed in a 100 mm noncoated tissue culture (Falcon) dish and allowed to incubate at 37°C overnight. Since hepatocytes display very low plating efficiency in serum-free medium onto plastic, they preferentially attach to the beads and not to the plate. Approximately 75% of the cells were observed to attach to the beads under these conditions. Prior to the beginning of the motility assay, beads were washed two to three times by allowing them to settle at 1*g* through 50 mls of MEM. It was important to visually inspect the bead mixture after washing to assure that nonadherent cells were not present and that a significant number of cells remained attached to the beads. Cells on microcarriers cannot be centrifuged since hepatocytes are very fragile and break under even minimal centrifugal force due to the collisions between beads. Washed, inspected beads were seeded equally onto collagen-coated 12-well cluster (16 mm diameter) plates and stimulated with either 50 ng/ml HGF or EGF for 24 hr. The assay is terminated by washing the plate free of beads (four to five wash cycles with MEM). Cells were fixed 10 min in 3% paraformaldehyde in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, then stained 10 min in 0.1% Coomassie blue in 10% methanol, and 10% acetic acid, then destained for the appropriate time in a 10% methanol, 10% acetic acid solution. Cells were counted in 15 random fields from three to six wells for each condition. Parallel ^{3}H-thymidine uptake bioassays were performed on identical cultures to assure that the cells that migrated to the plate were not due primarily to mitosis.

Boyden chamber filter assays. Modified Boyden chamber motogenicity assays were performed as described [Rosen et al., 1990b] on 12 μm pore tissue culture-treated Transwell inserts (Costar). Cells were plated at high density onto collagen I–coated inserts and allowed to attach for 2 hr in serum-free MEM. Nonadherent cells were removed, and attached cells were serum starved for 24 hr then stimulated with 50 ng/ml HGF or EGF for 24 hr. Cells on inserts were fixed and stained as described for the bead assay, but the cells on the top part of the chamber were completely removed by rubbing with a cotton-tipped applicator after staining. Cells that had migrated to the bottom of the membrane were counted and photographed.

Wound assay. Hepatocytes were seeded at near confluent density onto collagen-coated tissue culture dishes, then serum starved for 24 hr. Dishes were washed to remove floating cells, then a ~400 μm swath of the monolayer was scraped free of cells using a sterile Pasteur pipette. The visible scratch on the plate then serves as a reference point for cell migration. Monolayers were again washed free of disrupted cells, then fed with control, serum-free MEM (no addition) or MEM supplemented with either 50 ng/ml HGF or EGF. Cells were monitored at 24 hr and only those cells that migrated into the denuded area and touched the scratch were scored during the assay.

Morphometry. Hepatocyte "flatness" was determined as a factor of the surface area covered by individual cells. Control or stimulated cells in random fields were photographed at 48 hr postgrowth factor addition. Photographs of each field were printed on 8 × 10 inch sheets, and individual cells with clearly visible nuclei in each field were identified, cut out, and weighed. Average weight/cell for each condition was determined, then size increase over control cells was calculated. At least 100 cells for each condition from triplicate plates were evaluated. This procedure was repeated four times.

Signal transduction labeling. Cells plated at high density (3×10^4 cells/cm^2) as described above were serum starved for 24 hr, then phosphate starved for 1 hr in phosphate-free MEM supplemented with essential amino acids, pyruvate, and gentimycin (Gibco). Cells were metabolically ^{32}P loaded by addition of 100–500 μCi ^{32}P-orthophosphate (Amersham)/ml phosphate-free MEM (Gibco) and incubated for 4–5 hr at 37°C. ^{32}P-labeled cells were subjected to 50 ng/ml HGF or EGF for 1, 10, or 60 min. For each experiment all growth factors were carefully monitored so as to assure that mitogenic activity was present. Parallel bioassays for both mitogenicity and signal transduction were performed on sister cultures by ^{3}H-thymidine uptake as described above. Cells were plated and cultured identically to those used for experiments, including phosphate starvation. Growth factors were added at the identical concentrations used for the parallel phosphorylation experiments and incubated for 48 hr.

Cytoskeletal isolation. Cytoskeletons were isolated at 4°C from labeled or unlabeled cells as well as stimulated and unstimulated cells under identical conditions. Cells in culture were placed on ice and washed twice in phosphate-buffered saline (PBS). Monolayers were lysed by addition of 1 ml/100 mm dish of cytoskeletal isolation buffer that maintains phosphorylation states of isolated proteins (10 mM Tris, 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 30 mM Na pyrophosphate, 50 mM NaF, 100 μM sodium orthovanadate 0.1% azide, and 10 μg/ml each E-64, leupeptin, pepstatin, and 2 mM PMSF). After 5 min incubation, the entire lysate is removed from the dish by cell scraper and placed into 1.5 ml screwtop microfuge tubes. Cytoskeletons were pelleted from the lysate at 14,000*g*. This pellet typically represents 10–15% of the total hepatocyte protein. Supernatants and cytoskeletal pellets were then prepared for 2-D gel electrophoresis or immunoprecipitation as described below.

Protein electrophoresis and electroblotting procedures. Large format analytical two-dimensional gel electrophoresis was performed exactly as described in the Investigator 2-D Electrophoresis System Handbook (Millipore Corp., Bedford, MA). Cytoskeletal pellets were solubilized by sonication in cytoskeleton isolation buffer supplemented with 1% SDS. Two hundred micrograms of the cytoskeletal and supernatant fractions [protein quantitated by the bicinchoninic acid technique of Smith et al., 1985] was precipitated in 80% (v/v) acetone on ice for 30 min. Pellets were then resolubilized in 10 μl of 0.3% SDS, 200 mM dithiothreitol, and 50 mM Tris, pH 7.4. Often vigorous sonication on ice was necessary to completely solubilize the protein. Solubilized proteins were then diluted 1:4 in 9.9 M urea, 4% NP-40, 2.2% Ampholytes (3–10/2D, Millipore), and 100 mM dithiothreitol prior to addition of 25 μl (100 μg) of the sample to the first dimension isoelectric focusing gels. Discontinuous one-dimensional 10% gel electrophoresis was performed as described [Laemmli, 1970]. Large format 2-D gels were electrotransferred in 25 mM Tris, 192 mM glycine, 0.01% SDS, and 20% methanol to Immobilon-P PVDF membrane (Millipore), using the Millipore Graphite Electroblotter II (3 hr at 500 mA), and small format 1-D gels (Tall Mighty Small, Hoefer) were transferred overnight in the same blotting buffer at 250 mA using the Mini-Transphor Electroblotter from the same company. Blots were reversibly stained with Ponceau red to assure even transfer, then blocked in 5% Blotto (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5, 5% nonfat dry milk) for at least 1 hr prior to immunoblotting. Primary antibodies were diluted in 5% Blotto, but subsequent washes and incubations were performed in Blotto with 1% nonfat dry milk.

Analytical densitometry. Densitometric analysis of 2-D gels and autoradiographs was performed using the BioImage Analytical Scanning Densitometer (Millipore/BioImage, Bedford, MA) combined with the Two-Dimensional Gel Investigator software package. Analysis of one-dimensional gels, blots, and autoradiographs was accomplished with the Whole Band Analysis software package on the same system.

Immunoprecipitation, immunodetection, and other assays. Immunoprecipitation was performed exactly as described by Firestone and Winguth [1990] using a 1:1 mixture of Protein A/G sepharose (Sigma) as the immunoabsorbant. Prior to immunoprecipitation, cytoskeletal pellets were resuspended in the original volume of cytoskeleton supplemented with 0.1% SDS and 1% deoxycholate, then sonicated (Branson) on ice for 10–12 bursts at 50% power on setting #5. Particulate matter remaining after this procedure is removed by centrifugation at 14,000*g*. Antibodies [rabbit polyclonal anti-actin (Sigma, A2668), monoclonal anti-actin (Chemicon, MAB1501), rabbit polyclonal anti-MAP kinase (UBI, ERK1), monoclonal anti-MAP kinase (Biodesign, MK12)] were used at concentrations suggested by the supplier for immunoprecipitation. Anti-phosphotyrosine monoclonal antibodies were purchased from Sigma. Subsequent immunodetection on blots was performed using above primary and HRP-labeled secondary antibodies at half the recommended concentration, then visualized using standard chemiluminescence techniques (Amersham, NEN, or Kirkegaard and Perry). Immobilon-P membranes were stripped and reprobed with different antibodies as described by the manufacturer. Membranes, if dry, were rewet with methanol, washed in water, then incubated in a hybridization oven for 1 hr at 80°C in "Strippo" (0.2 M glycine, 0.05% Tween-20, pH 2.5). This process was repeated with fresh, 80°C Strippo for an additional hour prior to reprobing. Typically, membranes were reblocked in 5% Blotto for 1 hr prior to addition of another set of primary antibodies. Whole-cell extract supernatants for MAP kinase assays were prepared as described by Ray and Sturgill [1987]. Myelin basic protein peptide (UBI) was used as phosphoacceptor, as described by Clark-Lewis et al. [1991].

RESULTS

HGF and EGF Effects on Morphology, Motility, and Mitogenicity

Initially, mitogenic differences elicited by HGF and EGF were determined on primary rat hepatocyte cultures seeded onto collagen-coated plates at low and near confluent densities (Fig. 1). Hepatocytes plated at a low density of 10^4 cells/cm^2 respond similarly in 24 and 48 hr ^{3}H-thymidine uptake bioassays to 50 ng/ml HGF or EGF, but when normalized for molarity, HGF

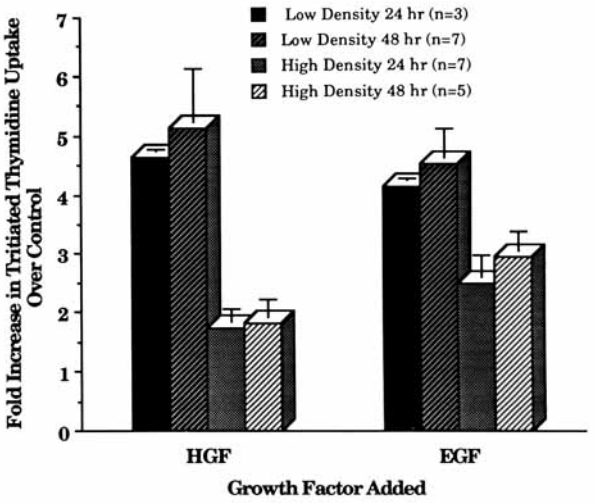

Fig. 1. Comparison of the mitogenic effects of HGF and EGF on primary cultures of rat hepatocytes. Low cell densities are hepatocytes that have been seeded onto collagen-coated plates at 10^4 cells/cm^2, and high cell densities are plated at near confluency of 5–6 × 10^4 cells/cm^2. Fifty nanograms per milliliter of HGF or EGF is at or above the concentration necessary for maximum effect to stimulate hepatocyte DNA synthesis in 48 hr and 24 hr bioassays. Since the mwt of HGF (97 kDa) is ~16 times that of EGF (6 kDa), HGF consistently exhibits a potency up to 16 times that of EGF on a molar basis. Low cell density cultures are stimulated to a greater degree (two to three times) than high cell density cultures for either HGF or EGF. EGF, however, gives greater stimulation than HGF in high cell density cultures. Values are given as mean ± SEM.

(mwt 97 kDa) is 10–18 times more potent a mitogen than EGF (mwt 6 kDa) for primary rat hepatocytes. Cells plated at near confluent densities (5–6 × 10^4 cells/cm^2) respond much less to either mitogen in 24 or 48 hr bioassays; however, EGF slightly but consistently stimulated cells to a greater degree than HGF.

The morphological response to HGF and EGF was also monitored. Figure 2 shows that at low density plating, HGF-treated cells displayed extensive, flattened lamellipodial processes when compared to either control or EGF-treated cells. The area over which cells spread was morphometrically determined as described in Methods and Materials, and HGF-stimulated cells were determined over ~34% more area than control cells, while EGF-treated cells covered only 10% more area than control cells (Table I).

The motogenic response of hepatocytes to HGF and EGF was examined using three different motility assays (summarized in Table I). Hepatocytes were first examined for motility using a 24 hr microbead migration assay as described. HGF stimulated a 1.8-fold increase in motility versus a 1.25-fold increase when stimu-

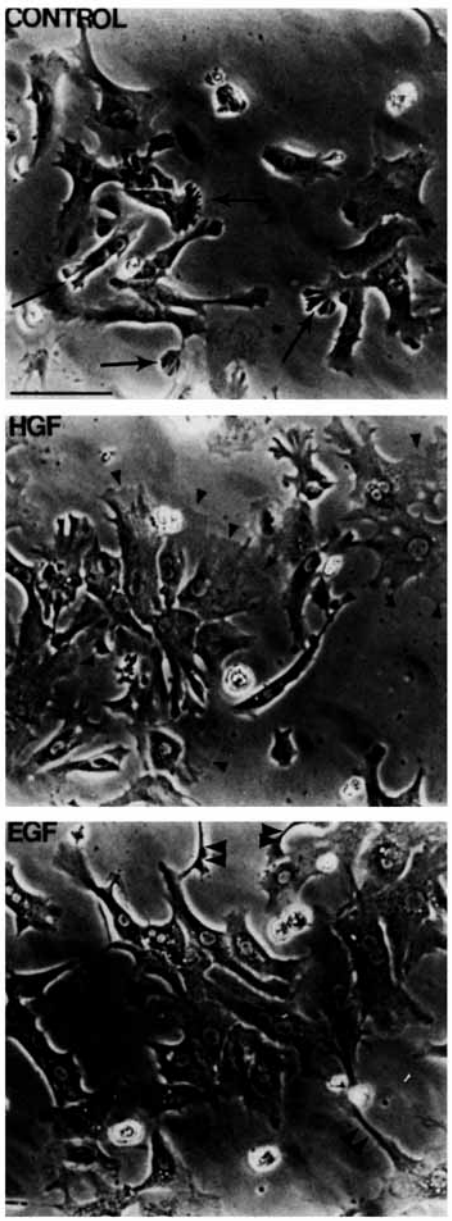

Fig. 2. Morphology of rat hepatocyte cultures stimulated with HGF or EGF. Cells were plated at low density (10^4 cells/cm^2) as described, then photographed 48 hr after growth factor addition; however, 24 hr incubations give identical morphologies. Typical morphologies of hepatocytes are shown in the micrographs. Control cells are less flattened or spread then sister cultures treated with either 50 ng/ml HGF or EGF (see also Fig. 5). Many control cells appear to have "half-open fan" lamellipodia (arrows), while HGF-treated cells have a pronounced flattened morphotype with extensive, flattened lamellipodia (arrowheads). EGF-treated cells are of intermediate flatness and rarely exhibit extensive fan-like or flattened lamellipodia. Many EGF-treated cells express long, thin processes (double arrowheads). Increased "flatness" or area of cells has been shown to be coincident with increased motility of epithelial cells treated with HGF or scatter factor (Table I). These observations also appear to hold true for hepatocytes in culture. Bar represents 100 μm.

lated by EGF. Interestingly, the hepatocytes required a collagen-treated substratum for bead to dish migration to occur (data not shown). A substratum of plain tissue culture plastic on the dish surface prohibited the migration of stimulated as well as unstimulated cells from the beads to the dish, suggesting that an extracellular matrix is crucial for the attachment as well as migration of hepatocytes.

Other motility assays were performed in order to examine the locomotion of hepatocytes over shorter (~ 10 μm) distances. Modified Boyden chamber assays [Rosen et al., 1990c] provided results of greater magnitude than those observed using the microbead assays (Fig. 3). HGF consistently evoked a greater motogenic response than EGF (5.01- vs. 3.28-fold over controls, respectively). Under these conditions, HGF and EGF were shown to cause numerous lamelli to migrate through 12 μm pores of collagen-coated polycarbonate filters, but the morphology of the lamelli was very distinctive, depending upon the growth factor used. EGF caused very thin processes to migrate to the opposite side of the filter, while HGF stimulated very wide, flattened lamellipodia to spread through the pores. These migratory lamelli mimicked those seen in HGF- and EGF-treated cells in Figure 2.

The third motogenicity assay was performed using a monolayer wounding assay (Fig. 4). Cells were plated at near confluent densities and allowed to attach overnight. A Pasteur pipette was used to clear a swath of ~ 400 μm in the monolayer (Fig. 4, $t = 0$). Under these conditions, HGF was observed to stimulate hepatocytes to migrate into the denuded areas in significantly greater numbers within 24 hr than cells stimulated with EGF (4.38- vs. 1.89-fold over controls, respectively). Similar results were observed at 48 hr postwounding, with HGF completely covering denuded areas. It was also observed that while EGF did not stimulate significant numbers of cells to migrate into the wound, a monolayer remodeling appeared to occur at areas of high, near confluent cell density distal to the wound.

Additionally, hepatocytes assumed subtle but visible morphological differences when near confluent monolayers were stimulated with HGF compared to EGF. Figure 5 shows that when hepatocytes are seeded at very high density, HGF-treated cells cover more surface area/cell than do control or EGF-treated cells (see also

TABLE I. Summary of Motogenic Characteristics of HGF or EGF on Primary Rat Hepatocytes*

	Growth factor added		
	HGF	EGF	*P* value
Motility assays (Fold over controls)			
Microcarrier beads ($n = 4$)	1.80 ± 0.25	1.25 ± 0.11	0.0157[a]
Boyden chamber ($n = 7$)	5.01 ± 1.13	3.28 ± 0.62	<0.0001[b]
Wound-filling assay ($n = 5$)	4.38 ± 1.82	1.89 ± 0.49	0.0076[b]
Morphometry			
Cell area (% increase over control) ($n = 4$)	33.97 ± 8.75	10.26 ± 2.44	0.0323[a]

*Motogenicity was determined using three different techniques: the microcarrier assay (samples done in three to six replicates, mean ± SEM), the Boyden chamber assay (three to six replicates, mean ± SEM; see Fig. 3), and the wound-filling assay (35 mm length of denuded swath counted per sample, four to five replicates, mean ± SEM). Samples were scored by counting those cells that migrated into denuded area and physically touched the scratch at 24 hr postwounding and growth factor addition (see Fig. 4). Each motility assay gave slightly different results due to the nature of the assay (see text), but HGF significantly induced greater motility than EGF in each assay. Cell size (mean ± SEM) was determined and HGF-treated cells consistently were ~34% "more spread" or flatter than control cells, while EGF displayed a morphotype of intermediate flatness. This degree of flatness or increase in cell area coincides well with the degree of motility seen in HGF- and EGF-treated cells compared to controls.

[a]Unpaired Student's *t*-test.

[b]Paired Student's *t*-test.

Fig. 2). However, monolayers treated with EGF (EGF, Fig. 5A) for 48 hr appear to become much more compact in nature, with well defined areas of cell–cell contact. HGF-treated monolayers (HGF, Fig. 5A), while flatter, show areas of cells that are multilayered and less ordered, with areas of cell–cell contact ill defined. Control monolayers display neither distinct cell–cell contact or multilayering. At slightly subconfluent areas of the monolayer, HGF-treated hepatocytes exhibit a profound increase in expression of processes and extensive, flattened lamellipo-

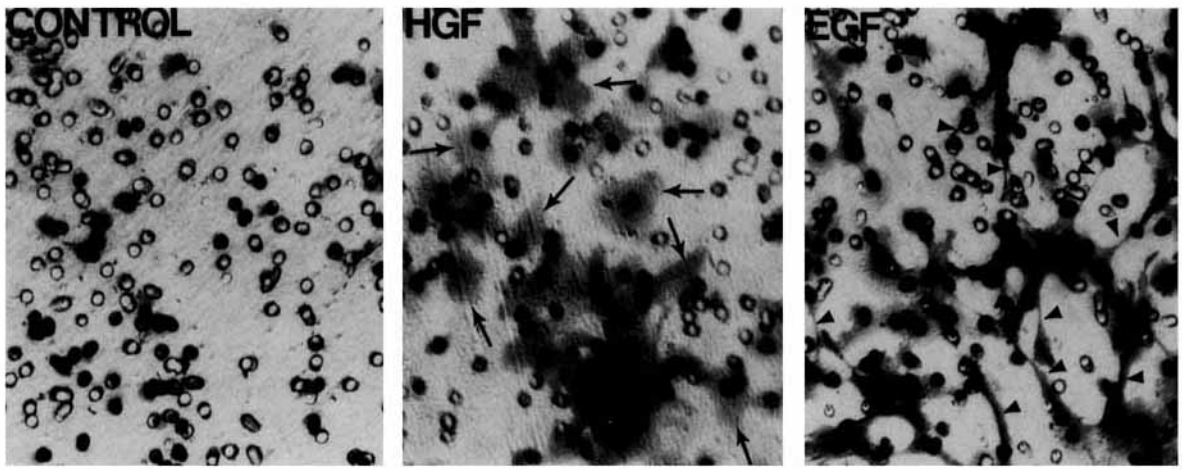

Fig. 3. Modified Boyden chamber motility assays of rat hepatocytes. Serum-starved rat hepatocytes were seeded at high density (3×10^4 cells/cm^2) onto collagen-coated 12 μm pore Transwell inserts as described in Methods and Materials. Cells were stimulated with 50 ng/ml HGF or EGF for 24 hr prior to processing. Both EGF and HGF induce migration of lamellipodial processes to the opposite side of the filter, but more spindle-like processes were formed by EGF-treated cells (arrowheads), while HGF-treated cells induce wider, flattened lamellipodial processes (arrows) similar to those seen in Figure 2. HGF consistently stimulated more migration then EGF.

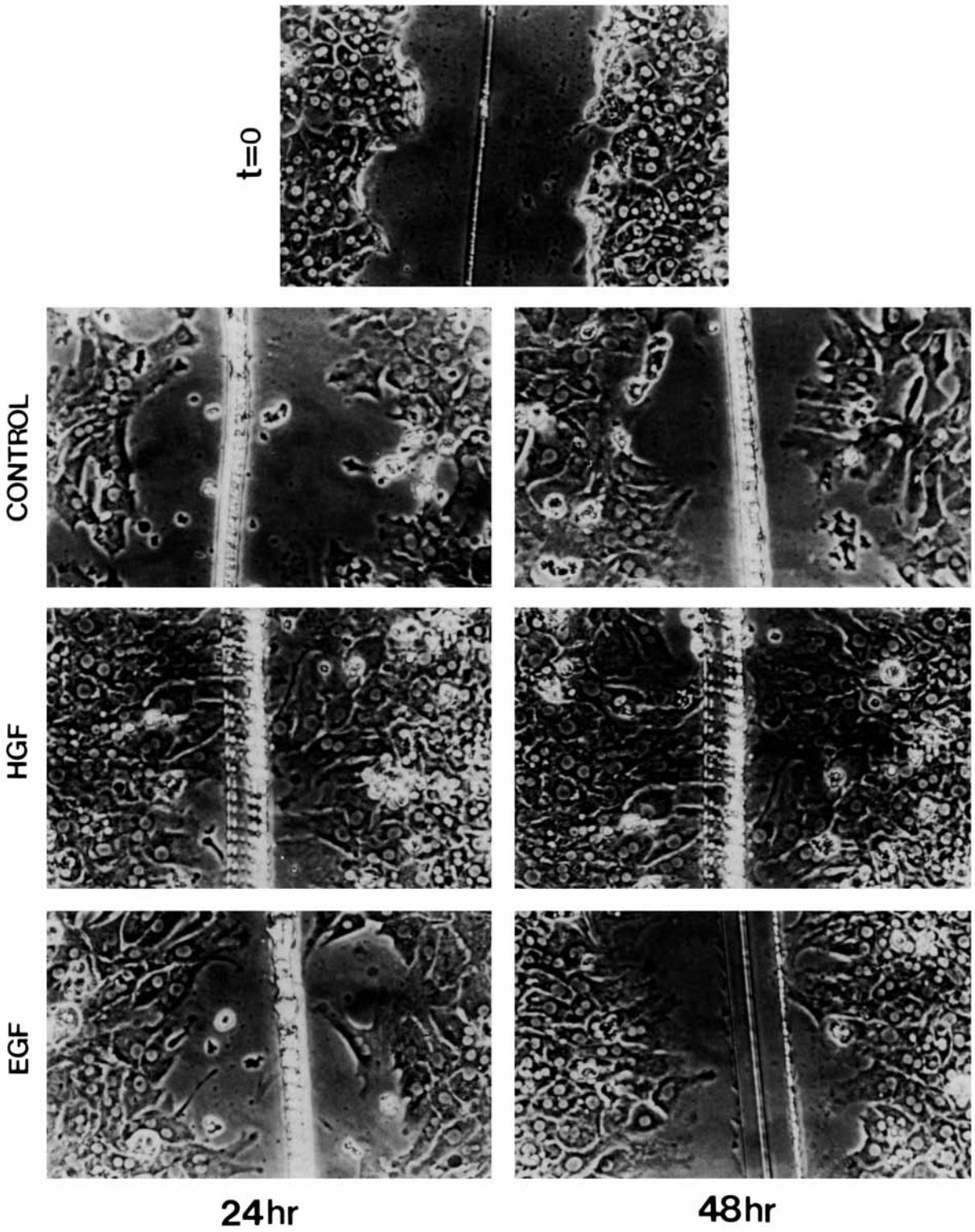

Fig. 4. Wound-healing migration of HGF- and EGF-treated monolayers. Approximately 400 μm wide wounds were prepared on confluent monolayers of hepatocytes as described in Methods and Materials. Micrographs were taken at 24 and 48 hr postwounding and growth factor addition ($t = 0$). HGF consistently induced the migration of hepatocytes to fill the denuded space, faster and more completely than EGF-treated cells. However, EGF appears to reorganize the confluent cells distal to the wound into a more compact epithelial-like morphology, especially at 48 hr, than HGF-treated cells.

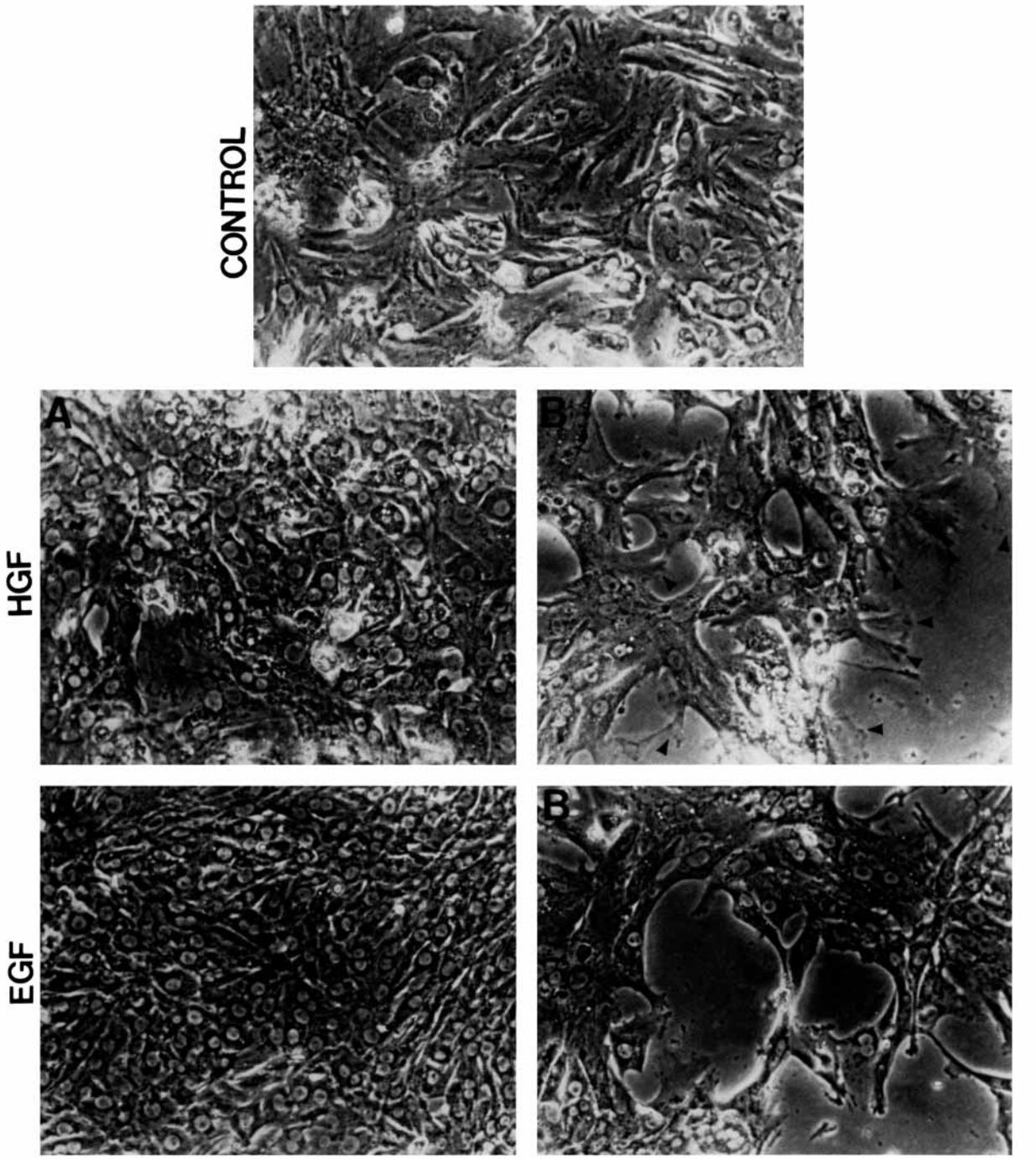

Fig. 5. Morphological effects of HGF and EGF on confluent and near confluent hepatocyte monolayers. Rat hepatocytes were seeded onto collagen-coated plates at very high, near confluent density ($5–6 \times 10^4$ cells/cm^2). Cells were then stimulated with 50 ng/ml HGF or EGF for 48 hr, after which micrographs were taken of cells within confluent areas as well as boundary areas of near confluency. Under confluent conditions, EGF (**EGF, A**) profoundly reorganizes the cells to resemble an ordered, compact epithelial-like monolayer, exhibiting well defined cell–cell contacts. HGF administration (**HGF, A**), on the other hand, appears to disorganize the monolayer and result in multilayering of hepatocytes exhibiting ill defined cell–cell contacts. Control cells display no apparent cell–cell contacts. At areas bordering near confluency, hepatocytes stimulated with HGF (**HGF, B**) express extensive flattened lamellipodia (arrowheads), while EGF-treated cells (**EGF, B**) do not.

dia (HGF, Fig. 5B), while EGF-treated cells lack extensive expression of such flattened lamellipodia (EGF, Fig. 5B). Increased flatness (Table I) as well as increased expression of flattened, leading-edge lamellipodia (Figs. 2, 5) are found to be coincident with increased motility of cells treated with HGF/SF [Li et al., 1992; Bhargava et al., 1993]. Since primary cultured hepatocytes exhibit a heterogeneous range of sizes and extent of nucleation, the large increases in cell area (twofold) seen by Li and colleagues [1992] for more size-homogeneous populations (e.g., MDCK cells) are diluted in heterogeneous hepatocyte cultures. However, the differences elicited by HGF and EGF strongly indicate different signal transduction cascades may be responsible for the morphological, mitogenic, and motility variations seen in hepatocyte cultures challenged with either cytokine.

Signal Transduction

In an attempt to examine if HGF and EGF transduce different signals to downstream targets within the cytoskeleton, cytoskeletal extracts of ^{32}P-loaded rat hepatocytes were prepared from HGF- and EGF-stimulated cells, then examined using large format 2-D gels. The results displayed in Figure 6 show that two proteins, p28 and p42, undergo differential phosphorylation in response to HGF or EGF. Additionally, actin rapidly became phosphorylated under a similar time course for both mitogens (Figs. 6, 7, 9). By comparing the F-actin with the G-actin in the Triton X-100 soluble fraction, phosphoactin was associated only with the cytoskeletal extract (Fig. 7), where it became rapidly phosphorylated upon either growth factor stimulation. The accompanying supernatants, also run on quantitative 2-D gels and silver stained, show that very little actin is present in the rat hepatocyte noncytoskeletal supernatant (Fig. 7), and a phosphorylated isoform was not detected in this fraction, even when gels were exposed to film for extended amounts of time (data not shown). Equal amounts of cytoskeletal and supernatant proteins were immunoprobed on blots, and 85% of the actin was found in the cytoskeletal pellet (data not shown). Figure 8 indicates the average fold increases over control of five proteins—p28, p42, two actin isoforms, and a control, nonstimulated phosphoprotein, p50—examined in quantitative 2-D autoradiographs of hepatocyte cytoskeletons.

To determine if the phosphoprotein that comigrated with actin was indeed phosphoactin, cytoskeletal extracts were solubilized and immunoprecipitated with a polyclonal anti-actin antibody. Figure 9 shows that the actin precipitated from the cytoskeletal extracts and immunodetected with anti-actin monoclonal antibody (Fig. 9A) was phosphorylated only when either growth factor was added to the cells (Fig. 9B). When this blot was stripped and reprobed with anti-phosphotyrosine antibodies, only the phosphorylated band in Figure 9A and B reacted positively to this antibody, suggesting tyrosine phosphorylation of actin when hepatocytes were stimulated with either HGF or EGF. Additional information was obtained by exposing the actin blot to 1 M NaOH at 40°C for 1 hr [Cheng and Chen, 1981] to hydrolyze phosphoserine and phosphothreonine residues (Fig. 9D). Under these conditions the actin band remained phosphorylated. Tyrosine phosphorylation of actin has recently been described to occur as the result of shape changes in *Dictyostelium discoidium* coincident with growth [Schweiger et al., 1992; Howard et al., 1993]. This is a novel finding in mammalian cells. Given that actin is highly conserved throughout nature, it may represent a parallel event connecting the motogenic and mitogenic properties of HGF and EGF on hepatocytes.

Finally we were interested to determine if the highly phosphorylated protein "p42" rapidly stimulated by HGF and not EGF was MAP kinase. MAP kinase is a downstream convergence point of many growth factor receptor tyrosine kinase signal transduction pathways [Thomas, 1992], and has been shown to be rapidly activated by both EGF [Rossomando et al., 1989] and HGF [Halaban et al., 1993; Faletto et al., 1993] in different cell types. Figure 10 shows the time course of MAP kinase phosphorylation stimulated by EGF and HGF in cytoskeletal and noncytoskeletal fractions. This phosphorylation time course is very different from the phosphorylation events of p42 shown in Figure 6 in that HGF and EGF stimulate MAP kinase nearly identically in Figure 10. MAP kinase assays were also performed on whole-cell lysates as described in Methods and Materials. After 10 min stimulation with either growth factor, hepatocyte extracts facilitated a similar two- to threefold increase of ^{32}P-radiolabeled acceptor peptide for HGF- or EGF-treated cells (data not shown). This increase is similar to values re-

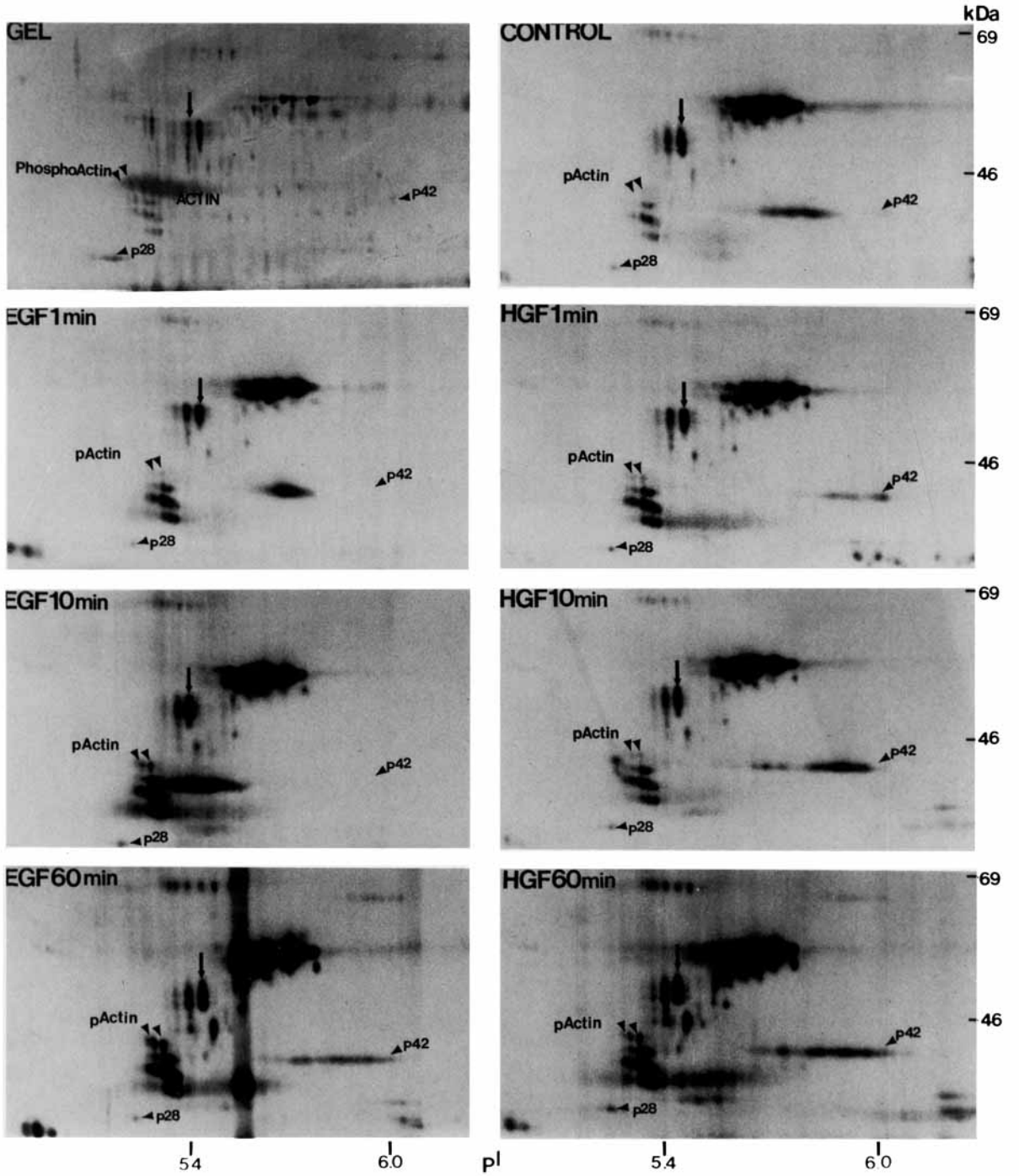

Fig. 6. Two-dimensional gel analysis of phosphorylated cytoskeletal proteins from rat hepatocytes stimulated with 50 ng/ml HGF or EGF for various amounts of time. Serum-starved rat hepatocytes were stimulated for the times indicated and their cytoskeletons were prepared. The positions of phosphoactin (pActin, double arrowheads), p42, and p28 are indicated on the gel and autoradiographs. p28 and p42 are two proteins that rapidly become phosphorylated with HGF and not EGF. Once normalized for protein loading (see Fig. 8), p42 becomes phosphorylated 20-fold over controls within 1 min and over 50-fold in 1 hr; while p42 eventually becomes phosphorylated with EGF stimulation, it takes between 10 min and 1 hr and becomes phosphorylated 20-fold over controls. p28 becomes phosphorylated fivefold over controls in 1 hr with HGF, while EGF causes an overall decrease in the phosphorylation of this protein. Both growth factors stimulate the phosphorylation of actin (double arrowheads) within 1 min. The position of a protein (mwt ~ 50 kDa) that does not undergo appreciable changes *in phosphorylation* is marked with a thick, vertical arrow. The panels displayed above are from 60 hr exposures, but are representative of three different experiments. One hundred micrograms of protein was loaded onto each first dimension focusing gel. Gels were then silver stained, dried between sheets of dialysis paper, then subjected to autoradiography (see also Fig. 8).

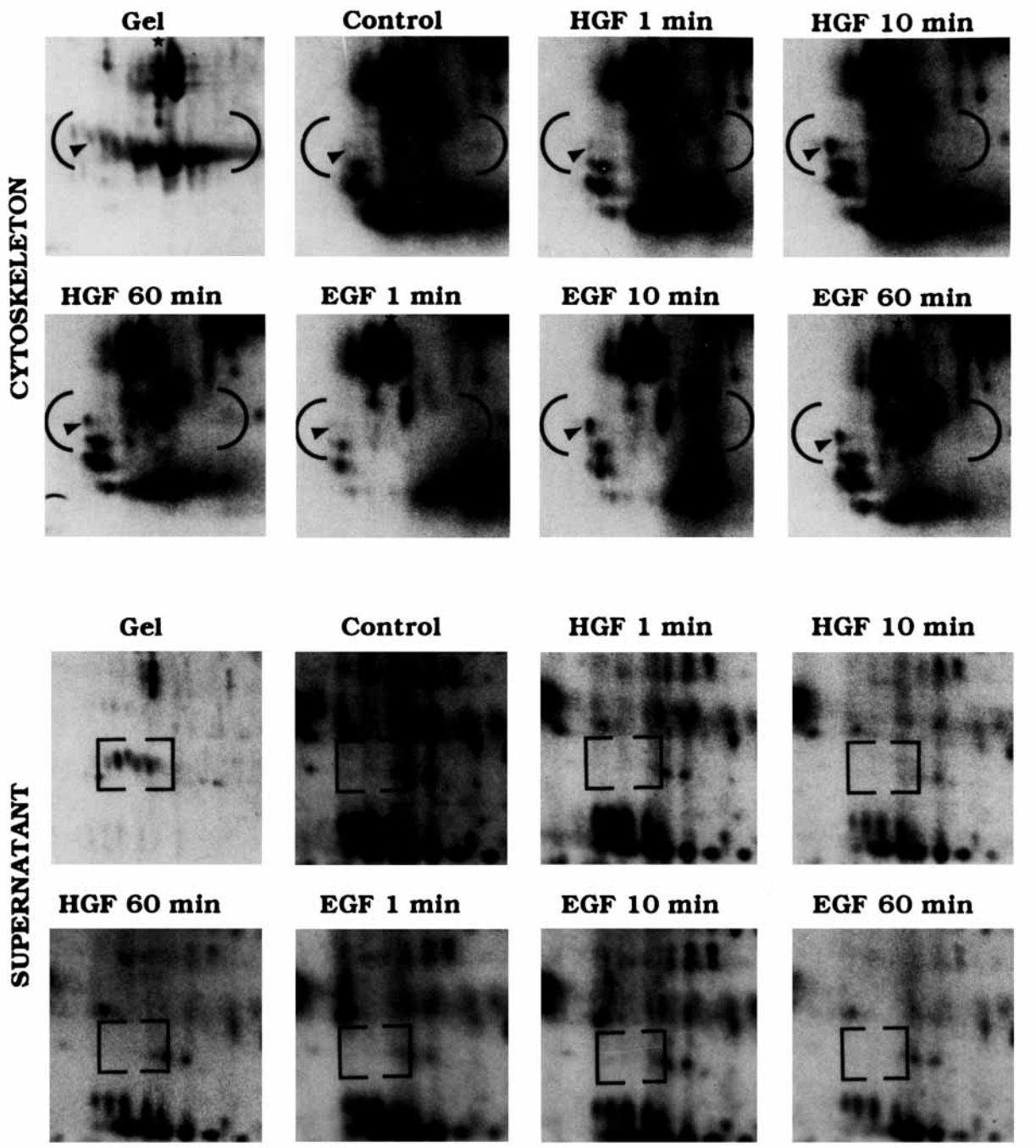

Fig. 7. Two-dimensional gel analysis of cytoskeletal-associated phosphoactin. Triton X-100 detergent resistant (cytoskeleton) and soluble (supernatant) fractions from control and HGF- or EGF-stimulated cells were analyzed for phosphoactin content. As early as 10 min, acidic isoforms of actin become phosphorylated in the cytoskeletal pellet (arrowhead, inside round brackets), while in the supernatant, G-actin (inside square brackets) does not become phosphorylated, even at 60 min. The star indicates p50, a protein whose phosphorylation does not change in response to either growth factor (see also Fig. 6, vertical arrow). The silver-stained gel representing the autoradiographs is labeled first in each series. One hundred micrograms of protein was loaded onto each large format first dimension focusing gel. Autoradiographs are 60 hr exposures.

ported using similar techniques on different cell lines [Ray and Sturgill, 1987].

In addition to the differences in phosphorylation kinetics, the position of MAP kinase in a 2-D gel is expected to be basically distal to the site at which p42 is located. p42 displays a pI of ~6.0 on the 2-D gels shown in Figure 6, but the literature pI value for MAP kinase is a more neutral 6.8–7.0 [Rossomando et al., 1989; Cooper, 1989]. Immunoblots of large format 2-D

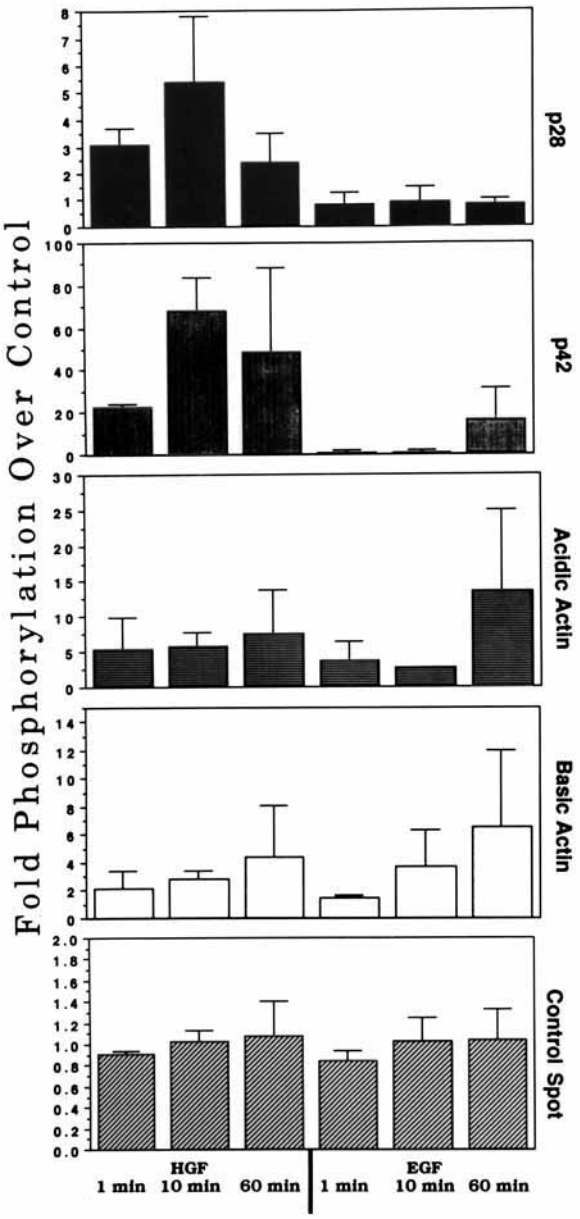

Fig. 8. Quantification of p28, p42, phosphoactin, and p50 signals in ^{32}P-labeled cytoskeletal extracts. Silver-stained gels of all the time points shown in Figure 6 were scanned and analyzed using the BioImage scanning densitometer equipped with the Investigator software package (Millipore). Loading ratios were determined for each silver-stained gel by comparing all the spots from proteins resolved in each of the stimulated samples to that of the control gel. Since similar protein amounts were loaded onto each first dimension (as determined by bicinchoninic acid protein quantitation), the variation was seen to be minimal (about 10% difference in total protein load among the seven gels in the set). These normalization values were then applied to the integrated optical densities obtained for each of the spots evaluated in the accompanying autorads. The values shown in each of the panels below are the average ± SD of three separate experiments. Acidic actin is the left phosphoactin isoform and basic actin is the right phosphoactin isoform shown in Figure 6. p50 is the control spot at 50 kDa whose signal does not change appreciably, and is marked with the thick vertical arrow in Figure 6, and a star in Figure 7.

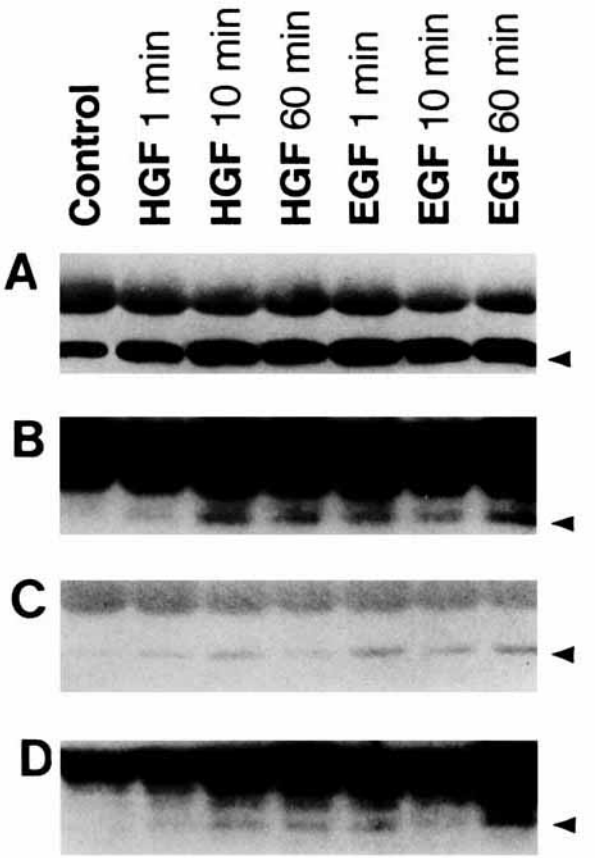

Fig. 9. Immunoprecipitation of phosphoactin from the cytoskeleton after stimulation with HGF or EGF. ^{32}P-labeled cytoskeletal fractions were obtained and immunoprecipitated as described in Methods and Materials. A rabbit polyclonal to actin was used. Immunoprecipitated proteins were resolved on 10% discontinuous 1-D minigels and blotted to Immobilon-P, PVDF membrane. These blots were then probed for actin (monoclonal #MAB1501, Chemicon) to assure equal loading and position on the blot. **A** shows that actin was precipitated from the Triton X-100 resistant supernatant. **B** shows the autoradiographic exposure of A, indicating that the precipitated actin is phosphorylated. **C** shows the blot from A reprobed with an anti-phosphotyrosine antibody, indicating that the actin from HGF- and EGF-stimulated cells is phosphorylated on tyrosine. **D** is the autoradiographic exposure of B after alkali hydrolysis, further indicating that actin is phosphorylated on tyrosine. The large spot at 50 kDa in A and C is the IgG heavy chain from the precipitation crossreacting with the 2° antibody during the immunoblot. The large spot in B and D at 50 kDa is a phosphoprotein that coprecipitates with all cytoskeletal fractions, regardless of the primary antibodies used. It does not undergo large changes in phosphorylation (see also Fig. 10E,F). Arrows indicate actin band location.

gels did not show crossreactivity of p42 with the anti-MAP kinase antibodies used in *our study* (data not shown). Our data show that four isoforms of MAP kinase are seen to exist in the rat hepatocyte; two that become phosphorylated but remain in the supernatant and, interestingly, two isoforms of MAP kinase which appear to

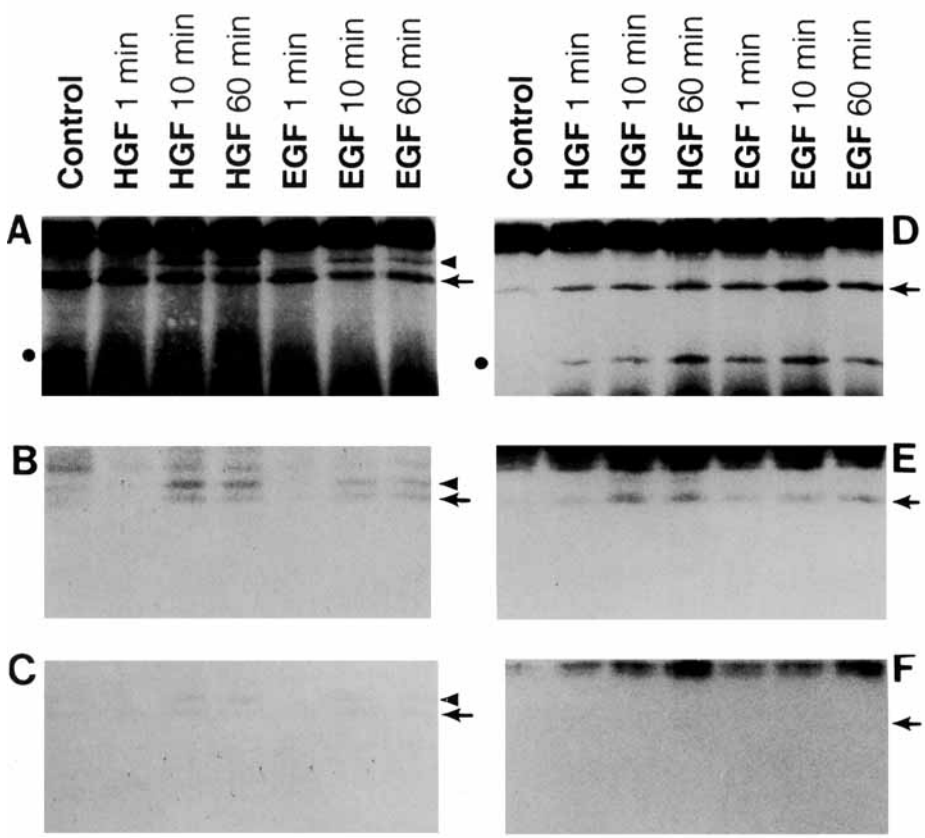

Fig. 10. Immunoprecipitation of MAP kinase from the cytoskeletal and the noncytoskeletal extracts after stimulation with HGF or EGF. Lysate fractions were obtained and immunoprecipitated as described in Methods and Materials. An equal mixture of rabbit polyclonal to rat MAP kinase (erk 1-CT, UBI, Lake Placid) and monoclonal to MAP kinase (MK12, Biodesign, Kennebunkport, ME) was used as the primary antibody preparation. Immunoprecipitated proteins were resolved on 10% discontinuous 1-D minigels and blotted to Immobilon-P. These blots were then probed for MAP kinase using the same antibody preparation described above to assure equal loading and position on the blot. **A** shows that MAP kinase was precipitated from the supernatant first as a low molecular form of about 43 kDa (arrow). Upon stimulation with either HGF or EGF, a dimer appears of molecular weights 43 and 44 kDa (arrowhead) at 10 min. **B** shows the autoradiographic exposure of A. The control MAP kinase is not phosphorylated, but the subsequent dimers become phosphorylated at the 10 min time point for both HGF and EGF. **C** shows the autoradiographic exposure of B after alkali hydrolysis, indicating that the phosphorylated dimers seen in B are probably phosphorylated on tyrosine. **D** shows the immunoprecipitation of MAP kinase from the cytoskeleton. It is precipitated as a single band of only the lower molecular weight isoform (43 kDa). It also appears that the MAP kinase quickly translocates to the cytoskeletal fraction of the preparation, since it is not present in control cytoskeleton. **E** is the autoradiographic exposure of D and indicates that at the time points of 1, 10, and 60 min after HGF or EGF stimulation, only the 43 kDa MAP kinase isoform becomes phosphorylated in the cytoskeletal fraction. **F** is the autoradiographic exposure of E after treatment with 1 N NaOH. The signal is completely hydrolysed, indicating that the MAP kinase which translocates to the Triton X-100 resistant phase is probably phosphorylated on serine and/or threonine. Interestingly, a 30 kDa protein (dot) that crossreacts with the MAP kinase antibody mixture in the chemiluminescence immunoblots completely translocates to the cytoskeletal fraction after HGF or EGF stimulation. This protein, unlike the other MAP kinase isoforms, does not become phosphorylated and has not yet been characterized. The large spot at 50 kDa in A and D is the IgG heavy chain from the precipitation crossreacting with the 2° antibody during the immunoblot. The large spot in E and F at 50 kDa is a phosphoprotein that coprecipitates with all cytoskeletal fractions, regardless of the primary antibodies used (see also Fig. 9B,D).

rapidly translocate to the Triton X-100 insoluble fraction upon HGF or EGF stimulation. One isoform at 43 kDa is phosphorylated rapidly and translocates from the supernatant to the Triton insoluble pellet within 1 min. The other isoform is about 30 kDa, also rapidly translocates to the pellet fraction from the supernatant, but is not observed to become phosphorylated. The translocation may be to the cytoskeleton or, more likely, the nucleus, since most Triton X-100 resistant fractions also enrich for the nucleus. By determining enrichment for DNA [Stolz and Jacobson, 1992], we find that DNA, and therefore the nucleus, is enriched sevenfold in cytoskeletal extracts over whole-cell lysates. Using other cell systems, others have demonstrated that MAP kinase isoforms translocate to the nucleus within 10 min

of mitogen stimulation [Chen et al., 1992; Sanghera et al., 1992]. Gonzales and colleagues have found that multiple isoforms of MAP kinase exist and are expressed differentially in human tissues, but their subcellular location and phosphorylation kinetics were not determined [Gonzales et al., 1992]. Further investigation into the nature of these four isoforms will be necessary to determine their roles in HGF and EGF signal transduction.

DISCUSSION

The results obtained from this study strongly indicate that both HGF and EGF are powerful mitogens, motogens, and morphogens for rat hepatocytes in vitro. However, each cytokine appears to act via different mechanisms. This suggests that the unique features manifested by each growth factor on primary rat hepatocytes may dictate information to different signal transduction pathways or elements, particularly in the cytoskeleton. Here we take the initial steps to decipher the differences seen in the mitogenic, motogenic, and morphogenic effects elicited by HGF and EGF by examining signal transduction to the cytoskeleton. HGF and EGF are frequently described in the literature as mitogens, but this describes only a small aspect of their total function.

Before its structure was determined to be identical to that of HGF, scatter factor was known as a potent motogen and morphogen for epithelial cells [Weidner et al., 1990; Rosen et al., 1990a; Stoker and Gherardi, 1991]. HGF was recently shown to have motogenic and mitogenic effects on endothelial cells [Bussolino et al., 1992; Grant et al., 1993], human keratinocytes [Matsumoto et al., 1991; Tajima et al., 1992], and human gastric adenocarcinoma cells [Shibamoto et al., 1992]. EGF, though described primarily as a mitogen, stimulates migration in rat intestinal epithelium [Blay and Brown, 1985] and migration and mitosis in keratinocyte cultures [Barrandon and Green, 1987]. Using three different motility assays, paired with DNA synthesis assays, we observe that both EGF and HGF are simultaneously motogenic and mitogenic for rat hepatocytes in vitro. However, the characteristics of the locomotion and morphogenic changes that accompany the movement are unique to the particular cytokine. HGF shows greater motogenicity than EGF when stimulated to migrate from microcarriers, across porous membranes, or into denuded areas of confluent monolayers. HGF also causes increased flatness by increasing cell spreading onto the collagen substratum, indicating greater motility [Li et al., 1992]. EGF, on the other hand, does elicit a motogenic response, although it is weaker than that of HGF. Since the extreme, flattened lamellipodia are not present on EGF-treated cells, the mechanisms by which the two motogens operate are probably different. Interestingly, in areas of near confluent density, HGF and EGF exert different morphogenic effects on hepatocyte monolayers. While EGF stimulates a greater mitogenic response than HGF under these conditions, it also facilitates the formation of a highly ordered, closely opposed monolayer, with distinct cell–cell contact. Conversely, HGF causes a less ordered, multilayered monolayer, with less defined areas of cell–cell contact and increased cell size, reminiscent of colony scattering. The observations described above strongly suggested that EGF and HGF did not stimulate a redundant chain of events in rat hepatocytes.

By examining the signal transduction events in cytoskeletal extracts from hepatocytes stimulated with either HGF or EGF, we have identified two proteins, p42 and p28, that become differentially rapidly phosphorylated by HGF and not EGF. This suggests that HGF and EGF transduce cytokine-specific downstream signals to unique cytoskeletally associated proteins as a means to different morphogenic, mitogenic, and motogenic ends.

The mechanisms by which growth factor receptor tyrosine kinases (RTK) regulate proliferation and/or motogenesis on target cells has been the subject of intense research over the past decade. Recently, linear amino acid sequences of SH2 (src-homology 2) motifs in the cytoplasmic targets of specific activated RTKs have predicted and revealed protein–protein interactions among a wide variety of downstream, cytosolic-signaling proteins [Koch et al., 1991; Cantley et al., 1991; Schlessinger and Ullrich, 1992; Pawson and Gish, 1992; Songyang et al., 1993]. Phospholipase Cγ (PLCγ), phoshotidylinositol 3-kinase (PI3K), *src*-related tyrosine kinases $p59^{fyn}$, as well as GRB2 and *ras*GAP, are among the downstream signal transducing proteins that have been shown to bind directly to specific linear sequences surrounding the phosphorylated tyrosine(s) of RTKs [Cantley et al., 1991; Songyang et al., 1993]. The HGF receptor c-*met* [Comoglio, 1993; Bardelli et al., 1992; Graziani et al., 1991] and the EGF receptor [Margolis et

al., 1990a,b; Yang et al., 1991; Lowenstein et al., 1992] have been shown to bind and/or phosphorylate one or more of these signal transducers in vitro. It is interesting to note that c-*met* has been shown to possess a consensus PI3K binding domain, but the EGF receptor has not [Cantley et al., 1991]. PI3K has recently been shown to coimmunoprecipitate with the tyrosine-phosphorylated HGF receptor [Graziani et al., 1991]. Although EGF has been observed to activate PI3K [Carter and Downes, 1992], it was not demonstrated that direct interaction between the two molecules exists. Thus, the direct binding and not activation alone may represent an important bifurcation in the signal transduction pathways between HGF and EGF. PI3K has also been implicated in actin filament rearrangement [Cantley et al., 1991] and this may suggest a link to HGF's well documented motogenic properties in other cell systems.

SH3 (src-homology 3) regions have also been implicated as signal transduction elements. They are also present on some cytoskeletally associated proteins like α spectrin [Musacchio et al., 1992]. Spectrin as well as another cytoskeletal protein, ezrin, have been shown to be downstream phosphorylation targets of the EGF receptor in A431 cells [Bretscher, 1989]. SH3 domains have also been implicated in membrane–cytoskeletal interactions [Drubin et al., 1990] although no direct evidence for this has been published. The small GTP binding proteins *rac* and *rho* have also been implicated in growth factor–induced cytoskeletal events of membrane ruffling [Ridley et al., 1992], focal adhesion assembly, and stress fiber formation [Ridley and Hall, 1992], but their exact role in the cytoskeletal signal transduction cascade remains to be elucidated. The sum of the data described above strongly suggests that growth factor–activated RTKs may physically bind specific cytoskeletally associated proteins, activating them via phosphorylation, leading ultimately to cytoskeletal remodeling.

Profilin represents yet another enticing connection between the RTKs and cytoskeletal signal transduction [Forscher, 1989; Haarer and Brown, 1990; Hoffman, 1991]. Profilin is a basic, 12–15 kDa protein that interacts with PIP2 (phosphotidylinositol 4,5-bisphosphate) [Lassing and Lindberg, 1985] and protects it from hydrolysis by PLCγ. Upon RTK stimulation with growth factors, subsequent activation of PLCγ via G-proteins results in PIP2 hydrolysis to IP3 (inositol 1,4,5-trisphosphate) and diacylglycerol (DAG). DAG can then activate protein kinase C (PKC) and IP3 translocates to the cytosol to release Ca^{2+} from intracellular stores [Irvine, 1992]. HGF and EGF have been shown to activate inositol breakdown and Ca^{2+} release in a variety of cells [Baffy et al., 1992; Kaneko et al., 1992; Osada et al., 1992a,b; Cunningham et al., 1989], resulting in negative regulation of c-*met* [Gandino et al., 1990, 1991] and the EGF receptor [Thompson et al., 1989], although concomitant activation of PKC has not been proven for all cell types. As profilin is released from the membrane, it subsequently binds to G-actin monomers, resulting in dynamic reorganization and elongation of the microfilament-based cytoskeleton at the barbed end of actin filaments [Pring et al., 1992; Goldschmidt-Clermont and Janmey, 1991; Aderem, 1992]. However, profilin, as well as actin, appears to be present in low amounts in normal liver [Buß and Jockusch, 1989], suggesting it may play a limited role in the transduction of signals to the microfilament cytoskeleton via RTKs in normal liver. Conversely, regenerating hepatocytes may upregulate the presence of these proteins in order to facilitate an enhanced proliferative response. Both EGF [Cunningham et al., 1989] and HGF [Graziani et al., 1991] have been shown to facilitate phosphotidylinositol breakdown, suggesting that profilin may provide a viable link from the RTK to the microfilament cytoskeleton signal transduction cascade during hepatocyte proliferation. This mode of action also needs to be addressed in future studies.

Finally, actin itself has been implicated in the direct binding of the EGF receptor [den Hartigh et al., 1992; Roy et al., 1991], liver cell membranes [Tranter et al., 1989], as well as the target of a tyrosine kinase in *D. discoidium's* cell shape response to changes in growth medium [Schweiger et al., 1992; Howard et al., 1993] or threonine phosphorylation by actin kinase in *Physarum polycephalum* [Furuhashi et al., 1992]. The tyrosine phosphorylation of a minor actin isoform in response to HGF and EGF stimulation may provide a link between the pleiotropic effects of mitogenicity and motogenicity by EGF and HGF on rat hepatocytes in vitro, and may also represent yet another link to the involvement of profilin. Experiments are currently underway to determine the mechanisms by which actin becomes phosphorylated and what role this phosphorylation may play in

HGF and EGF signal transduction in hepatocytes.

The information summarized above shows for the first time that HGF and EGF are both motogenic and mitogenic for hepatocytes in vitro. HGF and EGF also exert unique morphogenic effects of hepatocytes at both high and low density. Additionally, rapid phosphorylation of cytoskeletally associated proteins p28 and p42 by HGF and not EGF may represent an early bifurcation signal responsible for the morphogenic, mitogenic, and motility differences seen between HGF and EGF. The rapid phosphorylation of a minor actin isoform within the hepatocyte cytoskeleton is an important finding, suggesting that the cytoskeleton is indeed a viable target for signal transduction. Given that each growth factor stimulates all downstream signal transduction events via its own unique protein tyrosine kinase receptor, our data suggests that HGF's or EGF's unique density-dependent DNA synthesis, motility, morphology, and cytoskeletal protein phosphorylation occur downstream of the initial receptor tyrosine phosphorylation. Redundant signal transduction events are also readily apparent, since both HGF and EGF stimulate MAP kinase and actin phosphorylation with similar amplitude and temporal kinetics. The diversity of potential RTK associations and subsequent phosphorylation cascades among various growth factor RTKs has been postulated previously by Cantley and colleagues [Cantley et al., 1991; Songyang et al., 1993]. Here we show that HGF and EGF do indeed stimulate redundant as well as divergent signal transduction events. These observations may elucidate the separate, distinct roles HGF and EGF may play in liver regeneration.

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