Epidermal Growth Factor-induced Selective Phosphorylation of Cultured Rat Hepatocyte 55-kD Cytokeratin before Filament Reorganization and DNA Synthesis

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Abstract. We have reported previously that the addition of dexamethasone to cultured quiescent suckling rat hepatocytes in the presence of insulin, a culture condition which does not cause growth activation, induces a selective increase in the synthesis of the 49-kD/55-kD cytokeratin (CK49/CK55) pair over a 24-h period. This increased synthesis coincides with the formation of dense filament networks reminiscent of those observed in situ at the cell periphery (Marceau, N., H. Baribault, and I. Leroux-Nicollet. 1985. *Can. J. Biochem. Cell Biol.* 63:448–457). We show here for the first time that when EGF is added 48 h after insulin and dexamethasone, there is an early preferential phosphorylation of the CK55 of the CK49/CK55 pair, an induced filament rearrangement from the cell periphery to the cytoplasm, and a subsequent entry into S phase and mitosis after a lag period of 8 h. Indirect immunofluorescence microscopy with monoclonal antibodies to CK49 and CK55 indicate that, while before EGF treatment the cytokeratin filaments were mainly distributed near the cell periphery, the addition of EGF resulted in their reorganization to a predominantly cytoplasmic localization within <3 h. Anti-tubulin and anti-actin antibodies showed no detectable alteration in the distribution of microtubules and microfilaments. Pulse-chase measurements with [^{35}S]methionine showed no apparent change in the turnover of either CK49 or CK55 during the period that precedes the initiation of DNA synthesis. ^{32}P-labeling in vivo followed by SDS-PAGE demonstrated that CK55 was phosphorylated at a much higher level than CK49 in nonstimulated hepatocytes, and that the addition of EGF resulted in a selective stimulation of ^{32}P-CK55 labeling within <30 min. Comparative analyses by two-dimensional PAGE of [^{35}S]methionine and ^{32}P-labeled cytokeratins at various times after EGF stimulation demonstrated a rapid increase in a first phosphorylated form of CK55 and the appearance of a second phosphorylated form at 30 min poststimulation. The changes in the relative proportion of non-phosphorylated and phosphorylated forms were confirmed by immunoblotting with the anti-CK55 monoclonal antibody. Determinations of the ^{32}P-labeled phosphoamino acids of CK55 extracted from the gels demonstrated that the radioactivity was mostly in serine residues. Labeling of Triton-permeabilized hepatocytes with γ^{32}P-ATP after treatment with EGF for 30 min to 3 h at 37°C, also demonstrated a phosphorylation of CK55 and CK49 as well, implying that the EGF-responsive serine protein kinase is detergent insoluble and probably part of the surface membrane skeleton. We propose that early selective CK55 phosphorylation at serine residues and subsequent rearrangements of CK49/CK55 filaments from cell periphery to cytoplasm are EGF receptor driven cell surface reactions that are part of the cascade of events that culminate in the initiation of DNA synthesis and subsequent cell division of hepatocytes.

EPIDERMAL growth factor exerts profound effects on many cellular activities. Ever since the discovery by Cohen (1962, 1965) that EGF induces hyperkeratatic epidermis in vivo and in vitro, a wealth of studies have been performed in several laboratories to examine its mitogenic action not only on keratinocytes, the specialized epithelial cells of the epidermis (Green, 1977), but also on cells of various tissue origins, including simple epithelia (Buhrow et al., 1983; Carpenter et al., 1978; Chinkers et al., 1981; Delize et al., 1986; Hunter and Cooper, 1981; Haigler et al., 1978; Schlessinger and Geiger, 1981; Willingham et al., 1983; for review see Carpenter and Zendegui, 1986). The liver is a genuine representative of these epithelial tissues, and it is well-established that EGF is a potent growth promoting factor of rat hepatocytes both in vivo (Bucher et al., 1978; Bucher and McGowan, 1979; Earp and O'Keefe, 1981) and in primary culture (McGowan, 1986). In this regard, we have previously demonstrated a differential responsiveness

of cultured suckling and adult rat hepatocytes to EGF, which seems to be linked to their different states of differentiation (Baribault et al., 1985). Indeed, the differentiation pattern of hepatocytes is developmentally regulated (Guertin et al., 1983; Powell et al., 1984), and on the basis of the differential expression of AFP[1] and albumin, suckling rat hepatocytes are a homogenous population of differentiating epithelial cells (Marceau et al., 1986*b*). These cells can be readily set up in primary culture and when seeded in serum-free medium containing insulin and dexamethasone, they respond to EGF by entering into S phase in a synchronous manner after a lag period of 24 h (Baribault et al., 1985; Baribault and Marceau, 1986). Cultured suckling rat hepatocytes therefore offer a reliable in vitro model to examine the molecular events related to the action of EGF on nonkeratinizing epithelial cells.

Among the many changes generated by EGF after binding to its receptor (EGF-R) is the stimulated phosphorylation of several cellular proteins (Hunter and Cooper, 1981; for review, see Carpenter and Zendegui, 1986). An in vitro study on the endogenous phosphorylation of epidermal proteins demonstrated that EGF rapidly and predominantly stimulates the phosphorylation of cytokeratins (Aoyagi et al., 1983), the major differentiation gene products of keratinocytes (Sun et al., 1984). Interestingly, cytokeratins form the intermediate filaments (IFs), not only of keratinizing, but also nonkeratinized epithelial cells (Osborn and Weber, 1982; Franke et al., 1982; Lazarides, 1982; Kim et al., 1984; Sun et al., 1984). Cytokeratins are encoded by a multigene family of some 20 proteins which can be subdivided into two distinct classes, type I and type II (Fuchs et al., 1981; Moll et al., 1982; Fuchs and Marchuk, 1983; Sun et al., 1984; Kim et al., 1984). These two types are normally synthesized in pairs, subsets of which are differentially expressed in epithelial cell types of distinct lineages and differentiation status (Moll et al., 1982; Sun et al., 1984). Cytokeratin IFs are assembled from tetrameric complexes containing two molecules of each type (Quinlan et al., 1984). It is therefore of major interest to examine the effects of EGF on the phosphorylation status of cytokeratins of cultured simple epithelial cells.

Rat hepatocytes contain two cytokeratins of 49 and 55 kD, named here CK49 and CK55,[2] respectively (Franke et al., 1981; Marceau et al., 1983, 1986*b*). Previous work from our laboratory has indicated that dexamethasone in the presence of insulin induces a transient and selective increase in CK55 and CK49 synthesis during the first 24 h, which coincides with the formation of an IF network reminiscent of the filament arrangement in normal rat liver (Marceau et al., 1985). In the present study, neonatal rat hepatocytes were first exposed to insulin and dexamethasone for 48 h and then treated with EGF to assess their cellular response, with respect to the initiation of DNA synthesis and the modifications of IF organization, and cytokeratin metobolic stability and phosphorylation. The results show that the addition of EGF causes the initiation of DNA synthesis after a lag period of 8 h, a prominent rearrangement of CK49/CK55 IFs within 3 h, and an early preferential increase in ^{32}P-labeling of CK55 on serine residues within 30 min in the absence of significant change in the degradation of CK49 and CK55. γ-^{32}P-ATP labeling of EGF-treated, Triton-permeabilized hepatocytes suggests that the EGF-responsive serine protein kinase is part of the surface membrane skeleton. We propose that early selective CK55 phosphorylation and subsequent CK49/CK55 filament rearrangement are part of the cascade of events that culminate in the initiation of DNA synthesis in EGF-stimulated hepatocytes.

1. *Abbreviations used in this paper*: AFP, alpha-fetoprotein; CK49 and CK55, Cytokeratins of 49 and 55 kD; EGF-R, epidermal growth factor receptor; IF, intermediate filament.

2. CK55 and CK49 are the rat orthologues of human cytokeratins 8 and 18, respectively (Moll et al., 1982).

Materials and Methods

Materials

Fischer 344 rats were obtained from Charles River Breeding Laboratories, Inc. (Willington, MA). Experiments were performed on 14-d-old rats. The suckling rats remained with their mother until 1 h before they were killed. Collagenase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). The alpha modification of MEM was obtained from Gibco (Burlington, Ontario). Insulin was purchased from Calbiochem-Behring Corp. (La Jolla, CA). EGF was purified from mouse submaxillary glands according to an established procedure (Savage and Cohen, 1972). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Plastic dishes (100 mm, No. 5221; 60 mm, No. 3002; 35 mm, No. 3001) were from Falcon Labware (Montreal, Quebec), and tissue culture multiwell plates were from Flow Laboratories, Inc. (McLean, VA). ^{3}H-TdR 67.7 Ci/mMol [^{35}S]methionine, 1,000 Ci/mMol, ^{32}P-orthophosphoric acid (285 mCi/mg), and γ-^{32}P-ATP (3,000 Ci/mMol) were purchased from New England Nuclear (Montreal, Quebec).

Liver Cell Isolation and Hepatocyte Culture

Cells were isolated by the two-step collagenase perfusion method (Seglen, 1976) modified for adult and preweanling rats (Deschenes et al., 1980). The preparations contained at least 96% hepatocytes, the remaining 4% being endothelial and Kupffer cells (Baribault et al., 1985; Baribault and Marceau, 1986).

Cells were plated on fibronectin-coated 60- or 35-mm dishes in serum-free α-MEM containing streptomycin (0.1 mg/ml) and penicillin (200 U/ml) at 37°C in a 95% air–5% CO_2 atmosphere. The medium was changed 2 h after seeding and the attached cells were then cultured in serum-free medium supplemented with combinations of EGF (10 ng/ml), insulin (150 ng/ml), and dexamethasone (1 μM).

DNA Synthesis and Mitosis

Hepatocytes were pulse-labeled for 8 h with ^{3}H-TdR (1 μCi/ml) at various times after seeding. ^{3}H-TdR incorporation into DNA was measured by liquid scintillation as described previously (Marceau et al., 1982; Baribault et al., 1985; Baribault and Marceau, 1986). Cells in mitosis were monitored using the Hoechst staining (Chen, 1976; Baribault et al., 1985).

Immunofluorescence Microscopy

We used two monoclonal antibodies generated against rat hepatocyte cytokeratins, which react specifically with CK49 and CK55 (Leroux-Nicollet et al., 1983; Marceau et al., 1985). Analysis by indirect immunofluorescence staining of ethanol-fixed hepatocytes in culture was performed as described before (Marceau et al., 1985). Cells were washed three times at room temperature with PBS, treated in cold ethanol (100%), 10 min at −20°C, and then rinsed again with PBS. Cells were overlaid with an appropriate dilution of mouse ascitic monoclonal antibody, and kept for 30 min at room temperature. After washing, the cells were overlaid with fluorescein isothiocyanate–conjugated goat anti-mouse Ig diluted 1:50 in PBS. After a 30-min incubation at room temperature, the coverslips were rinsed and mounted on microscope slides in the presence of glycine–glycerol buffer. The cells were observed with a Leitz microscope equipped with epifluorescence illumination and photographed with Kodak Tri-X film.

When indirect immunofluorescence was used in combination with ^{3}H-TdR autoradiography or with Hoechst staining, cells were fixed with 100% ethanol at −20°C for 10 min and processed for immunofluorescence first, then the liquid emulsion was added and the cultures were further processed for autoradiography or staining with the Hoechst reagent (Chen, 1976), as described before (Baribault et al., 1985).

[^{35}S]Methionine Protein Labeling

Cytokeratin Synthesis. Hepatocytes cultured for 46 h in the presence of dexamethasone and insulin were incubated in methionine-free medium containing 17.5 μCi/ml of [^{35}S]methionine (New England Nuclear), and then stimulated at 48 h with EGF for different times. At the end of the incubation period, total cellular proteins were solubilized by SDS, heated for 3 min at 90°C, and precipitated by ethanol at −20°C overnight. The samples were assayed for total protein content and their radioactivity measured with a scintillation counter. Some experiments were performed in the presence of 10 μg/ml cycloheximide.

Cytokeratin Degradation. Hepatocytes cultured for 48 h in the presence of insulin and dexamethasone were pulse labeled with [^{35}S]methionine for 15 min. EGF (10 ng/ml) and cycloheximide (10 μg/ml) were then added and proteins were fractionated by SDS-PAGE at 0, 1, 3, and 8 h after labeling. Control cultures without EGF were processed at the same times. In some experiments, cytoskeletal fractions enriched in cytokeratins were prepared by extraction of the cells with Triton X-100 (see below).

^{32}P-Protein Labeling

Intact hepatocytes. Hepatocytes were incubated in phosphate-free medium containing insulin, dexamethasone, 100 μCi/ml ^{32}P-orthophosphate for 4 h before EGF stimulation. After incubation with EGF for various times, the cell monolayers were processed to examine the ^{32}P-labeling of total proteins or of Triton-resistant cytoskeletons (see below).

Permeabilized Hepatocytes. Hepatocytes were cultured in 60-mm dishes for 48 h in the presence of dexamethasone and insulin, and then EGF was added to fresh medium for different times at 37°C. The cells were then washed twice with a 20-mM Hepes, pH 7.4, buffer containing 250 mM sucrose, 1 mM $MgCl_2$, 1 mM benzamidine, 2 mM sodium fluoride, 2 mM sodium vanadate, 20 mM sodium molybdate, 200 KIU/ml aprotinine, and 1 mM PMSF. The cells were permeabilized in the same buffer supplemented with 0.5% Triton X-100 for 2 min at 4°C. Before phosphorylation, the cells were washed in buffer without the inhibitors. The ^{32}P-labeling was done directly in the dishes, in a volume of 1.0 ml for 5 min at 22°C. The final concentrations of buffer constituents were 25 mM Hepes, pH 7.0, 1 mM $MgCl_2$, 5 mM γ-^{32}P-ATP, and 10 ng/ml EGF. Phosphorylation was stopped by washing the cells twice with the 20 mM Hepes, pH 7.4, buffer containing the inhibitors, and the proteins then solubilized in 200 μl of 2× concentrated electrophoresis buffer (Kay et al., 1986; Rubin and Earp, 1983).

Preparations of Hepatocyte Cytoskeletons

Hepatocytes cultured in 60-mm dishes were washed twice with 10 mM phosphate buffer, 140 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM sodium vanadate, 200 KIU/ml aprotinine, and 1 mM PMSF. The cells were then incubated for 5 min in this buffer containing 1% Triton X-100 and then for 20 min in the same buffer containing 0.5% Triton X-100 and 1.5 mM KCl. All of the steps were done at 4°C. The cytoskeleton preparations were washed twice at 4°C with the phosphate buffer and finally solubilized in 2.3% SDS electrophoresis buffer (Franke et al., 1981). The reliability of this procedure for the purification of cytokeratins is well-established (Moll et al., 1982; Sun et al., 1984). It is based on the fact that no excess pool of cytokeratin monomers is maintained in cells and that de novo synthesis cytokeratin pairs results in the formation of hetero tetramers necessarily assembled into IFs (Domenjourd et al., 1988). Thus, the Triton-insoluble cytokeratins constitute virtually all of the available cellular cytokeratins.

Gel Electrophoresis

The solubilized proteins were fractionated by one-dimensional 10% SDS-PAGE according to Laemmli (1970). The gels were stained with Coomassie blue and then processed for autoradiography with XAR-5 Kodak film. Protein standards used were myosin heavy chain (200 kD), β-galactosidase (116.5 kD), phosphorylase B (92.5 kD), BSA (66.2 kD), and ovalbumin (45 kD). Two-dimensional PAGE was performed as described by O'Farrell et al. (1977), except that CHAPS was used instead of NP-40 and SDS (Perdew et al., 1983). Ampholines of pH 5-7:3-10:3-5 (7:2:1) were mixed to yield a pH gradient that ranged from 4.5 to 7.0.

Immunoblot Analysis

Proteins were electrophoretically transfered to nitrocellulose sheets using a blotting apparatus (3 h at 14°C: Bio-Rad Laboratories, Cambridge, MA), according to procedures described in detail before (Towbin et al., 1979; Marceau et al., 1986*a*). Sheets were saturated in 5% skimmed milk powder. The blots were then incubated with the various antibodies and the bound antibodies were detected by autoradiography after a reaction with ^{125}I-labeled goat anti-mouse Ig. Immunoblotting assays with the anti-CK55 and anti-CK49 antibodies on total proteins and Triton-resistant fractions of cultured hepatocytes have already established the identity of CK55 and CK49, respectively (Marceau et al., 1985, 1986*a*).

Phospho-Amino Acid Analysis

The band corresponding to the ^{32}P-labeled CK55 was extracted from the gels. The protein was hydrolyzed with 6 M HCl at 110°C for 2 h. The hydrolysate was frozen and lyophilized over NaOH pellets. The yellow residue was dissolved in water and shaken with Dowex AG1-X8 resin at room temperature for at least 4 h. Phospho-amino acids were eluted from the resin with 1 M HCl. Two-dimensional thin-layer electrophoresis (model CANAG HVE; Terochem, Mississauga, Ontario) was carried out on cellulose thin-layer plates at pH 1.9 with 88% formic acid/glacial acetic acid/H_2O (50:156:1,794) for 45 min at 2.5 KV and at pH 3.5 with pyridine/glacial acetic acid/H_2O (10:100:1890) for 30 min at 2.0 KV (Cooper et al., 1983).

Results

Addition of EGF to Quiescent Rat Hepatocytes Results in Entry into S Phase

We have shown that the addition of EGF and insulin, with or without dexamethasone, to quiescent differentiating rat hepatocytes results in the stimulation of DNA synthesis after a lag period of 24 h and then mitosis (Baribault et al., 1985). In the present study, freshly isolated hepatocytes from 14-d-old rats were seeded on fibronectin-coated plastic dishes in serum-free medium containing insulin and dexamethasone for 48 h and then stimulated with EGF. Without EGF, the hepatocytes remained quiescent, whereas in the presence of EGF they entered into S phase after a lag period of 8 h (Fig. 1). Using this method, we examined cytokeratin-related events associated with the EGF mitogenic action.

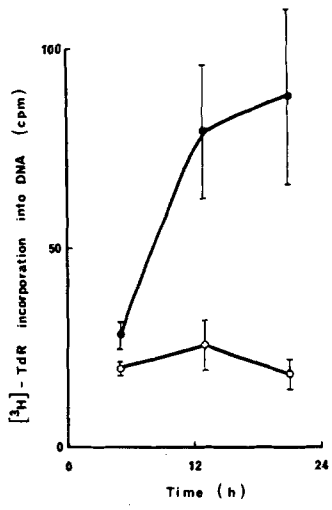

Figure 1. Effect of EGF on the initiation of DNA synthesis in cultured suckling rat hepatocytes. Cells were cultured in the presence of 150 μg/ml insulin and 1 μM dexamethasone for 48 h before the addition of 10 μg/ml EGF (●) or no addition (○). ^{3}H-TdR was then added for 8 h at various times. Cells were solubilized and the amount of ^{3}H-TdR incorporated into DNA was counted. Data correspond to the midpoints of the 8-h labeling periods. Values are the means of four separate experiments of triplicate dishes. Bars represent SEM.

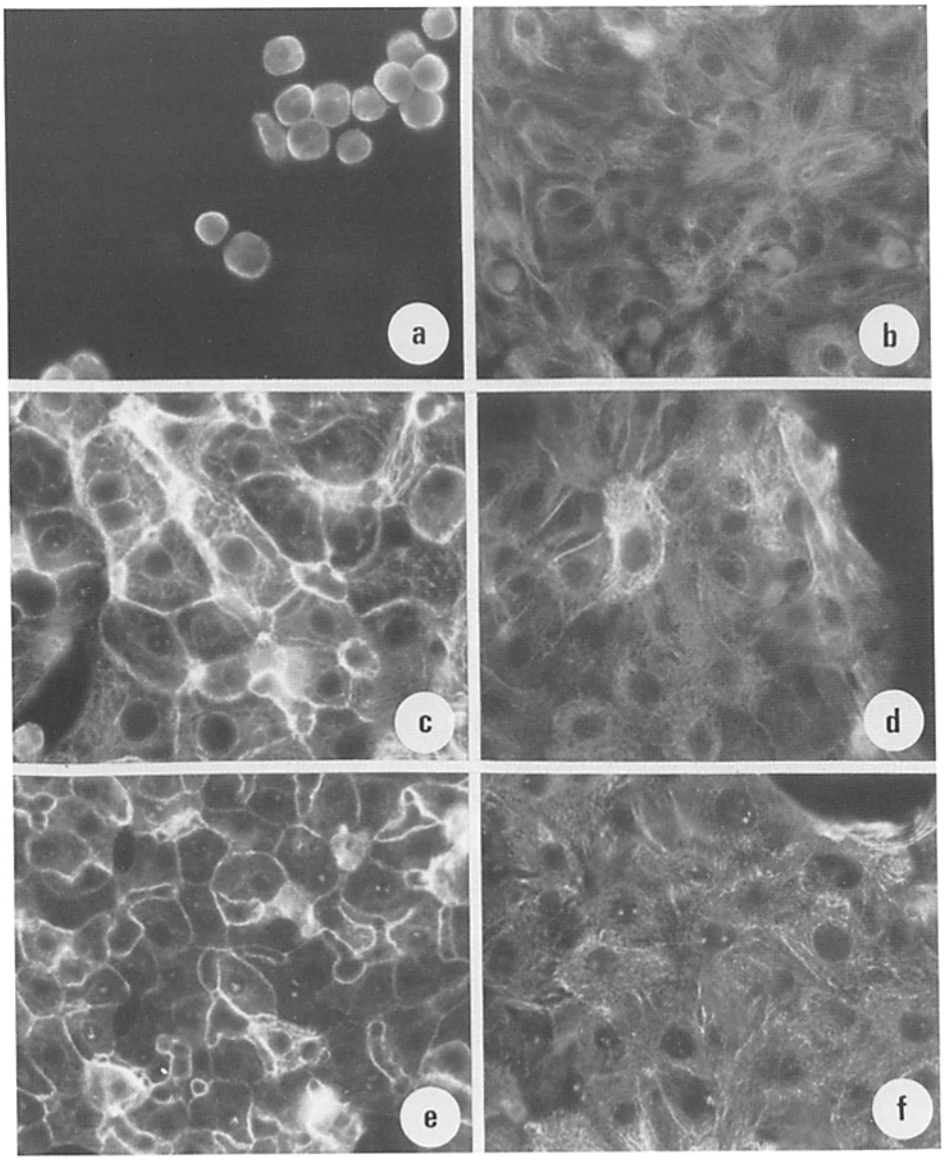

Figure 2. Effect of EGF on the organization of CK49 and CK55 filaments. Freshly isolated hepatocytes (*a*) and cultured hepatocytes (*b–f*) were fixed, stained with anti-CK55 (*a–d*) or anti-CK49 (*e* and *f*) antibodies, and examined by indirect immunofluorescence microscopy. Hepatocytes were cultured for the first 48 h after plating the medium containing insulin and dexamethasone with (*b*) or without (*c–f*) EGF. In *d* and *f* EGF was added at 48 h as described in Fig. 1 and the cells were fixed 3 h later.

EGF Selectively Induces Cytokeratin Filament Rearrangement Early during the Prereplicative Period but Not at Mitosis

Previous data from indirect immunofluorescence microscopy with monoclonal antibodies to CK55 and CK49 have shown that, in the presence of insulin and dexamethasone only, cytokeratin IFs of cultured rat hepatocytes are mainly distributed near the surface membrane (Marceau et al., 1985). The present data showed that the addition of EGF at 48 h after seeding resulted in a reorganization of both CK55- and CK49-containing filaments from the surface membrane to the cytoplasm within <3 h (Fig. 2). This cytoplasmic distribution remained intact even through mitosis, as demonstrated by the data from double labeling with ^{3}H-TdR autoradiography or Hoechst 33258 and immunolocalization with CK55 (Fig. 3). These EGF-induced alterations of the cytoskeleton were specific to cytokeratin IFs since immunocytological analyses with antibodies to tubulin and actin revealed no detectable change in the organization of microtubules and microfilaments. Furthermore, drugs such as colchicine and cytochalasin B which are known to disrupt microtubules and microfilaments, had no effect on cytokeratin filament redistribution (data not shown).

EGF Preferentially Stimulates the Phosphorylation of CK55 among the CK49/CK55 Pairs

Since many cytokeratins are phosphoproteins (Baffet et al., 1985; Celis et al., 1983; Traub, 1985) and EGF influences the phosphorylation of several cellular proteins (Aoyagi et al., 1983; Hunter and Cooper, 1981), we examined whether the degree of phosphorylation of rat hepatocyte cytokeratins could be affected by EGF.

Pilot experiments on the relative contribution of dexamethasone and insulin to the ^{32}P-labeling of cytokeratins at 48 h after seeding revealed that dexamethasone or insulin alone had no stimulating effect (data not shown) whereas their combined addition resulted in a relatively low level of ^{32}P-labeling of CK55 on gels of fractionated total proteins

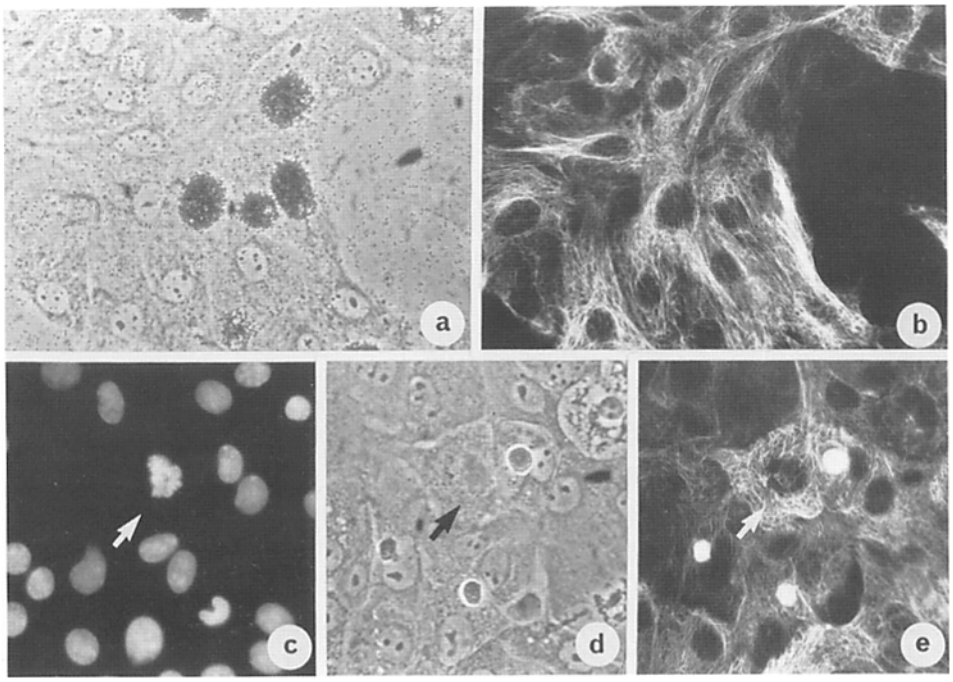

Figure 3. Arrangement of CK55 containing filaments during S phase and mitosis. Cells were fixed and stained for immunofluorescence (*b* and *e*) with anti-CK55 and then processed for autoradiography (*a*) or Hoechst staining (*c*). *a* and *b* are views of the same field in phase contrast (*a*) and immunofluorescence (*b*); *c*, *d*, and *e* are views of the same field in Hoescht staining, phase contrast, and immunofluorescence, respectively. The arrows indicate hepatocytes in mitosis.

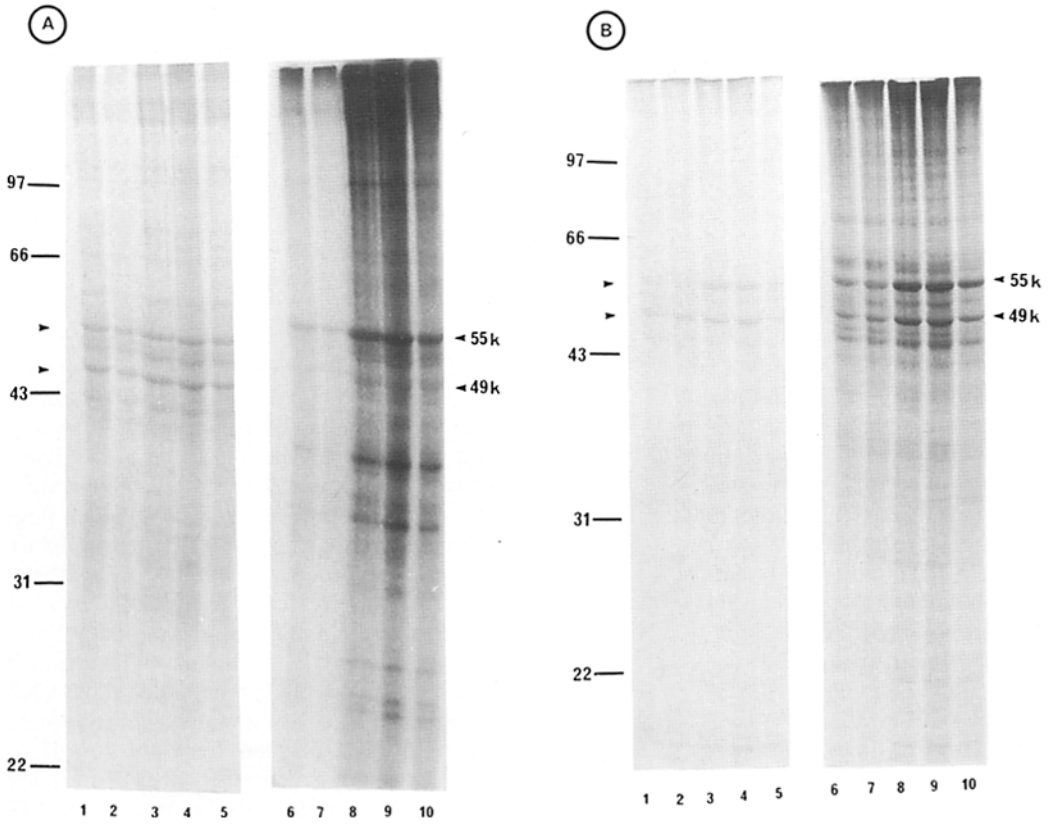

Figure 4. Time course of the ^{32}P (*A*) and [^{35}S]methionine (*B*) labelings of proteins in response to EGF stimulation. Hepatocytes were cultured in the presence of insulin and dexamethasone and growth stimulated by EGF as reported in Fig. 1. They were labeled in vivo with ^{32}P-orthophosphate or [^{35}S]methionine and the proteins were fractionated by SDS-PAGE. Gels were exposed for 5 and 16 h, respectively. Lanes *1–5* show the Coomassie stainings whereas lanes *6–10* show the ^{32}P-labeled (*A*) and ^{35}S-labeled (*B*) proteins at 0, 15 min, 30 min, 1 h, and 3 h, respectively.

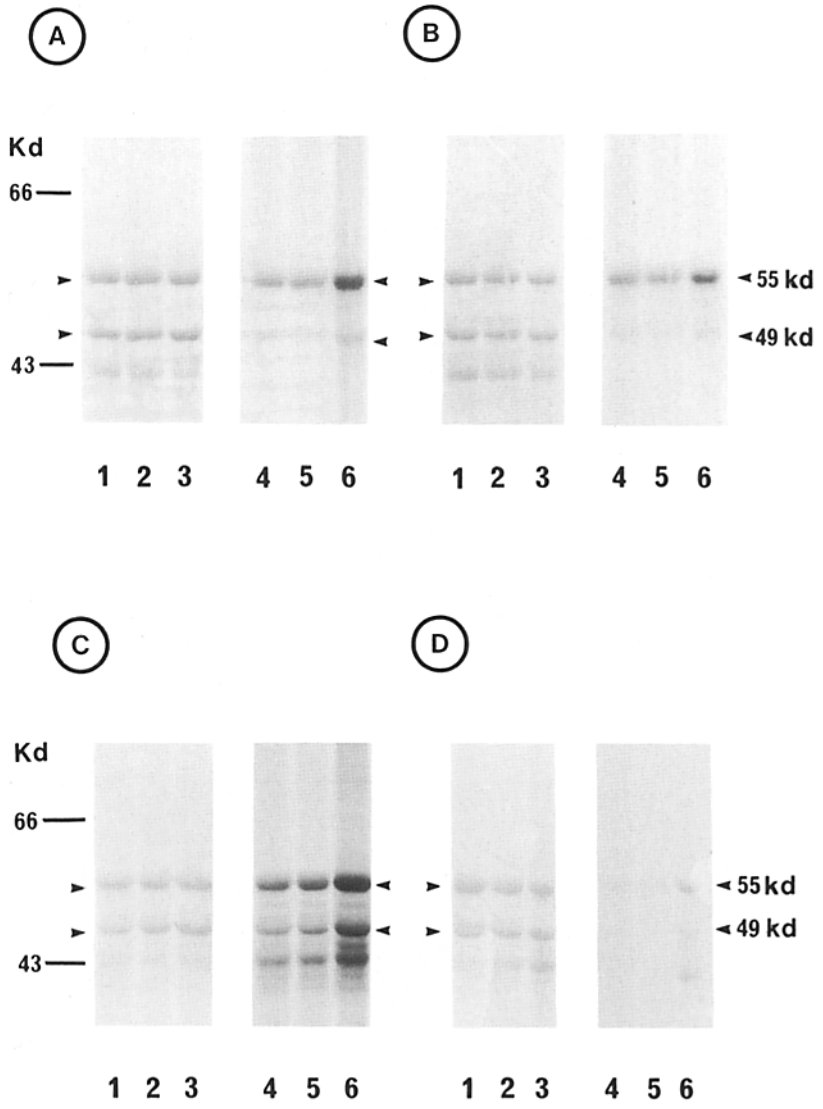

Figure 5. Time course of the ^{32}P labeling (*A* and *B*) and [^{35}S]methionine labeling (*C* and *D*) of CK49 and CK55 in absence (*A* and *C*) and presence (*B* and *D*) of 10 μg/ml cycloheximide after EGF stimulation. Hepatocytes were stimulated with EGF and labeled with ^{32}P-orthophosphate or [^{35}S]methionine as described in Fig. 4. Triton-resistant cytoskeletons were prepared and the proteins were fractionated by SDS-PAGE. Gels were exposed for 5 and 16 h, respectively. Lanes *1–3* show the Coomassie stainings whereas lanes *4–6* show the ^{32}P labelings or [^{35}S]methionine labelings of CK49 and CK55 at 0, 15, and 30 min after stimulation.

(Fig. 4 *A*, lanes *1* and *6*). When hepatocytes were first cultured in the presence of dexamethasone and insulin for 48 h and growth stimulated for up to 3 h with EGF, the level of ^{32}P-labeling of CK55, when compared to the protein staining, rapidly increased to reach a plateau at 30 min (Fig. 4 *A*, lanes *3* and *8*). Comparison of the ^{32}P-autoradiograms with the protein staining intensities revealed that the increase in the ^{32}P-labeling of CK55 after 30 min of EGF treatment was at least threefold. It is worth noticing that proteins in the range of 90, 35, and 22 kD were similarly stimulated in response to EGF. Since a 4-h ^{32}P-prelabeling time before EGF may not be sufficient to saturate the intracellular ATP pools, it is possible that part of the increase in the level of CK55 phosphorylation with increasing time of exposure to EGF could be due to changes in the specific activity of the ATP pools. However, longer prelabeling periods were deleterious to the hepatocyte viability. Still, the most important data was that in comparison with CK55, the relative level of ^{32}P-labeling of CK49 remained low (Fig. 4 *A*). The selective stimulation of phosphorylation of CK55 of the CK49/CK55 pair by EGF was even more apparent after SDS-PAGE fractionation of Triton-resistant cytokeratins (Fig. 5 *A*). Analyses of the proteins labeled with [^{35}S]methionine indicated a slight increase in the labeling of many proteins after a 30-min stimulation (Fig. 4 *B*), including those of the Triton-resistant fractions (Fig. 5 *C*). The observed increase of ^{32}P-labeling of CK55 in response to EGF stimulation largely occurred in preexisting IFs since the addition of cycloheximide resulted in a relatively small reduction of ^{32}P-labeling of Triton-resistant CK55 (Fig. 5 *B*), in spite of a very substantial inhibition of protein synthesis (Fig. 5 *D*). Pulse–chase measurements during EGF stimulation revealed no significant change in the rate of degradation of either CK55 or CK49, thus indicating that they were metabolically stable during the time of IF rearrangement (data not shown).

To assess in more detail the induced changes in the degree of phosphorylation of CK55 and CK49 in response to EGF stimulation, Triton X-100–resistant cytokeratins were prepared at 0, 30 min, and 3 h after stimulation from hepatocytes labeled with either ^{32}P-orthophosphate or [^{35}S]methionine, and then analyzed by two-dimensional PAGE. Coomassie blue staining revealed that CK55 was resolved into

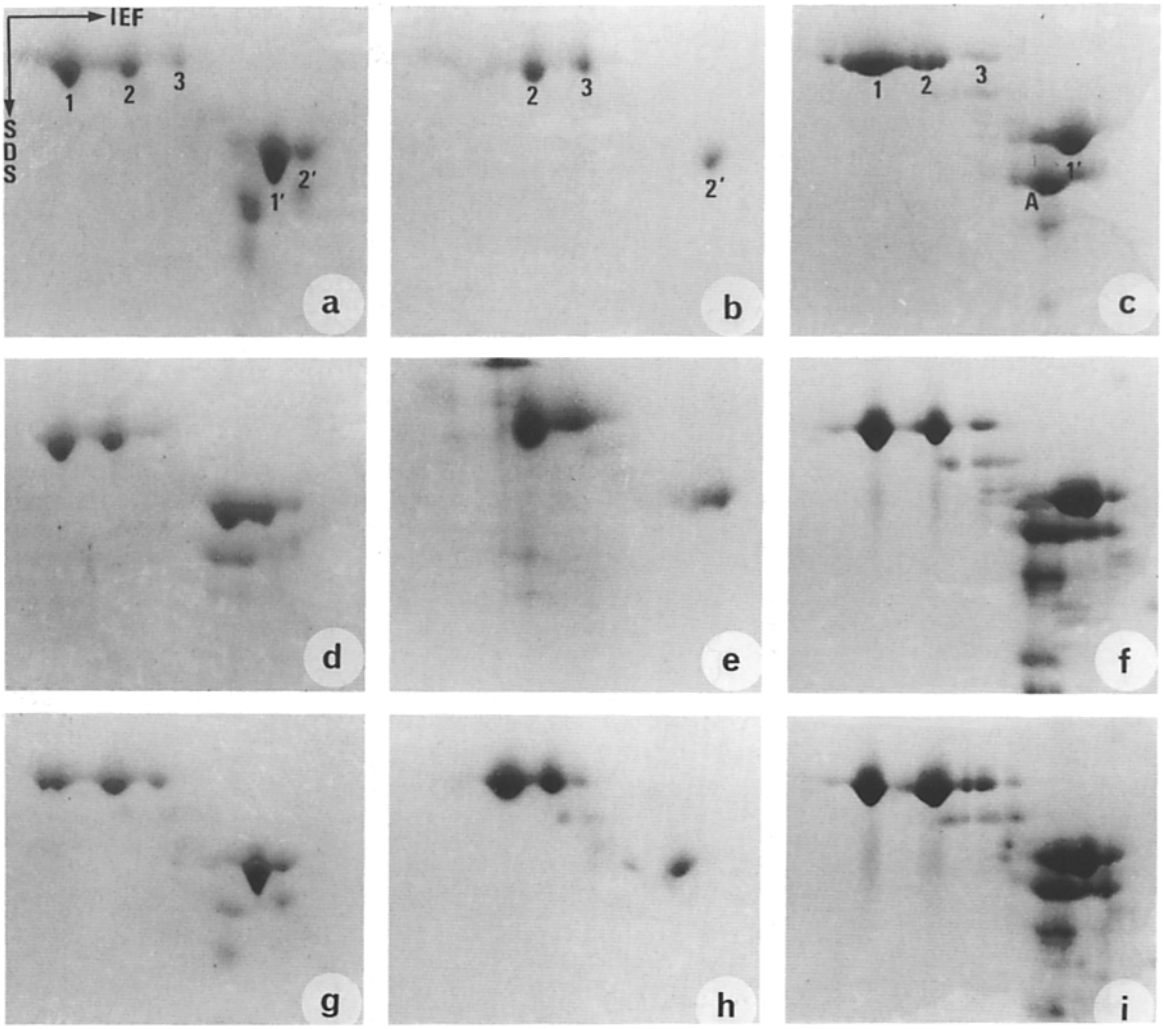

Figure 6. Two-dimensional PAGE analyses of ^{32}P and [^{35}S]methionine labeled CK49 and CK55 after EGF stimulation. Hepatocytes were treated as in Fig. 4, and the extraction was done 0, 30 min, and 3 h after the addition of EGF. *A*, *D*, and *G* are the Coomassie blue stainings for the time 0, 30 min, and 3 h, respectively. *B*, *E*, and *H* are the corresponding autoradiograms of the ^{32}P labelings. The exposure time was 16 h. *C*, *F*, and *I* are the autoradiograms of the [^{35}S]methionine labelings. The exposure time was 24 h. *1*, *2*, and *3*, the three CK55 species; *1'* and *2'*, the two CK49 species; *A*, actin. The IEF migration was toward anode.

three species (1,2,3) and CK49 into two species (1',2') (Fig. 6, *a*, *d*, and *g*). At all times species 1 and 1' were not phosphorylated (Fig. 6, *b*, *e* and *h*). However, at time 0, species 2 was more phosphorylated than species 3 (Fig. 6 *b*); and at 30 min and 3 h after stimulation, there was a relative increase in the ^{32}P-labeling of species 2 and 3 (Fig. 6 *e*). The data from parallel ^{35}S-labeling experiments (Fig. 6, *c*, *f*, and *i*) showed changes in labeling proportional to the amount of the protein (Fig. 6), indicating that significant changes in the phosphorylation levels of CK55 were associated with the EGF stimulation. In contrast, species 2' (CK49) was labeled to a low level, and the addition of EGF induced little change in the extent of phosphorylation (Fig. 6).

Immunoblotting analyses of Triton-resistant fractions obtained at 0, 30 min, and 3 h after stimulation with EGF using the anti-CK55 antibody confirmed the observation that species 1, 2, and 3 were all CK55 (Fig. 7). These data were further used to obtain information on the stoichiometry of CK55 phosphorylation by scanning the autoradiograms and calculating the changes in the phosphorylated CK55 forms relative to the nonphosphorylated form. From work reported by others on the phosphorylation of IF proteins (Celis et al., 1983; Franke et al., 1982; Evans, 1989), it is very likely that spots 2 and 3 represent the addition of 1 and 2 phosphates to CK55, respectively. Thus, the amount of phosphorylated CK55 at time 0 (spots 2 and 3; Fig. 7 *a*) represents 40% of the total CK55. After 30 min of EGF treatment (Fig. 7 *b*) the phosphorylated forms represent 65%; an overall increase of 1.6-fold.

All of the data on cytokeratin phosphorylation suggest that EGF causes selective changes in CK55 phosphorylation before CK49/CK55 IF reorganization.

The EGF-responsive Serine Protein Kinase Is Resistant to Triton-Permeabilization

The EGF-R of various EGF responsive cells, including the A431 cells, is associated with the detergent-cytoskeleton

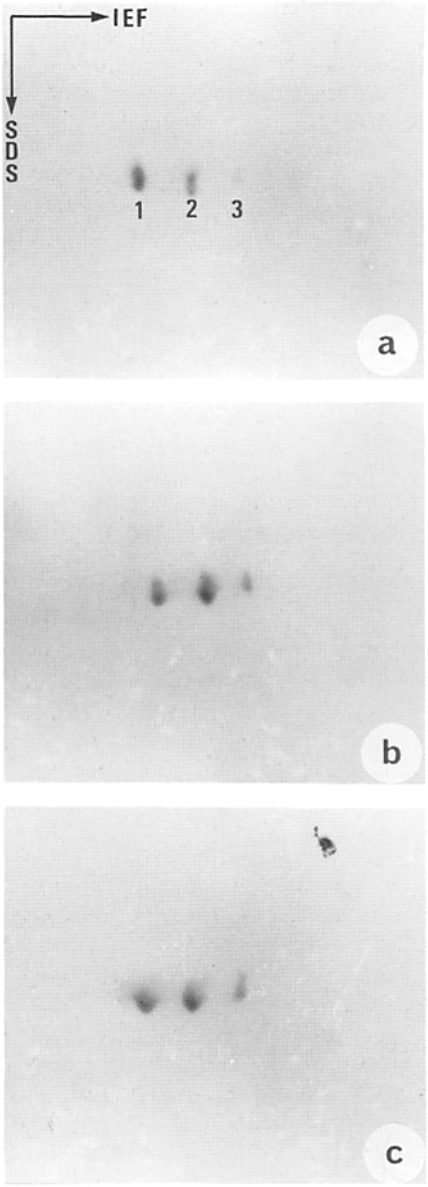

Figure 7. Immunoblotting of unlabeled cytoskeletal proteins with anti-CK55 antibody. Hepatocytes were stimulated with EGF as described in Fig. 4; the cytoskeletal proteins were extracted after 0, 30 min, and 3 h (*A*, *B*, and *C*), and then fractionated by two-dimensional PAGE. The proteins were transfered on a nitrocellulose sheet and then incubated with the anti-CK55 monoclonal antibody followed by the anti-mouse Ig^{125}I antibody. The exposure time was 18 h.

(Landreth and Rieser, 1985; Landreth et al., 1985; Wiegant et al., 1986). To determine if the protein kinase responsible for the preferential CK55 phosphorylation was part of the detergent-resistant cytoskeleton, we measured the phosphorylation of cytokeratins of EGF-stimulated Triton-permeabilized hepatocytes. After labeling with γ-^{32}P-ATP, both CK55 and CK49 were phosphorylated at comparable levels in the nonstimulated hepatocytes. At 30 min, and particularly at 3 h after stimulation, CK49 and CK55 became more labeled, but the increase was slightly higher in CK55 (Fig. 8). Those observations suggested that the EGF-responsive serine kinase was resistant to the permeabilization with detergent, but that such a treatment rendered CK49 accessible to the protein kinase.

Phospho-Amino Acid Analyses of CK55 Demonstrates Phosphorylation on Serine Residues

Total proteins of ^{32}P-prelabeled EGF-stimulated or nonstimulated hepatocytes were subjected to SDS-PAGE, the CK55 bands were excised from the gels, and then their phospho-amino acid compositions were determined. The data revealed the presence of only ^{32}P-labeled serine residues in CK55 (Fig. 9, *A* and *B*), and the same results were obtained by using CK55 isolated from Triton-resistant cytokeratins (data not shown). Comparative phospho-amino acid analyses on total cellular proteins yielded ^{32}P-labeled serine, threonine, and tyrosine (Fig. 9, *C* and *D*), thus indicating that the enhanced phosphorylation of CK55 by a EGF-responsive serine protein kinase in intact hepatocytes was rather specific.

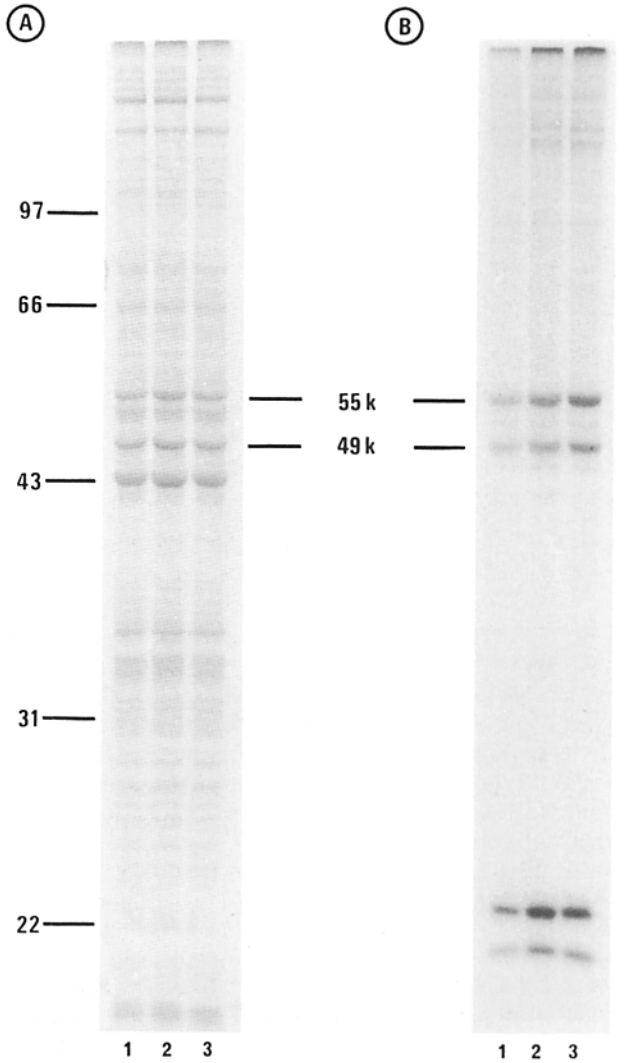

Figure 8. γ-^{32}P-ATP labeling of CK55 and CK49 in permeabilized hepatocytes. After the addition of EGF for 0, 30 min, and 3 h (lanes *1*, *2*, and *3*), hepatocytes were processed as described in Materials and Methods. Phosphoproteins were fractionated by SDS-PAGE. The exposure time was 3 h. (*A*) Coomassie blue stainings of the gels; (*B*) the corresponding autoradiograms.

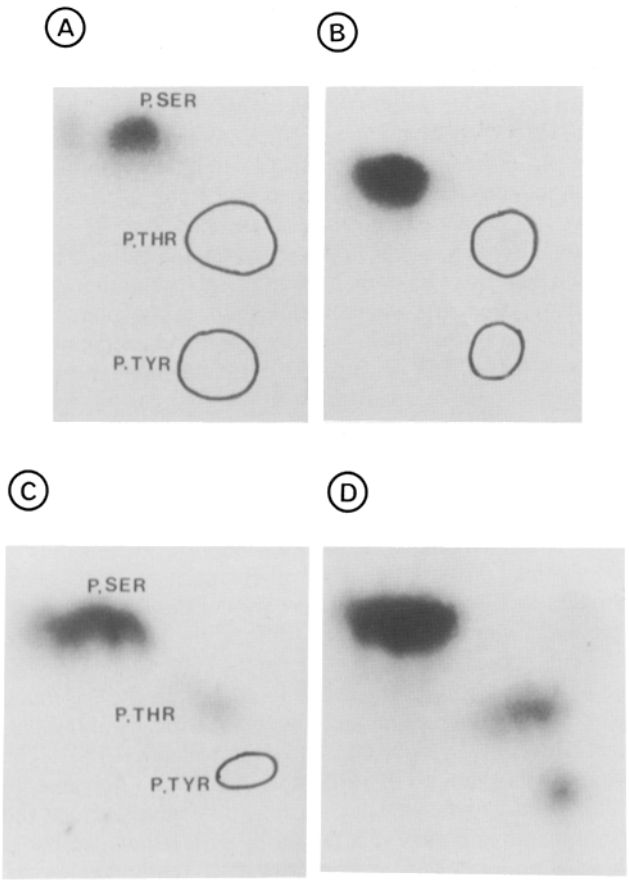

Figure 9. Amino acid analyses of CK55 extracted from SDS-PAGE gels (*a* and *b*) and total cellular proteins (*c* and *d*) after in vivo labeling in the absence (*a* and *c*) or presence (*b* and *d*) of EGF. Extracted phospho–amino acids were separated by two-dimensional thin-layer electrophoresis. Plates were exposed for 6 d.

Discussion

These results show for the first time that EGF stimulates a selective increase in the phosphorylation of CK55 on serine residues. This phosphorylation precedes the early cytoplasmic redistribution of CK49/CK55 filaments which occurs during the prereplicative period of growth-stimulated differentiating rat hepatocytes. The rapid redistribution of cytokeratin filaments from the surface membrane to the cytoplasm may be an important and necessary organizational event, perhaps linked to the need to disrupt the pericanalicular sheath, so that hepatocytes can initiate DNA synthesis and subsequently divide.

Much of the work reported on the mechanisms of action of EGF has been performed on A431 human epidermoid undifferentiated carcinoma cells cultured in serum-supplemented medium (Ushiro and Cohen, 1980). Although this tumor cell line is an attractive model to examine EGF biological action, mainly because the cells express an unusually large number of EGF-Rs (Haigler et al., 1978; Schlessinger, 1988), the addition of EGF at concentrations that are normally mitogenic for other cell types results in a marked inhibition of the growth of A431 cells in insulin-containing serum-free medium (Barnes, 1982). Our data show that in cultured neonatal rat hepatocytes, a physiological concentration of EGF in the presence of insulin and dexamethasone is sufficient to induce their entry into S phase and mitosis. This cell system is therefore very appropriate to examine EGF-dependent events during the initiation of DNA synthesis and cell division in normal, differentiating epithelial cells.

Some growth factor receptor genes and many retroviral oncogenes encode tyrosine-specific protein kinases (Hunter and Sefton, 1979; Ushiro and Cohen, 1980; Hunter and Cooper, 1981). So far, only a few cellular targets have been identified as in vivo and/or in vitro substrates for these tyrosine protein kinases. Among them are p35 and p36 now designated as lipocortins I and II (Brugge, 1986; Huang et al., 1986), which are localized in the lamina region underlying the surface membrane (Gould et al., 1984; Keski-Oja and Alitalo, 1985), the microtubule-associated protein (Nishika et al., 1987), and type I DNA topoisomerase (Tse-Dinh et al., 1984). The data from the phospho–amino acid analyses of total cellular proteins suggest the presence of a tyrosine-protein kinase activity in EGF-stimulated hepatocytes (Fig. 9, *C* vs. *D*), but the targets remain to be identified. Qualitative evaluation of the labeled phosphotyrosine relative to the total labeled phospho–amino acids yielded a value of ~4%, which is higher than usual for EGF-stimulated cells (Hunter and Cooper, 1981). At any rate, of particular interest in the context of the present study is the finding that CK55 is not a cellular target for this type of kinase activity.

In addition to the phosphorylation of tyrosine residues by the EGF-R kinase, EGF stimulation can also lead to a rapid phosphorylation of serine (and threonine) residues of various cellular proteins including ribosomal protein S6 and histone H_1, presumably via one or more EGF-responsive serine protein kinases (Halegona and Patrik, 1980; Lastick and McConkey, 1980). From the present SDS-PAGE analyses performed on EGF-stimulated intact hepatocytes, it is clear that CK55 and several other cellular phosphoproteins (Fig. 4 *A*) can undergo a rapid increase in phosphorylation, but only CK55 seems to be part of the Triton-resistant cytoskeleton (Fig. 5 *A*). Also of interest is the finding that CK49, the normal partner of CK55 in rat hepatocytes (Franke et al., 1981), is phosphorylated to a very low level (Figs. 4 *A* and 5 *A*). Considering that CK49 can be readily phosphorylated under in vitro conditions (Fig. 8), it is unlikely that the increase in CK55 phosphorylation can be due to a significant EGF-induced augmentation of the specific activity of the ATP pool. At any rate, the induced selective phosphorylation refers here solely to a selective effect on CK55 among the CK49/CK55 pair. Moreover, the phosphoamino acid analyses (Fig. 9, *A* vs. *B*) suggest that as in the case of S6 and H1, the phosphorylation of CK55 involves the participation of an EGF-responsive serine protein kinase.

The observation that in comparison to CK55, CK49 is phosphorylated to a very low level in intact nonstimulated hepatocytes (Fig. 5), suggests that the serine residues of CK55 among the CK49/CK55 pair act predominantly as substrates. One possible explanation could be that such a selective effect is due to differences in serine composition and amino acid sequences in the well-defined domains of these IF proteins. Although the amino acid sequences of CK49 and CK55 are unknown, it has been reported that Xenopus 55.7-kD cytokeratin (equivalent to rat CK55; Franz and Franke,

1986) has 24 and 21 serine residues at the amino- and carboxy-terminal domains, respectively, compared to 16 and 4 serine residues in the case of the mouse Endo B (equivalent to rat CK49; Singer et al., 1986). Current models of IF structure assume that the filament assembly is primarily based on the interaction of the central domains of the protein pairs, implying that the terminal domains are free to interact with other cellular components (for review, see Steinert et al., 1985). Under in vivo conditions CK55 is much more phosphorylated than CK49, but in Triton-permeabilized hepatocytes both cytokeratins become phosphorylated. On this basis, the large difference in their phosphorylation level would suggest that most serine residues of CK49, likely of the carboxy-terminal domains, are not accessible in vivo. It would be interesting to determine which serine(s) and which domain(s) are in fact phosphorylated under in vivo and in vitro conditions.

The next question to consider concerns the identity of the protein kinase involved in the in vivo CK55 serine phosphorylation. While the in vivo phosphorylation of cytokeratins is well-accepted (Baffet et al., 1985; Celis et al., 1983; Traub, 1985), the possible involvement of specific kinases has not often been considered. However, recent data on the in vitro phosphorylation of vimentin and desmin have provided evidence indicating that the catalytic subunit of cAMP-dependent protein kinase A can readily phosphorylate serine sites in their amino terminal domains (Inagaki et al., 1987; Geisler and Weber, 1988). In this regard, it is worth noticing that an increase in the cellular level of cAMP leads to a rapid stimulation of vimentin phosphorylation in quiescent cultures of Swiss 3T3 cells (Escribano and Rozengurt, 1988). However, other studies using cell cytosol and Triton-resistant cytoskeleton preparations from fibroblasts exhibited a discrete kinase activity for vimentin that is cAMP and Ca^{++} independent (Evans, 1989). It would therefore be of interest to determine which of these kinases is involved in the phosphorylation of cytokeratins in epithelial cells.

In the context of the present data, the evidence for the association of EGF-R with the Triton-resistant cytoskeleton (Landreth et al., 1985; Wiegant et al., 1986) is pertinent. That CK55 is phosphorylated when EGF-treated Triton-permeabilized hepatocytes are incubated with γ-^{32}P-ATP and that CK49/CK55 IFs are localized at the cell periphery at the time of EGF stimulation indicates that the EGF-responsive serine protein kinase is part of the surface membrane skeleton. However, in Triton-permeabilized hepatocytes CK49 becomes phosphorylated as well. This means that until one can show that the sites phosphorylated in vitro are the same as those phosphorylated in EGF-stimulated intact hepatocytes, the possibility remains that the enzyme that phosphorylates CK49 and CK55 in vitro is not the serine protein kinase that phosphorylates CK55 in vivo.

Although the functional significance of cytokeratin phosphorylation is unclear, there are data suggesting that it is not necessarily linked to filament stability (Celis et al., 1983), but may somehow be involved in modulating the association of the filaments with other cellular structures (Fuchs et al., 1986; Knapp et al., 1983). In this regard, we recently looked at the CK49/CK55 IFs of rat hepatocytes in whole mount, unembedded Triton-extracted rat liver slices, using double immunoelectron microscopy. Examination of the pericanalicular region revealed that IFs containing both CK55 and CK49 form a continuous sheath around the bile canaliculi (Katsuma et al., 1988). While both CK55 and CK49 were localized at the cell border and in the cytoplasm, the staining pattern for CK49 in the cytoplasm was punctuate, whereas that of CK55 was filamentous. The immunofluorescence micrographs of neonatal rat hepatocytes cultured without EGF reveal staining patterns similar to that in situ, particularly around the canalicular structures (see Fig. 2). The data on the stoichiometry of CK55 phosphorylation indicate only a 1.6-fold increase. However, depending on the number of sites phosphorylated, and whether EGF induces phosphorylation at a new site(s), this might be sufficient to induce the detachment of CK49/CK55 IFs from the pericanalicular sheath and their subsequent reorganization in the cytoplasmic space.

Cytokeratin IFs of growing epithelial cells are cytoplasmic, but their regional distribution and stability vary with cell types and whether the cells are in interphase or mitosis. For example, in MDCK cells at interphase, cytokeratins form elaborate filament networks within the cytoplasm (Fey and Penman, 1985) while in others such as T51B cells, cytokeratin IFs are mainly found around the nucleus (Marceau and Swierenga, 1985). In some epithelial cell lines the filaments appear as intact bundles in the plane of the cleavage furrow during cytokinesis, while in other cells there is much more filament disruption and the cytokeratins exhibit a pattern of discrete cytoplasmic dots (Lane et al., 1982). The present data show no disruption of cytokeratin filaments in cultured rat hepatocytes during mitosis, which supports the above mentioned view that the early EGF-stimulated phosphorylation of CK55 is confined to a rearrangement of CK49/CK55 IFs for membrane to cytoplasm, a necessary event for the growth activation of cultured quiescent hepatocytes.

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