Rapid Phosphorylation and Reorganization of Ezrin and Spectrin Accompany Morphological Changes Induced in A-431 Cells by Epidermal Growth Factor

Anthony Bretscher

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

Abstract. Addition of EGF to human carcinoma A-431 cells is known to induce membrane ruffling after ~2 min (Chinkers, M., J. A. McKanna, and S. Cohen. 1979. *J. Cell Biol.* 83:260–265) and the phosphorylation of a protein referred to as p81, a known substrate for various protein-tyrosine kinases (Cooper, J. A., D. F. Bowen-Pope, E. Raines, R. Ross, and T. Hunter. 1982. *Cell.* 31:263–273). Ezrin, a M_r~80,000 cytoskeletal protein of the isolated chicken microvillar core, is present in actin-containing cell surface structures of a wide variety of cells (Bretscher, A. 1983. *J. Cell Biol.* 97:425–432). Ezrin was then found to be homologous to p81 and to be phosphorylated on tyrosine in response to EGF (Gould, K. L., J. A. Cooper, A. Bretscher, and T. Hunter. 1986. *J. Cell Biol.* 102:660–669). Here, the purification of ezrin from human placenta is described. Antibodies to human ezrin, together with antibodies to other microfilament-associated proteins, were used to follow the distribution and phosphorylation of these proteins in A-431 cells after EGF treatment. EGF induces the formation of microvillar-like surface structures on these cells within 30 s and these give way to membrane ruffles at ~2–5 min after EGF addition; the cells then round up after ~10–20 min. Ezrin is recruited into the microvillar-like structures and the membrane ruffles, and is phosphorylated on tyrosine and serine in a time course that parallels the formation and disappearance of these surface structures. Spectrin is recruited into the membrane ruffles and shows a similar rapid kinetics of phosphorylation, but only on serine residues, and remains phosphorylated through the rounding up of the cells. The microvillar-like structures and membrane ruffles are also enriched in fimbrin and α-actinin. Myosin becomes rapidly reorganized into a striated pattern that is consistent with it playing a role in cell rounding. These results show that two cortical proteins, ezrin and spectrin, become phosphorylated in a time course coincident with remodeling of the cell surface. The results are consistent with the notion that ezrin phosphorylation may play a role in the formation of cell surface projections whereas spectrin phosphorylation may be involved in remodelling of more planar areas of the cell surface.

CELLS in multicellular organisms respond to external signals in a variety of ways. Among the effectors that impinge on higher cells are growth factors that induce a number of changes in their target cells (reviewed in Carpenter and Cohen, 1979; Carpenter, 1987). The response of the human carcinoma A-431 cell line to added EGF has been extensively studied as it contains an abundance of surface receptors for this growth factor (Fabricant et al., 1977). The only known enzymatic activity induced by the binding of EGF to its receptor is the induction, on the cytoplasmic surface of the plasma membrane, of its intrinsic tyrosine kinase activity (Ushiro and Cohen, 1980). The response of the cells to EGF is complicated; very early responses (in <30 s) include an increase in intracellular-free Ca^{2+} and the generation of specific inositol phosphates (Moolenaar et al., 1986; Wheeler et al., 1987; Hepler et al., 1987; Gonzalez et al., 1988; Wahl and Carpenter, 1988; Wahl et al., 1988). Some proteins, including the receptor itself, become phosphorylated on tyrosine over a period of minutes to hours (Cohen et al., 1980; Hunter and Cooper, 1981; Sawyer and Cohen, 1985). Over the first several minutes the cells undergo a series of morphological changes that eventually result in their rounding up and the endocytosis of the bulk of the receptors (reviewed in Carpenter, 1987). Long term effects include the stimulation of DNA synthesis and cell division.

We are especially interested in understanding the molecular details of the rapid changes in the microfilament cytoskeleton in A-431 cells in response to EGF. Chinkers et al. (1979, 1981) were the first to show that treatment of A-431 cells with EGF induced membrane ruffling after 2–5 min and ultimately induced the rounding up of these cells. Coincident with these early morphological changes is a rapid increase in pinocytic activity (Haigler et al., 1979). With the discov-

ery of the ligand-inducible protein-tyrosine kinase activity of the receptor, it became important to identify substrates for the receptor kinase as possible clues to its mode of action. Fava and Cohen (1984) identified a 35-kD polypeptide (known as p35) that was a substrate for the receptor kinase in vitro and Sawyer and Cohen (1985) found that p35 started to become phosphorylated in vivo ~30 min after EGF addition. Subsequently this polypeptide was identified as a lipocortin (Pepinsky and Sinclair, 1986). Proteins identical and closely related to the A-431 cell p35 (Huang et al., 1986; Saris et al., 1986; see Brugge, 1986, for review) are localized under the plasma membrane in a pattern largely coincident with that of spectrin (Radke et al., 1983; Nigg et al., 1983; Greenberg and Edelman, 1983; Mangeat and Burridge, 1984). Another substrate, known as p81, was identified in A-431 cells by Hunter and Cooper (1981) and found to be phosphorylated 2 min after addition of EGF, although its function and location was unknown.

Over the last 10 yr we have been studying the molecular structure of the microvillar cytoskeleton of chicken intestinal epithelial cells (Bretscher, 1986*a*). Among the proteins of this structure that we have purified is a minor component with an apparent molecular mass of 80,000 D that we have termed ezrin (Bretscher, 1983, 1986*b*). Using antibodies to ezrin we have shown that it is present, as expected, in the microvilli of fixed intestinal epithelial cells, and also in other cells in surface structures that contain an actin cytoskeleton (Bretscher, 1983). The study of chicken intestinal ezrin has been hampered in part because the available protocols for its purification yield very small amounts of material (Bretscher, 1983, 1986*b*); consequently nothing is currently known about its function in this structure. The p81 protein-tyrosine kinase substrate identified by Hunter and Cooper (1981) turns out to be identical or very closely related to chicken intestinal ezrin (Gould et al., 1986). Thus there appears to be a close correlation between the EGF-induced phosphorylation of p81 in A-431 cells noted by Hunter and Cooper (1981) and the rapid morphological changes induced by EGF in A-431 cells as described by Chinkers et al. (1979, 1981). We wished to study this correlation in more detail, and investigate the possible participation of other key microfilament-associated proteins in these morphological changes. Although our antibodies to chicken ezrin cross react with the human protein in A-431 cells, they react much more weakly than with the chicken protein. It was therefore desirable to purify ezrin from a human source, preferably in quantities sufficient for biochemical studies, and develop antibodies to it. In this study we report the purification of ezrin from human placenta and the preparation of antibodies to it. We also describe in more detail the defined series of morphological changes that occur in these cells in response to EGF, the localization of actin, ezrin, spectrin and myosin, and the coincident phosphorylation of ezrin and spectrin during these changes.

Materials and Methods

Purification of Human Ezrin

A key property of chicken and human ezrin is that they bind to hydroxyapatite under conditions where most other proteins do not; a hydroxyapatite column was an essential feature of ezrin purification from chicken brush borders (Bretscher, 1983, 1986*b*) and is an essential feature of ezrin purification from human placenta. Human placenta obtained after Caesarian delivery was frozen as soon as possible and stored at −70°C. Proteolysis is a problem during the purification of ezrin from placenta, so all steps were performed at 4°C and all buffers were made 0.5 mM in PMSF and 0.5 mM in benzamidine before use. 80 g of frozen placenta was thawed and homogenized in 320 ml 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, in a blender (model 7011, Waring Products Div., New Hartford, CT) operating at full speed for 15 s. The homogenate was clarified at 47,000 *g* for 20 min and the supernatant recovered and 100 μl of diisopropyl fluorophosphate (Aldrich Chemical Corp., Milwaukee, WI) added to reduce proteolysis. The extract was made 40% saturation (22.6 g/ml) in ammonium sulfate and the precipitated material removed by centrifugation. The ammonium sulfate concentration was increased to 65% (by addition of 15.3 g/100 ml) and the resulting precipitated material collected by centrifugation and dissolved in 20 ml of 20 mM NaCl, 1 mM DTT, 10 mM Imidazole-HCl, pH 6.7, and dialyzed for 3 h against this buffer. During this period a precipitate formed that was removed by centrifugation at 47,000 *g* for 20 min. The clarified material was made 100 mM in phosphate from a 0.8-M KH_2PO_4-K_2HPO_4, pH 7.0, stock solution and applied to a 10-ml hydroxyapatite column (HA-Ultogel; LKB Instruments, Inc., Gaithersburg, MD) equilibrated with 100 mM phosphate buffer. The column was developed with a 40-ml gradient of 100–800 mM potassium phosphate, pH 7.0, buffer. Fractions were monitored by SDS-PAGE on 7% minigels and fractions rich in ezrin pooled and dialyzed against 20 mM NaCl, 1 mM DTT, 20 mM Bis-tris-propane, pH 6.7, for 3 h. The material was then filtered and applied to a Mono Q FPLC column (Pharmacia Fine Chemicals, Piscataway, NJ) and developed with a 40-ml gradient 20 mM to 1 M NaCl in the dialysis buffer. Fractions rich in ezrin were pooled, dialyzed against 20 mM NaCl, 1 mM DTT, 20 mM MES, pH 6.7, and applied to a Mono S column (Pharmacia Fine Chemicals) and developed with a 40-ml gradient in this buffer from 20 to 1,000 mM NaCl. Homogeneous ezrin eluted at ~440 mM NaCl. Essentially identical results were obtained when Q-Sepharose and S-Sepharose columns were substituted for the Mono Q and Mono S columns, respectively.

Cell Culture, EGF Treatment, and ^{32}P- and ^{35}S-labeling of Cells

A-431 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DME supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY). Subconfluent cells were used in all experiments. For each experiment, cells in 100-mm plates were washed four times in filter-sterilized ABS buffer (6.4 g/liter NaCl; 0.3 g/liter $CaCl_2$–$2H_2O$; 0.4 g/liter KCl; 4 g/liter $NaHCO_3$; 0.2 g/liter $MgSO_4$–$7H_2O$; 1 g/liter glucose; 0.1 g/liter Na pyruvate; 2.13 g/liter BES, pH 7.4, with NaOH) at 37°C and then incubated for 1 h at 37°C in 4 ml ABS. When appropriate, 0.5 mCi of ^{32}P (Amersham Corp., Arlington Heights, IL; carrier-free) was added per 100-mm plate and incubation continued for 1 h at 37°C. EGF (Boehringer Mannheim Biochemicals, Indianapolis, IN; receptor grade) was then added to a final concentration of 200 ng/ml and the cells incubated for appropriate times at 37°C. At the various time points, cells were quickly washed with 3 ml ABS at 4°C and scraped into 0.9-ml extraction buffer (0.6 M KCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 0.15 M NaCl, 1 mM EDTA, 25 mM Tris, 100 μM $NaVO_4$, 50 mM NaF, 0.25 mM PMSF, 0.5 mM benzamidine, pH 7.4). The samples were then clarified by centrifugation at 50,000 *g* at 4°C for 1 h before being subjected to immunoprecipitation. For uniform ^{35}S-labeling of cells, cells were washed as described above, 200 μCi [^{35}S]methionine (Translabel; Amersham Corp.) added per dish, incubated for 30 min at 37°C, and then the removed growth medium returned and the cells incubated overnight at 37°C. The next day extracts were made as described above.

Samples for phosphoamino acid analysis were immunoprecipitated, the respective bands excized from the gels, electrophoretically eluted, lyophilized, hydrolyzed, and subjected to one-dimensional analysis as described (Cooper et al., 1983).

Cytoskeletal preparations were made by adding 0.9 ml of room temperature 0.5% Triton X-100, 75 mM KCl, 0.1 mM $MgCl_2$, 1 mM EGTA, 10 mM Imidazole-HCl, pH 7.4, to a 100-mm plate, recovering the soluble material, and then dissolving the cytoskeletal preparations as described for whole cells above. Both the soluble material and dissolved cytoskeletal residues were used for immunoprecipitation.

Immunoprecipitations

100 μl of extract was mixed with 20 μl of the appropriate antiserum and

200 μl of a 25% suspension of protein A-Sepharose (Pharmacia Fine Chemicals; LKB Instruments Inc.) in RIPA-VF buffer (0.1% SDS, 1% Triton X-100, 1% deoxycholate, 0.14 M NaCl, 1 mM EDTA, 25 mM Tris, 100 μM $NaVO_4$, 50 mM NaF; 0.25 mM PMSF, 0.5 mM benzamidine, pH 7.4). The mixture was shaken for 2 h at 4°C and then the immunoprecipitate washed 10 times by briefly sedimenting the beads in a microfuge, removing the supernatant, and resuspending the beads in 0.8 ml RIPA-VF buffer. The beads were then washed once in 25 mM Tris-HCl, pH 7.5, resuspended in 50 μl of double strength Laemmli sample buffer (Laemmli, 1970), boiled, centrifuged, and the supernatants recovered for gel electrophoresis. To elicit antibodies to human ezrin, p81 from the Mono S column was subjected to preparative SDS gel electrophoresis and used to immunize rabbits as described in detail (Bretscher, 1983). In outline, rabbits were injected with 0.5 mg of ezrin in Freund's complete adjuvant, then boosted with 0.5 mg ezrin in Freund's incomplete adjuvant on days 21 and 42. 10 d later the rabbits were found to have antibodies to ezrin as determined by double diffusion analysis and were sacrificed for their serum. The spectrin antiserum has been described (McOsker and Bretscher, 1985); the myosin antiserum was generated against the heavy chains of brush border myosin purified as described (Krizek et al., 1987), and excised from SDS gels. In immunoprecipitation experiments on extracts of A431 cells using this antiserum, myosin heavy chain was precipitated, but no coprecipitating light chains could be detected. Antiserum to chicken ezrin has been described (Bretscher, 1983).

Immunofluorescence Microscopy

Antigen affinity purified antibodies to brush border actin and spectrin were as described (Bretscher and Lynch, 1985; McOsker and Bretscher, 1985). Antibodies to human ezrin and myosin were purified from their antisera on purified immobilized antigens as described (Bretscher, 1983). For immunofluorescence microscopy, cells were grown on coverslips and treated exactly as described above for labeling the cells, except that the ^{32}P was omitted. They were then plunged into −20°C ethanol for 5 min, and rehydrated in PBS (Ca^{2+}-, Mg^{2+}-free). Antibodies in PBS was added to the coverslip and incubated at room temperature for 30 min. The primary antibodies were used at the following concentrations: anti-actin, 50 μg/ml; anti-myosin, 50 μg/ml; anti-spectrin, 50 μg/ml; anti-human ezrin, 5 μg/ml. The coverslips were washed with PBS, then with PBS made pH 9.0 by the addition of 10 mM Tris base, and again in PBS. The second antibody, fluorescein-labeled goat anti-rabbit IgG (Miles Scientific Division, Naperville, IL) that had been preabsorbed on fixed A-431 cells, was added at a dilution of 1:20. The coverslips were incubated at room temperature in the dark for 30 min, washed in PBS, and mounted in medium containing phenyldiamine. The cells were observed in a Zeiss Universal fluorescence microscope and cellular distributions recorded on Tri-X film (Eastman Kodak Co., Rochester, NY).

Results

Purification of Human Placental Ezrin and Characterization of Ezrin Antibodies

Ezrin has previously only been purified from chicken intestinal epithelial cells (Bretscher, 1983, 1986*b*). Polyclonal antibodies to this protein have identified a p81 polypeptide in human carcinoma A-431 cells that is immunologically and chemically related to chicken ezrin (Gould et al., 1986). These antibodies have also revealed that it is present in human placental microvilli (Edwards and Booth, 1986). We therefore adapted our method for the isolation of chicken ezrin to allow for its purification from human placenta; polypeptide compositions at each step in the preparation are shown in Fig. 1. Both chicken and human ezrin bind strongly to hydroxyapatite and this is the key step in the purification of the two proteins. After ammonium sulfate fractionation and dialysis of a placental extract, the material (Fig. 1, lane *A*) was applied to a hydroxyapatite column under conditions where the vast majority of proteins did not bind. After extensive washing, the column was eluted with a phosphate gradient and two polypeptides, with apparent molecular masses of 81 and 77 kD eluted towards the end of the gradient (lanes *E–H*). Fractions rich in the 81-kD polypeptide were pooled (lane *I*), dialyzed, and chromatographed on a Mono Q anion exchange column (lanes *J–O*). This yielded a highly enriched preparation of the 81- and the 77-kD polypeptides and some minor species, most of which were proteolytic degradation products. Enriched fractions were pooled (lane *P*) and chromatographed on a Mono S cation exchange resin which yielded homogeneous 81-kD polypeptide (lane *Q*) and 77-kD polypeptide slightly contaminated with the 81-kD species (lane *R*). From 100 g of placenta we routinely obtain several

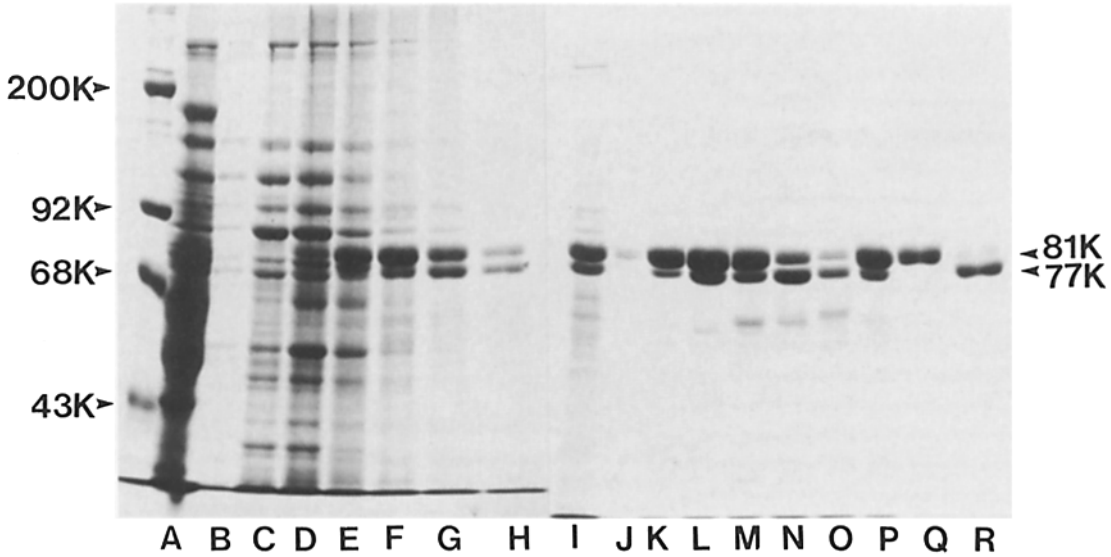


Figure 1. 7.5% SDS polyacrylamide gel of the steps in the purification of human placental ezrin. Lane *A*, proteins applied to hydroxyapatite column; lane *B–H*, peak fractions eluting from the hydroxyapatite column; lane *I*, pooled fractions that were applied to the Mono Q column; lanes *J–O*, peak fractions from Mono Q column; lane *P*, pooled fractions that were applied to the Mono S column; lane *Q*, peak p81 fraction from Mono S column; lane *R*, peak p79 fraction from Mono S column. Molecular mass standards are shown at left.

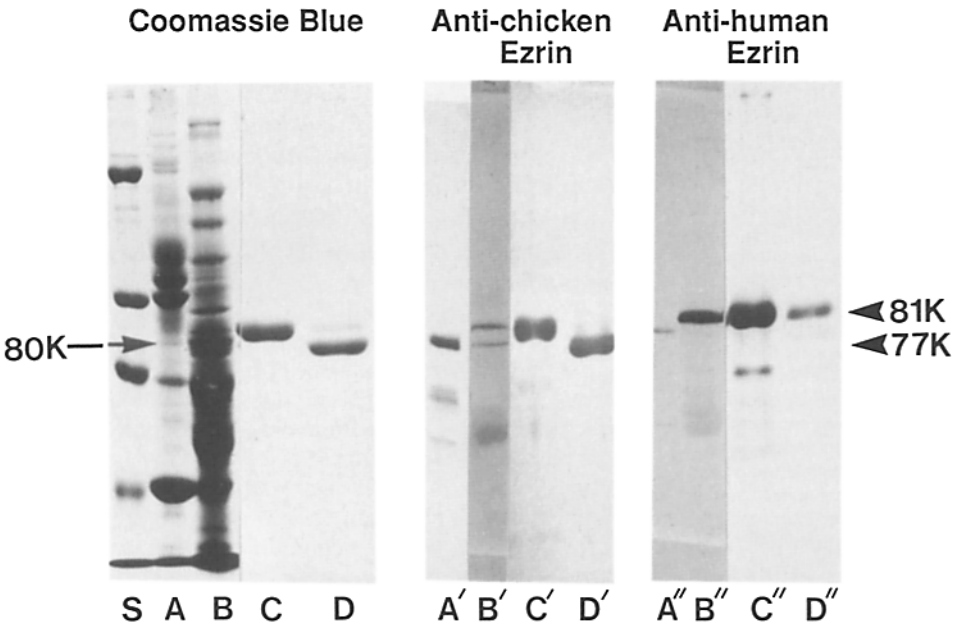


Figure 2. Characterization of the antibody to human ezrin. Coomassie Blue–stained 7.5% SDS gel is shown with (lane *A*) microvillar cores, (lane *B*) placental extract loaded on hydroxyapatite column, (lane *C*) purified p81, (lane *D*), purified p77. Lanes *A′–D′* and *A″–D″* are immunoblots probed with antibody raised against chicken intestinal ezrin (*A′–D′*) and human placental (81 kD) ezrin (lanes *A″–D″*). Lane *S* at left were the same molecular mass standards as shown in Fig. 1. Chicken ezrin is indicated as 80K at left and the human p81 and p77 at right.

milligrams of the Mono Q fractions, but poor recovery has been observed from the Mono S column yielding less than a milligram of each polypeptide species. Antibodies were elicited in rabbits to the Mono S purified 81 kD and characterized by immunoblotting (Fig. 2). Antibodies to the chicken protein recognize both the 81- and 77-kD polypeptides in crude placental extracts (lane *B′*) and purified fractions (lanes *C′* and *D′*). Antibodies to the 81-kD protein recognize chicken ezrin, although rather weakly (lane *A″*), and the placental 81-kD polypeptide (lanes *B″* and *C″*) but not, surprisingly, the 77-kD polypeptide (lanes *B″* and *D″*, the slight contamination of the 77-kD polypeptide with the 81-kD species is responsible for the reactivity seen in lane *D″*). These results indicate that the placental 81-kD is human ezrin; the 77-kD polypeptide might be a degradation product, or more likely, a protein closely related to the 81-kD human ezrin.

Ezrin and Spectrin Become Specifically Phosphorylated after EGF Treatment of A-431 Cells

Since we knew that ezrin is a substrate for a tyrosine kinase activity after treatment of these cells with EGF (Gould et al., 1986), we wanted to explore the state of phosphorylation as a function of time after the addition of EGF. In addition, we chose to examine the phosphorylation state of two other proteins, spectrin and myosin, believed to play key roles in cellular shape changes.

The antisera to human ezrin, myosin heavy chain, and spectrin were first characterized with respect to their ability to immunoprecipitate the relevant proteins from ^{35}S-labeled A-431 cells (Fig. 3). A single polypeptide of 81 kD was immunoprecipitated by the antiserum to human ezrin (lane *A*), a single polypeptide of 200 kD by the antiserum to myosin heavy chain (lane *C*), and a doublet at ~240K by the spectrin antiserum (lanes *B* and *D*). Although this antiserum only reacts with the 240K subunit of spectrin (McOsker and Bretscher, 1985), immunoprecipitation conditions were chosen to maintain the 235K/240K spectrin heterodimer; hence both chains were recovered in the immunoprecipitates.

To explore the effect of EGF treatment on protein phosphorylation, A-431 cells were prelabeled with ^{32}P for 1 h and proteins then analyzed for ^{32}P content at various times after EGF addition. A time course after EGF addition re-

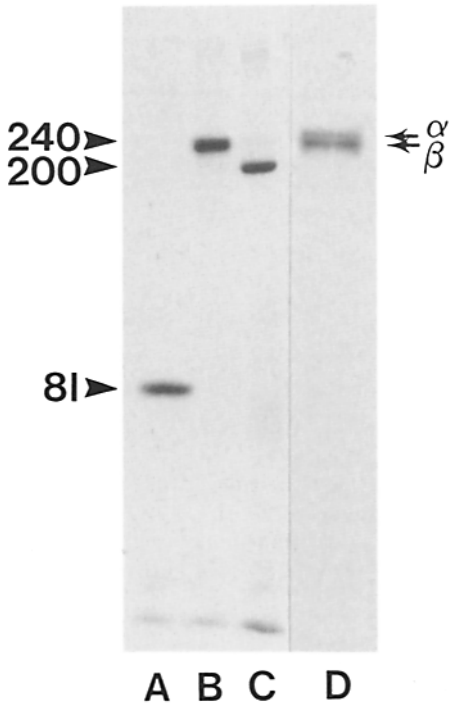


Figure 3. Immunoprecipitation of ezrin, spectrin, and myosin from A-431 cells. ^{35}S-labeled cells were harvested, extracts made, and were subjected to immunoprecipitation. The resulting immunoprecipitates were resolved on a 6.5% SDS gel and subjected to autoradiography. Lane *A*, material precipitated with antiserum to human ezrin; lanes *B* and *D*, material precipitated with antiserum to spectrin; lane *C*, material precipitated with antiserum to myosin heavy chains. Lane *D* is part of the gel with a lower loading of the immunoprecipitate to show that both the α and β chains of nonerythroid spectrin, as indicated, are immunoprecipitated.

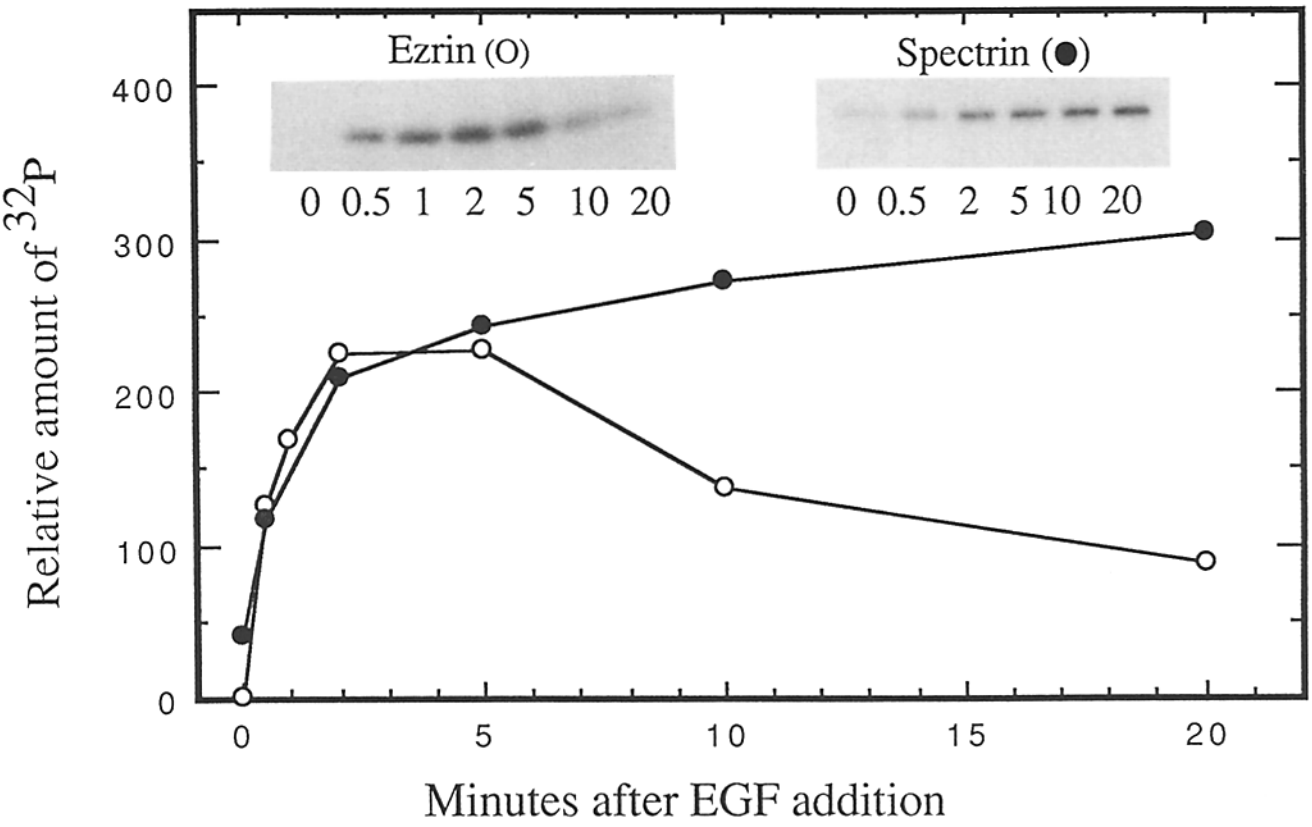


Figure 4. Phosphorylation of ezrin and spectrin as a function of time after addition of EGF to A-431 cells. Cells were incubated in ^{32}P for 1 h and then EGF was added. At each time point the cells were harvested, and the relevant proteins immunoprecipitated from extracts. The figure shows the relative amount of ^{32}P in ezrin (○) and spectrin (●) as a function of time as determined by scanning densitometry of the autoradiograms shown. The zero time point was just before addition of EGF to the culture. Resolution of the spectrin immunoprecipitates on lower percentage gels revealed that all the radioactivity was found in the β chain.

vealed no obvious changes in phosphorylation pattern when total phosphoproteins were analyzed by gel electrophoresis (not shown). However, by immunoprecipitation we found that both spectrin and ezrin became phosphorylated in a time-dependent manner (Fig. 4). Before addition, no radioactivity was associated with the immunoprecipitated ezrin or myosin heavy chains, and a small amount with spectrin. Samples taken at various times after EGF addition revealed that ezrin became significantly phosphorylated after 30 s, peaking at ~2–5 min, and then the phosphorylation level declined until the end of the experiment at 20 min. Spectrin phosphorylation also rapidly increased upon EGF addition, rising rapidly over the first 2 min, and then reaching a plateau at 5–20 min. Resolution on lower percentage gels revealed that all the label was incorporated into the 235K β-subunit of nonerythroid spectrin (not shown). No phosphorylation of the myosin heavy chains was detected. In parallel experiments with ^{35}S-labeled cells, the amounts of ezrin, spectrin, and myosin did not change during EGF treatment (not shown).

Earlier work has revealed that human ezrin is phosphorylated on tyrosine and threonine in response to EGF in A-431 cells (Gould et al., 1986). We therefore analyzed the phosphoamino acids present in ezrin 2 min after EGF addition and in spectrin 5 min after EGF addition (Fig. 5). In hydrolysates of ezrin, labeled phospho-serine and phospho-tyrosine were found, but only phospho-serine in hydrolysates of spectrin.

Localization of Actin, Ezrin, Spectrin, and Myosin in A-431 Cells after Treatment with EGF

A-431 cells are known to undergo a series of rapid morphological changes in response to the addition of EGF (Chinkers

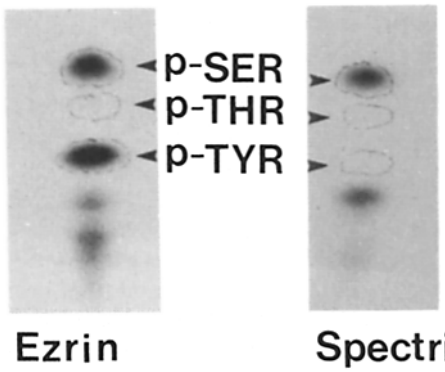
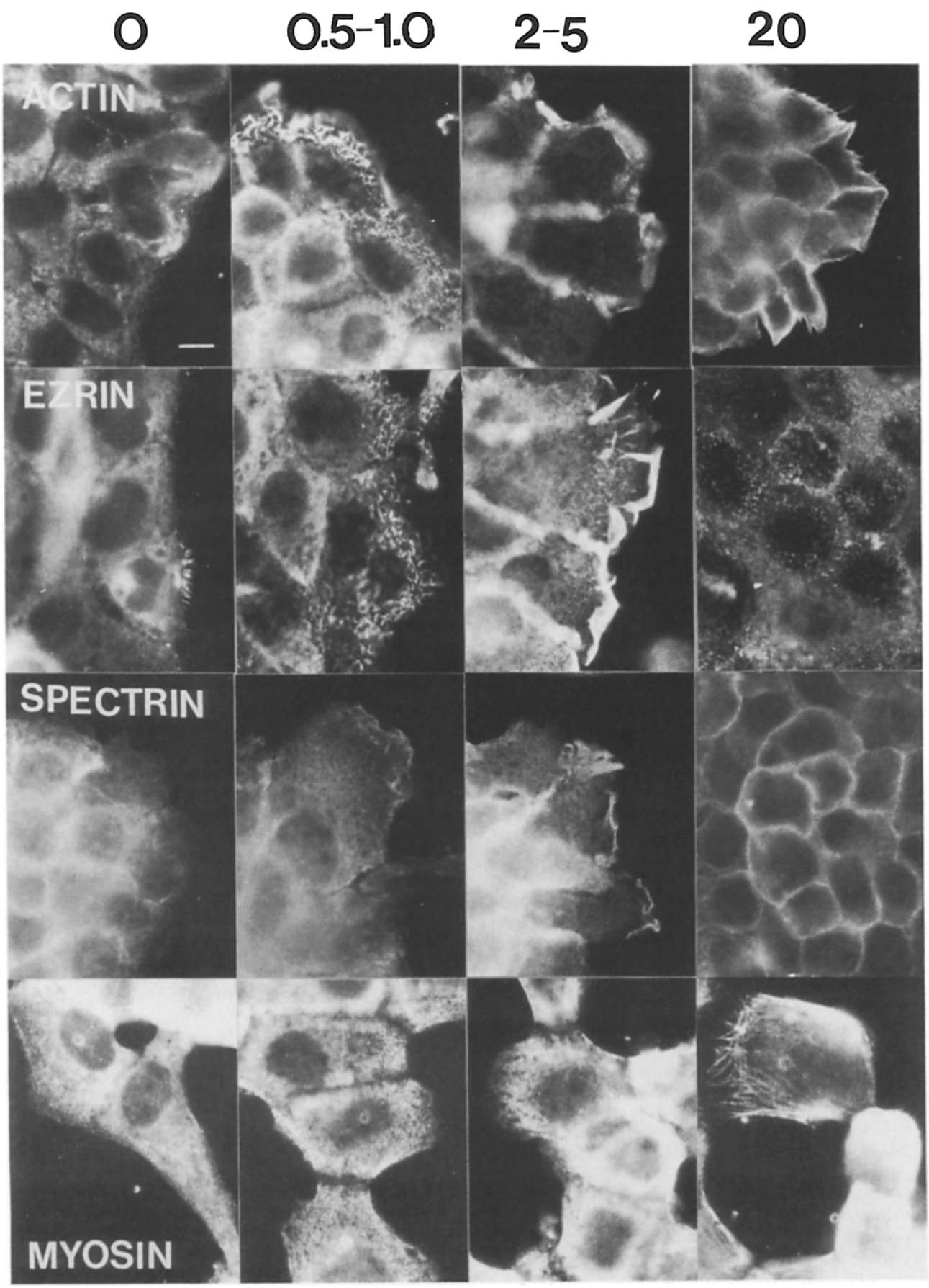


Figure 5. Phosphoamino acid analysis of ezrin 2 min after EGF addition, and spectrin 5 min after EGF addition. Ezrin contains phospho-serine and phospho-tyrosine, whereas only phospho-serine is evident in the spectrin sample.


0
0.5-1.0
2-5
20
ACTIN
EZRIN
SPECTRIN
MYOSIN

et al., 1979, 1981). We therefore wished to examine the distribution of some key microfilament-associated proteins by indirect immunofluorescence microscopy during these changes. The distribution of actin, myosin, ezrin, and spectrin was examined by immunofluorescence microscopy in A-431 cells before or after treatment with EGF. These cells are very poor subjects for the localization of proteins by immunofluorescence microscopy as they are rather rounded, the typical morphology of transformed cells. However, some aspects of the localization of these proteins could be clearly determined from these experiments. An overview of these events is shown in Fig. 6. Higher resolution micrographs of early events are shown in Fig. 7.

The localization of actin revealed a complicated series of surface events immediately after EGF treatment (Fig. 6, *top row*). Unstimulated cells revealed a rather featureless image. 30 s after EGF addition (the earliest reliable time point), an abundance of surface structures resembling large microvilli were found covering the entire surface of the cells. These structures then gave way to membrane ruffles that initiated at the edge of cells and were very prominent at 2–5 min after EGF addition; these are presumably the membrane ruffles described by Chinkers et al. (1979). The surface activity then declined and the cells rounded up; this was essentially complete at 20 min (*top row, last column*). Immunofluorescence microscopy was then used to explore the distribution of the selected proteins during the remodeling of the cell surface. Ezrin was clearly recruited into both the structures found at 30 s and the membrane ruffles that came after (Fig. 6, *second row, second column*; Fig. 7, *A–E*). It was then found in a very finely punctate pattern on the surface of the rounded cells at 20 min. By contrast, spectrin was not detectable in the surface structures generated after 30 s, but was clearly present in the membrane ruffles later in the morphological sequence (Fig. 6, *third row, third column*). At 20 min, spectrin reveals its typical submembranous localization in the rounded cells. Experiments with antibodies to fimbrin and α-actinin revealed that, as expected (Bretscher and Weber, 1980; Lazarides and Burridge, 1974), these proteins were also recruited into the structures that appeared after 30 s and into the membrane ruffles (data not shown). Myosin was not found in any of the cell surface structures. Before addition of EGF it was present in a rather uniform distribution (Fig. 6, *last row, first column*) but by 30 s and at later time points it is clearly arranged in a striated pattern (Fig. 6, *remaining columns*; Fig. 7, *F*).

To explore any possible correspondence between phosphorylation and association of specific proteins with the cytoskeleton, we examined the distribution of both ^{35}S-labeled proteins and ^{32}P-labeled phospho-proteins between the cytoskeleton and soluble fractions of cells before and at various times after treatment with EGF. Since essentially the same results were obtained before and at each time point after EGF addition, only the two minute time point is shown (Fig. 8). As described earlier (Gould et al., 1986) about half the ezrin is cytoskeletally associated, and this does not change significantly during EGF stimulation; the phosphorylated form does not show any significant preferential association with the soluble or cytoskeletal fractions. Essentially all the spectrin was recovered with the cytoskeletal fractions at all times after EGF addition, and no selective solubilization of phosphorylated spectrin could be detected.

Discussion

In this study, we describe the purification of human ezrin, a known substrate for several protein-tyrosine kinases including the products of certain oncogenes (Cooper et al., 1982), the generation of antibodies to this protein, and their use in following its distribution and phosphorylation in A-431 cells before and after treatment with EGF. We have also followed the distribution and phosphorylation of spectrin and myosin in these cells. Myosin undergoes a reorganization into an ordered striated pattern that is consistent with its probable role in the cell rounding that occurs during the several minutes after EGF treatment. A major result of this study is the remarkable correspondence between the rapid phosphorylation of ezrin and spectrin and their appearance in transitory surface structures that contain these proteins. This is in contrast to the relatively slower rate of phosphorylation of p35, where phosphorylation is barely detectable 5 min after EGF treatment and increases up to at least 4 h (Sawyer and Cohen, 1985).

The purification of ezrin from chicken brush borders has yielded variable results (Bretscher, 1983, 1986*b*), whereas the purification of ezrin from human placenta reported here reliably yields milligram quantities of protein. Two proteins were recovered, with apparent molecular masses of 81 and 77 kD, that cross reacted with antibodies to chicken ezrin. Antibodies to the human 81-kD protein reacted with chicken ezrin, but not the 77-kD protein. Peptide maps of chicken ezrin and human placental 81 kD are very similar, and in fact many of the peptides with similar mobilities have identical sequences (K. Gould, T. Hunter, and A. Bretscher, unpublished data); we have therefore designated this protein as human ezrin. The 77-kD protein is always recovered as part of the preparation and is found in the initial extracts of placenta (Fig. 2) and in certain cultured mouse and rat lines (Bretscher, 1983); it is therefore unlikely to be a degradation product. It binds tightly to hydroxyapatite, elutes slightly behind the 81-kD polypeptide from Mono Q and is clearly resolvable from the 81-kD polypeptide on Mono S. The antiserum to chicken ezrin cross reacts with it and the 81-kD polypeptide equally well (Fig. 2). Based on these findings we believe that the 77-kD is a distinct isoform of human ezrin,

Figure 6. Immunofluorescence microscopy localization of actin, ezrin, spectrin, and myosin in A-431 cells at various times, indicated in minutes above the micrographs, after addition of EGF. The plane of focus was adjusted to record the most prominent features for each antibody: for actin, ezrin, and spectrin at 0, 0.5–1.0, and 2–5 min, and also for actin at 20 min; the plane of focus is on the upper surface near the cell periphery; for ezrin and spectrin at 20 min the focal plane is on the upper surface in the center of the cells; for myosin all micrographs show its distribution on the lower surface of the cells. Bar, 10 μm.

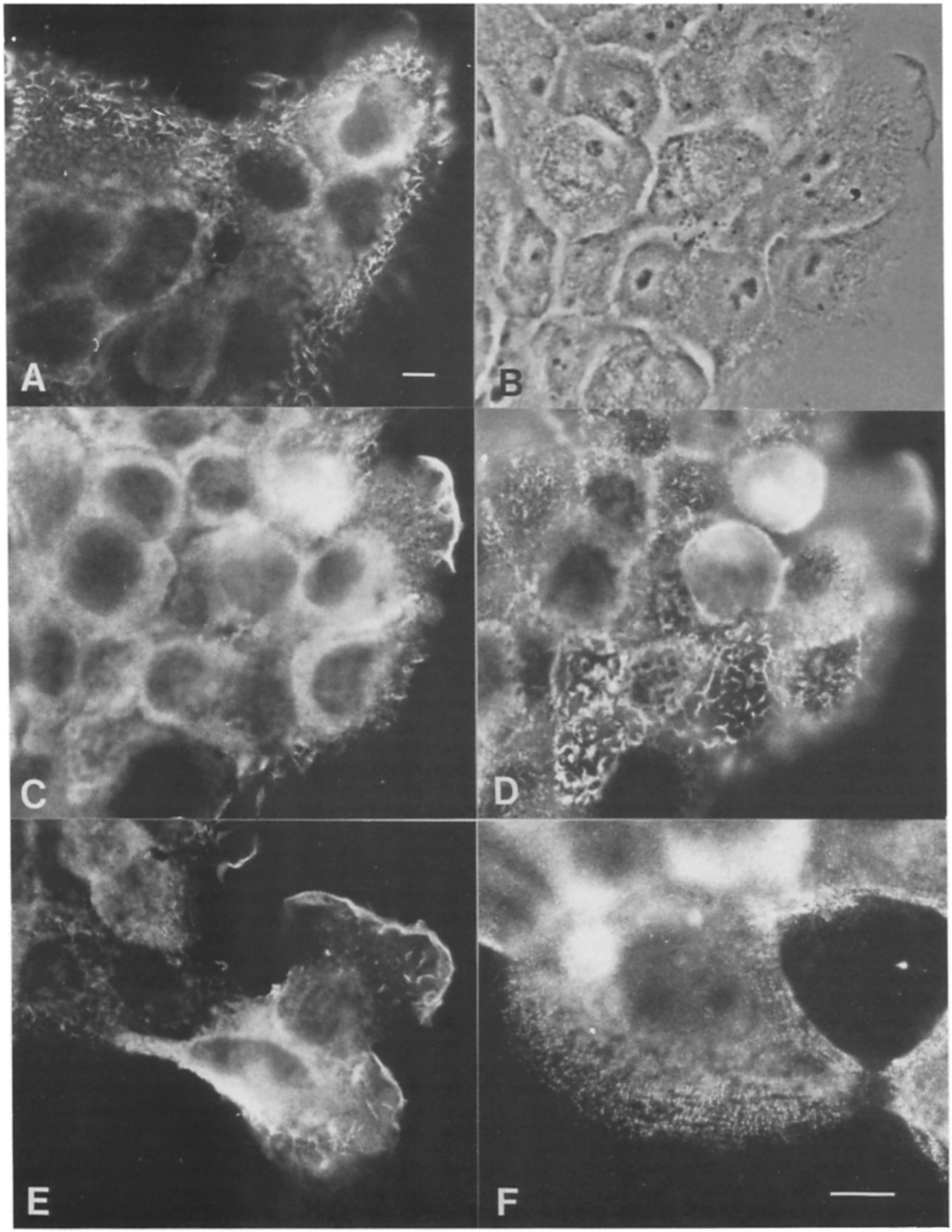


Figure 7. Micrographs of cells stained with antibodies to ezrin (*A–E*) and the heavy chains of myosin (*F*). *A* shows the ezrin-containing surface structures generated 30 s after addition of EGF. *B–D* show a group of cells 1 min after EGF addition as viewed by phase-contrast (*B*) and by immunofluorescence with ezrin antibody as seen in two focal planes (*C*, just above the substratum, and *D*, on the upper surface

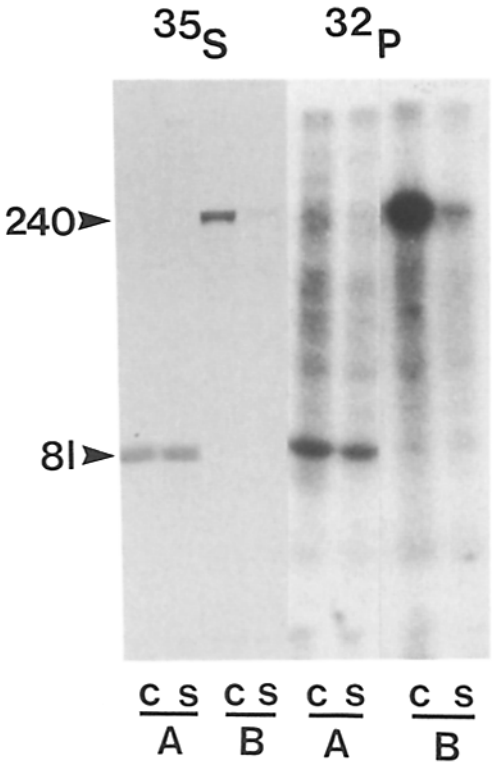


Figure 8. Distribution of ^{35}S-labeled ezrin (*A*) and spectrin (*B*) and ^{32}P-labeled ezrin (*A*) and spectrin (*B*) between soluble (*S*) and cytoskeletal (*C*) fractions in A-431 cells 2 min after EGF addition. Duplicate sets of plates were labeled with ^{35}S or ^{32}P and soluble fractions and cytoskeletal fractions recovered before or at various times after EGF addition. Each fraction was then subjected to immunoprecipitation with the respective antibodies. No changes were found during the time course in the distribution of ^{35}S as a result of EGF treatment, and the relative distribution of the ^{32}P label paralleled the ^{35}S label; hence only one time point is shown here.

although protein chemical evidence, currently in progress, is needed to establish this relationship.

Ezrin and β-spectrin rapidly become phosphorylated in A-431 cells in response to EGF. It is likely that the EGF receptor directly contributes to the tyrosine phosphorylation of ezrin as it appears to be in a similar location in these cells (see below). Clearly the phosphorylation of ezrin and spectrin on serine must be a result of some other protein kinase(s) that remain to be identified; a likely candidate is protein kinase C activated by diacylglycerol that is believed to be rapidly produced in A-431 cells in response to EGF (Carpenter and Cohen, 1979; Pike and Eakes, 1987; Wahl and Carpenter, 1988). The reason for the apparent discrepancy between this study where EGF was found to stimulate ezrin phosphorylation on tyrosine and serine, whereas previously it had been reported to stimulate phosphorylation on tyrosine and threonine (Gould et al., 1986), is not clear. It is worth noting, however, that very different conditions were used in the two studies (buffer vs. complete medium), and A-431 cells with apparently rather different morphologies were used. We have not determined the stoichiometry of ezrin and spectrin phosphorylation after EGF treatment. However, a previous estimate indicated that at least 10% of ezrin is phosphorylated on tyrosine 2 min after EGF treatment (Gould et al., 1986). The ratio of ^{32}P to ^{35}S, which measures relative phosphorylation levels, indicated that spectrin was phosphorylated to a greater extent than ezrin 2 min after EGF addition (see Fig. 8). We therefore suspect that both proteins become phosphorylated to a significant degree after EGF addition.

Using antibodies to actin and ezrin we have documented the very rapid appearance of cell surface structures in A-431 cells in response to EGF. Within 30 s, structures that look like giant microvilli appear on the cell surface and contain actin, ezrin, α-actinin, and fimbrin. In a recent study, Carpentier et al. (1987) localized the phosphorylated form of the EGF receptor in A-431 cells using a phosphotyrosine antibody. 1 min after EGF addition, they found that the receptor was fully phosphorylated and localized to cell surface structures similar to those described here; they were not able to observe the induction of these structures by EGF since the receptor is not phosphorylated before addition of EGF and therefore could not be visualized. Earlier studies indicate a random distribution for the receptor on unstimulated A-431 cells (Boonstra et al., 1985; Carpentier et al., 1986). A combination of their (Carpentier et al., 1987) and our results indicate that the receptor is also recruited into these surface structures and is therefore in a location near ezrin. The resolution of our experiments do not allow us to determine whether ezrin phosphorylation occurs before the surface structures appear, as would be expected if phosphorylation induces their formation, or is a consequence of both the receptor and ezrin being recruited to these structures. It is noteworthy that the EGF receptor in A431 cells has been reported to be associated with cytoskeletal elements (Wiegant et al., 1986), so a colocalization with certain cytoskeletal proteins is not unexpected. Nevertheless, the striking coincidence of the recruitment of ezrin into cell surface structures and the transitory nature of the phosphorylation strongly suggests that these two phenomena are related. In the absence of any knowledge of the function of ezrin, and no clear role for spectrin (Mangeat and Burridge, 1984), it is difficult to predict how these phosphorylations may alter their properties. It is difficult to believe that three proteins, ezrin, spectrin, and p35 (lipocortin), that likely play important roles in cell surface structure and dynamics are gratuitously phosphorylated in response to EGF. Rather, it is tempting to speculate that cell surface changes involving a filamentous actin core may respond to ezrin phosphorylation and the more barren areas respond to phosphorylation of the spectrin and p35 in the underlying cortical network. In the case of ezrin, the new method described here for its purification opens the way for a systematic study of this protein with the goal of elucidating its function and eventually assessing the possible role of phosphorylation on this function.

of the cells). Notice that structures over the entire cell surfaces and the appearance of the first membrane ruffle at the cell periphery. *E* is another group of cells 1 min after EGF addition showing the presence of some surface structures and membrane ruffles; micrographs such as these suggest that membrane ruffles are not generated by coalescence of the earlier surface structures. *F* shows the striated appearance of myosin distribution 2 min after EGF addition. Bar, 10 μm.

The author is greatly indebted to Janet Krizek who spent the better part of half a decade trying to improve the purification of ezrin from chicken intestinal cells and then found the placenta preparation relatively easy. She also helped extensively with all the other biochemical aspects of this work.

This work was supported by Public Health Service grant GM36652.

Received for publication 27 September 1988 and in revised form 4 November 1988.

References

Boonstra, J., P. Van Maurik, L. H. K. Defize, S. W. de Laat, J. L. M. Leunissen, and A. J. Verkley. 1985. Visualization of epidermal growth factor receptor in cryo-sections of cultured A-431 cells by immuno-gold labeling. *Eur. J. Cell Biol.* 36:209–216.

Bretscher, A. 1983. Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in non-muscle cells. *J. Cell Biol.* 97:425–432.

Bretscher, A. 1986*a*. The molecular structure of the microvillar cytoskeleton. Ion Gradient-coupled Transport. *Inserm (Inst. Nat. Sante Rech. Med.) Symp.* 26:13–21.

Bretscher, A. 1986*b*. Purification of the intestinal microvillus cytoskeletal proteins villin, fimbrin and ezrin. *Methods Enzymol.* 134:24–37.

Bretscher, A., and W. Lynch. 1985. Identification and localization of immunoreactive forms of caldesmon in smooth and nonmuscle cells: a comparison with the distributions of tropomyosin and α-actinin. *J. Cell Biol.* 100:1656–1663.

Bretscher, A., and K. Weber. 1980. Fimbrin: a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335–340.

Brugge, J. S. 1986. The p35/p36 substrates of protein-tyrosine kinases as inhibitors of phospholipase A_2. *Cell.* 46:149–150.

Carpenter, G. 1987. Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem.* 56:881–914.

Carpenter, G., and S. Cohen. 1979. Epidermal growth factor. *Annu. Rev. Biochem.* 48:193–216.

Carpentier, J.-L., A. R. Rees, R. Gregoriou, R. Kris, J. Schlessinger, and L. Orci. 1986. Subcellular distribution of the external and internal domains of the EGF receptor in A-431 cells. *Exp. Cell Res.* 166:312–326.

Carpentier, J.-L., M. F. White, L. Orci, and R. C. Kahn. 1987. Direct visualization of the phosphorylated epidermal growth factor receptor during its internalization in A-431 cells. *J. Cell Biol.* 105:2751–2762.

Chinkers, M., J. A. McKanna, and S. Cohen. 1979. Rapid induction of morphological changes in human carcinoma cells A-431 by epidermal growth factor. *J. Cell Biol.* 83:260–265.

Chinkers, M., J. A. McKanna, and S. Cohen. 1981. Rapid rounding of human epidermoid carcinoma cells A-431 induced by epidermal growth factor. *J. Cell Biol.* 88:422–429.

Cohen, S., G. Carpenter, and L. King. 1980. Epidermal growth factor-receptor-protein kinase interactions. *J. Biol. Chem.* 255:4834–4842.

Cooper, J. A., D. F. Bowen-Pope, E. Raines, R. Ross, and T. Hunter. 1982. Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. *Cell.* 31:263–273.

Cooper, J. A., B. M. Sefton, and T. Hunter. 1983. Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.* 99:387–402.

Edwards, H. C., and A. G. Booth. 1986. Calcium-sensitive, lipid-binding cytoskeletal proteins of the human placental microvillar region. *J. Cell Biol.* 105:303–312.

Fabricant, R. N., J. E. DeLarco, and G. J. Todaro. 1977. Nerve growth factor receptors on human melanoma cells in culture. *Proc. Natl. Acad. Sci. USA.* 74:565–569.

Fava, R. A., and S. Cohen. 1984. Isolation of a calcium-dependent 35-kilodalton substrate for the epidermal growth factor receptor/kinase from A-431 cells. *J. Biol. Chem.* 259:2636–2645.

Gonzalez, F. A., D. J. Gros, L. A. Heppel, and W. W. Webb. 1988. Studies on the increase in cytosolic free calcium induced by epidermal growth factor, serum, and nucleotides in individual A431 cells. *J. Cell. Physiol.* 135:269–276.

Greenberg, M. E., and G. M. Edelman. 1983. The 34 kd $pp60^{src}$ substrate is located at the inner face of the plasma membrane. *Cell.* 33:767–779.

Gould, K. L., J. A. Cooper, A. Bretscher, and T. Hunter. 1986. The protein-tyrosine kinase substrate, p81, is homologous to a chicken microvillar core protein. *J. Cell Biol.* 102:660–669.

Haigler, H., J. A. McKanna, and S. Cohen. 1979. Rapid stimulation of pinocytosis in human carcinoma cells A-431. *J. Cell Biol.* 83:82–90.

Hepler, J. R., N. Hakahata, T. W. Lovenberg, J. DiGuiseppi, B. Herman, H. S. Earp, and T. K. Harden. 1987. Epidermal growth factor stimulates the rapid accumulation of inositol (1,4,5)-trisphosphate and a rise in cystolic calcium mobilized from intracellular stores in A431 cells. *J. Biol. Chem.* 262:2951–2956.

Huang, K.-S., B. P. Wallner, R. J. Mattaliano, R. Tizard, C. Burne, A. Frey, C. Hession, P. McGray, L. K. Sinclair, E. P. Chow, J. L. Browning, K. L. Ramachandran, J. Tang, J. E. Smart, and R. B. Pepinsky. 1986. Two human 35 kd inhibitors of phospholipase A_2 are related to substrates of $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase. *Cell.* 46:191–199.

Hunter, T., and J. A. Cooper. 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A-431 human tumor cells. *Cell.* 24:741–752.

Krizek, J., L. M. Coluccio, and A. Bretscher. 1987. ATPase activity of the microvillar 110 kDa polypeptide-calmodulin complex is activated in Mg^{2+} and inhibited in K^+EDTA by F-actin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 225:269–272.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lazarides, E., and K. Burridge. 1975. α-Actinin: immunofluorescent localization of a muscle structural protein in non-muscle cells. *Cell.* 6:289–298.

Mangeat, P. H., and K. Burridge. 1984. Immunoprecipitation of nonerythrocyte spectrin within live cells following microinjection of specific antibodies: relation to cytoskeletal structures. *J. Cell Biol.* 98:1363–1377.

McOsker, C. C., and A. Bretscher. 1985. Fodrin is part of a filamentous cortical sheath of the detergent resistant cytoskeleton of cultured cells before and after cytochalasin treatment. *Eur. J. Cell Biol.* 39:321–327.

Moolenaar, W. H., R. J. Aerts, L. G. J. Tertoolen, and S. W. deLaat. 1986. The epidermal growth factor-induced calcium signal in A431 cells. *J. Biol. Chem.* 261:279–284.

Nigg, E. A., J. A. Cooper, and T. Hunter. 1983. Immunofluorescent localization of a 39,000-dalton substrate of tyrosine-protein kinase to the cytoplasmic surface of the plasma membrane. *J. Cell Biol.* 96:1601–1609.

Pepinsky, R. B., and L. K. Sinclair. 1986. Epidermal growth factor-dependent phosphorylation of lipocortin. *Nature (Lond.).* 321:81–84.

Pike, L. J., and A. T. Eakes. 1987. Epidermal growth factor stimulates the production of phosphatidylinositol monophosphate and the breakdown of polyphosphoinositides in A431 cells. *J. Biol. Chem.* 262:1644–1651.

Radke, K., V. C. Carter, P. Moss, P. Dehazya, M. Schliwa, and G. S. Martin. 1983. Membrane association of a 36,000 dalton substrate for tyrosine phosphorylation in chicken embryo fibroblasts transformed by avian sarcoma viruses. *J. Cell Biol.* 97:1601–1611.

Saris, C. J. M., B. F. Tack, T. Kristensen, J. R. Glenney, and T. Hunter. 1986. The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multidomain protein with internal repeats. *Cell.* 46:201–212.

Sawyer, T., and S. Cohen. 1985. Epidermal growth factor stimulates the phosphorylation of the calcium-dependent 35,000-dalton substrate in intact A-431 cells. *J. Biol. Chem.* 260:8233–8236.

Ushiro, H., and S. Cohen. 1980. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membrane. *J. Biol. Chem.* 255:8363–8365.

Wahl, M., and G. Carpenter. 1988. Regulation of epidermal growth factor-stimulated formation of inositol phosphates in A-431 cells by calcium and protein kinase. *J. Biol. Chem.* 263:7581–7590.

Wahl, M. I., T. O. Daniel, and G. Carpenter. 1988. Antiphosphotyrosine recovery of phospholipase C activity after EGF treatment of A-431 cells. *Science (Wash. DC).* 241:968–970.

Wheeler, L. A., G. Sachs, G. deVries, D. Goodrum, E. Woldemussie, and S. Muallem. 1987. Manoalide, a natural sesterterprenoid that inhibits calcium channels. *J. Biol. Chem.* 262:6531–6538.

Wiegant, F. A. C., F. J. Blok, L. H. K. Defize, W. A. M. Linnemans, A. J. Verkley, and J. Boonstra. 1986. Epidermal growth factor receptors associated to cytoskeletal elements in epidermoid carcinoma (A431) cells. *J. Cell Biol.* 103:87–94.