

The Protein-Tyrosine Kinase Substrate, p81, Is Homologous to a Chicken Microvillar Core Protein

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Abstract. p81, a protein-tyrosine kinase substrate previously identified in epidermal growth factor-treated A431 cells, is demonstrated to be homologous to ezrin, an 80-kD component of microvillar core proteins. p81 has been characterized using antiserum raised against purified chicken intestinal ezrin. p81, located by indirect immunofluorescent staining, is concentrated in surface projections of A431 cells such as microvilli and retraction fibers. None of the conditions of biochemical cell fractionation tested completely solubilizes p81; the insoluble p81 partitions as if associated with the cytoskeleton. The soluble form of p81 behaves as a monomer in all extraction proce-

dures studied. EGF-stimulated phosphorylation of p81 does not appear to change its intracellular location. p81 exhibits a wide tissue distribution with highest levels of expression in small intestine, kidney, thymus, and lung. Intermediate levels are found in spleen, thymus, lymph nodes, and bone marrow, with low levels in brain, heart, and testes. p81 is undetectable in muscle and liver. In A431 cells, p81 is phosphorylated on serine and threonine residues. Upon EGF treatment, ~10% of p81 becomes phosphorylated on tyrosine, and the phosphorylation of threonine residues increases.

PHOSPHORYLATION of proteins on tyrosine residues is a rare event in most normal cells (33). However, cells respond to the growth factors, epidermal growth factor (EGF)[†] and platelet-derived growth factor, with an increase in protein-tyrosine kinase activity as manifested by an elevation of phosphotyrosine in cellular protein (10, 34). There is a similar 5–10-fold increase in the phosphotyrosine content of cells transformed by acutely oncogenic retroviruses that encode protein-tyrosine kinases (33, 35, 50). Incubation of some normal cell lines with sodium orthovanadate, an inhibitor of phosphotyrosine-specific phosphatases, also leads to an increase in cellular phosphotyrosine and to transformation (39). These data suggest that phosphorylation of cellular proteins on tyrosine is involved in controlling cell growth and transformation (50). Some protein-tyrosine kinase substrates are likely to be proteins directly involved in regulating these processes.

Few substrate proteins for protein-tyrosine kinases have been identified. Three, lactate dehydrogenase, phosphoglycerate mutase, and enolase, are phosphorylated in transformed

chick cells and have known enzymatic functions (15). However, it has not yet been possible to demonstrate a change in function accompanying their phosphorylation. Other cellular proteins found to contain phosphotyrosine are vinculin (51, 52), and five proteins identified by their apparent molecular masses: p50 (5, 33, 46), p42 (18, 26, 40, 43), p36 (9, 10, 21, 34, 47), p35 (23), p81 (12, 13, 16, 34, 36), and p41 (10; Freed, E., personal communication). Functions have not yet been assigned to these latter proteins, and thus it has been difficult to assess the effect, if any, of their phosphorylation on cellular phenotype. Much work has been directed toward understanding the function of these proteins to ascertain whether their phosphorylation plays a role in transformation, mitogenesis, or differentiation.

In this study we demonstrate that p81 is homologous to an 80-kD protein purified from chicken intestinal epithelial cells (3, 4). This 80-kD protein, now called ezrin, is a minor component of the isolated microvillar cores of these brush border cells (3). Its location suggests it plays a structural role, but its exact function is not yet clear. Ezrin is present in a wide variety of cultured cells where it has been localized to cell surface structures (3). Phosphorylated p81 (pp81) was first detected in A431 cells, a human epidermoid carcinoma cell line, where a large increase in its phosphotyrosine content was observed after EGF treatment (34). Phosphotyrosine-

[†] Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; pp81, phosphorylated p81; RIPA-VF, buffer containing 10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% SDS, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1% Trasylol, 2 mM EDTA, 100 μ M Na₃VO₄, and 50 mM NaF.

containing pp81 was also detected in Snyder-Theilen-feline sarcoma virus (ST-FeSV) transformed A431 cells and ST-FeSV transformed NIH 3T3 cells (16, 36). The homology between ezrin and p81 has allowed us to study the intracellular location and tissue distribution of p81 and compare it with that of another protein-tyrosine kinase substrate, p36. In addition, we have characterized the phosphorylation sites in pp81 from A431 cells.

Materials and Methods

Cells and Antisera

A431 cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum. LIM1215 cells (55), a human colonic tumor line, were grown in RPMI supplemented with 10% fetal calf serum, 0.6 $\mu\text{g}/\text{ml}$ insulin, 1 $\mu\text{g}/\text{ml}$ dexamethasone, and 10^{-5} M α -thioglycerol. Rabbit anti-ezrin serum was prepared against purified chick intestinal ezrin as previously reported (3). Antiserum was used at a 1:200 dilution for Western blotting, and immunoprecipitations were performed with antiserum in excess. For use in indirect immunofluorescence and indirect immunoperoxidase staining, anti-ezrin Ig was affinity purified as described and used at 32 $\mu\text{g}/\text{ml}$ (3). Anti-p36 serum was prepared against partially purified chicken fibroblast protein and used as previously reported (14). Anti-fodrin serum was prepared against purified porcine brain fodrin (28) and was used in excess for immunoprecipitation.

Biosynthetic Labeling and Immunoprecipitation

Subconfluent cells were labeled by incubation with 50 $\mu\text{Ci}/\text{ml}$ or 1 mCi/ml for peptide mapping [^{35}S]methionine ($>1,000$ Ci/mmol, Amersham Corp., Arlington Heights, IL) at 37°C for 18 h in methionine-free DME supplemented with 5% calf serum and 5% DME or by incubation with 2–3 mCi/ml ^{32}P -orthophosphate (ICN Radiochemicals, Irvine, CA) at 37°C for 16–18 h in phosphate-free DME supplemented with 4% calf serum. Where indicated, EGF (Potter, S., The Salk Institute) was added at 60 ng/ml for 10 min at the end of the labeling period. Cells were washed three times in cold Tris-buffered saline before solubilization in either 400 μl for each 35-mm dish of cold RIPA-VF buffer (10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% SDS, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1% Trasylol (Mobay Chemical Corp., Pittsburgh, PA), 2 mM EDTA, 100 μM Na_3VO_4 , and 50 mM NaF) or 100 μl for each 35-mm dish of SDS lysis buffer (10 mM sodium phosphate, pH 7.2, 0.5% SDS, 1 mM EDTA, 1 mM dithiothreitol, and 1% Trasylol). In the latter case, the lysate was prepared at room temperature and heated for 2 min at 100°C. 3 vol of cold RIPA-VF was then added before centrifugation (20,000 g for 60 min at 4°C) and immunoprecipitation of the supernatant. Immunoprecipitations were performed as previously described (49). Alkali treatment of SDS gels was performed as detailed earlier (17).

Quantitation of p81 in A431 Cells

p81 was immunoprecipitated from lysates of A431 cells labeled for 18 h with [^{35}S]methionine using excess anti-ezrin serum. Immunoprecipitates were resolved on SDS polyacrylamide gels that were impregnated with 2,5-diphenylazole. p81 was detected by fluorography, and gel bands corresponding to p81 were cut out and dissolved in 0.2 ml 70% perchloric acid and 0.4 ml 30% H_2O_2 overnight at 60°C before scintillation counting. The ratio of recovered counts in each protein band to the total acid-precipitable counts used for immunoprecipitation gave an estimate of the abundance of p81.

Gel Electrophoresis and Western Blots

Two-dimensional gel sample preparation and one- and two-dimensional electrophoresis were performed as reported elsewhere (8, 24, 30). Isoelectric focusing was performed with pH 6–8 or pH 6–8/10 range ampholytes. A detailed account of the Western blotting procedure was described previously (30). SDS gels were silver stained as reported (42).

Phosphoamino Acid Analysis and Tryptic Peptide Mapping

Immunoprecipitated proteins were resolved on one-dimensional gels, extracted, and subjected to either phosphoamino acid analysis or tryptic digestion (2, 17, 33). Phosphoamino acids were resolved in two dimensions by electrophoresis on 100- μm cellulose thin-layer plates at pH 1.9, followed by electrophoresis at right angles at pH 3.5. Phosphopeptides were resolved in two dimensions on

100- μm cellulose thin-layer plates by electrophoresis at pH 1.9 for 25 min at 1 kV followed by chromatography (33). [^{35}S]Methionine-labeled peptides were resolved by electrophoresis at pH 4.7 for 27 min at 1 kV followed by chromatography (2).

Immunofluorescence Staining

A431 cells were cultured on 22-mm square glass coverslips (some precoated with poly-D-lysine) for 40 h before use. They were fixed in phosphate-buffered saline (PBS) containing 3% formaldehyde for 10 min at 25°C, washed three times (5 min each wash) with PBS, permeabilized for 5 min in ice cold 20 mM Hepes, pH 7.4, 0.5% Triton X-100, 300 mM sucrose, 3 mM MgCl_2 , 50 mM NaCl, and rinsed three times for 5 min with PBS. The coverslips were incubated with 20–30 μl of affinity-purified anti-ezrin Ig at 32 $\mu\text{g}/\text{ml}$ for 30 min at room temperature, washed three times with PBS for 20 min, and then incubated with 20–30 μl of a 1:15 dilution of affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO). The coverslips were washed three times with PBS for 20 min and mounted on glass slides with 10 mM Tris-HCl, pH 7.4, 0.2 M NaN_3 , and 90% glycerol. They were viewed with a Nikon Optiphot microscope (Nikon Inc., Garden City, NY) with episcopic fluorescence attachment through a Plan Apo 40 \times oil objective. Photographs were taken with Kodak Tri-X film.

Cell Fractionation into Soluble and Particulate Fractions

A431 cell cultures (35-mm dishes) were washed twice with cold Tris-buffered saline. The cells were allowed to swell for 10 min at 4°C in 0.4 ml of a solution containing 20 mM sodium phosphate pH 7.0, 10 mM NaCl, 10 mM NaF, 100 μM Na_3VO_4 , 1% Trasylol, 14 mM 2-mercaptoethanol, and either 1 mM EDTA or 1 mM MgCl_2 . They were scraped off the dish and transferred to a Teflon tissue grinder (Dual size 20, Kontes Co., Vineland, NJ) and homogenized with 25 strokes. Cell breakage was monitored by light microscopy. The homogenate was transferred to airfuge tubes and centrifuged in an Airfuge (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 g for 15 min. The pellets were resuspended in the original volume of swelling buffer, and both pellet and supernatant fractions were adjusted to RIPA-VF conditions.

Cell Fractionation into Detergent Soluble and Insoluble Fractions

Three procedures were used to define detergent soluble and insoluble fractions: (a) A431 cells (35-mm dishes) were extracted on the dish for 3 min at 4°C with 100 μl of ice-cold 10 mM Pipes pH 6.8, 1% Triton X-100, 100 mM KCl, 300 mM sucrose, 50 mM NaF, 100 μM Na_3VO_4 , 2.5 mM MgCl_2 , 1 mM CaCl_2 , and 1% Trasylol. Dishes were drained and washed once with 300 μl of additional buffer. Insoluble material was scraped into 400 μl buffer. (b) Same as a except that the extraction buffer was ice-cold 10 mM Pipes pH 6.8, 0.5% Nonidet P-40, 100 mM KCl, 300 mM sucrose, 50 mM NaF, 100 μM Na_3VO_4 , 3 mM MgCl_2 , and 1% Trasylol. (c) Same as a except that the extraction buffer was ice-cold 10 mM 2-(*N*-morpholino)ethane sulfonic acid pH 6.2, 10 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4 , 1.5 mM MgCl_2 , 10% glycerol, and 1% Trasylol (buffer M) with 1% Nonidet P-40, 1 mM EGTA, and 5 mM dithiothreitol. All fractions were adjusted to RIPA-VF conditions.

Detergent-insoluble material resulting from procedure c was fractionated further by a modification of the procedure reported by Staufienbiel and Deppert (54): a 15-min treatment at 37°C with 400 μl buffer M containing 50 $\mu\text{g}/\text{ml}$ DNase I, a 15-min treatment at 4°C with 400 μl buffer M containing 1 mM CaCl_2 , a 30-min treatment at 4°C with 400 μl buffer M containing 2 M NaCl, 1 mM EGTA, and 5 mM dithiothreitol and finally the insoluble material was dissolved in 400 μl RIPA-VF buffer. One-half of each extract was adjusted to RIPA-VF buffer for immunoprecipitation. The other halves were loaded onto 5 ml 10–30% linear glycerol gradients prepared in the appropriate buffer and centrifuged in a Beckman SW 50.1 rotor at 40,000 rpm for 19 h. Parallel gradients were prepared with the molecular weight standards ovalbumin (43 kD), bovine serum albumin (BSA) (68 kD), and rat immunoglobulin G (150 kD). Twenty 250- μl fractions were collected from each gradient. 25 μl of each fraction was analyzed directly by SDS PAGE, and the remainder was adjusted to RIPA-VF conditions and immunoprecipitated with anti-ezrin serum.

Immunoperoxidase Staining

The procedures for frozen tissue preparation and immunoperoxidase staining were exactly as described previously (30). Photographs were taken with Kodak technical black and white film.

Results

Ezrin Is Homologous to the Protein-Tyrosine Kinase Substrate, p81

The unphosphorylated form of the phosphotyrosine-containing 81-kD protein (p81) in A431 cells has been previously identified by its migration on two-dimensional gels (13). Because the position of p81 relative to actin on our two-dimensional gels was very similar to that reported for intestinal ezrin (3), we decided to investigate a possible homology. To do this we made use of an antiserum raised against chick intestinal ezrin that had been shown to react with a similar protein in a wide variety of species (3).

We first determined whether A431 cells contained a protein that could be immunoprecipitated by anti-ezrin serum. [³⁵S]-Methionine-labeled A431 cells were lysed in RIPA-VF buffer and subjected to immunoprecipitation with either nonimmune serum or anti-ezrin serum. After SDS polyacrylamide gel electrophoresis, we observed a predominant, specifically immunoprecipitated, protein with an apparent molecular mass of 81,000 (Fig. 1) that comprised ~0.03% of the total [³⁵S]methionine-labeled protein (see Materials and Methods). A minor protein with an apparent molecular mass of 120,000 was also specifically immunoprecipitated (Fig. 1). When lysates were prepared in SDS lysis buffer rather than RIPA-VF buffer, the 120-kD protein was no longer apparent in immunoprecipitates nor was it detected on Western blots. Analysis of glycerol gradients, in which RIPA-VF lysates of [³⁵S]methionine-labeled A431 cells had been sedimented, showed the 81-kD protein to have the sedimentation rate expected for a protein this size (data not shown). The 120-kD protein exhibited a higher sedimentation value than did the 81-kD protein, indicating that it was not complexed with this protein but rather was recognized independently by the antiserum. Two-dimensional tryptic peptide analysis of the [³⁵S]methionine-labeled 120-kD protein revealed that it was not related to the 81-kD protein (data not shown). Furthermore, affinity purified anti-ezrin Ig did not immunoprecipitate the 120-kD protein. Thus, a single 81-kD protein in A431 cells appeared to be homologous to ezrin.

Two different experiments were performed to test the relatedness of the 81-kD protein immunoprecipitated by anti-ezrin serum and p81. For reference, the spot on a two-dimensional gel of [³⁵S]methionine-labeled A431 cells that corresponds to the previously identified unphosphorylated form of p81 is indicated with an arrow in Fig. 2A (13). In the first experiment, immunoprecipitates of [³⁵S]methionine-labeled A431 cell proteins, prepared either with a nonimmune serum or with anti-ezrin serum, were mixed with total unlabeled A431 cell proteins and resolved by two-dimensional gel electrophoresis. The gels were first silver-stained to display the pattern of unlabeled proteins (Fig. 2B), and labeled proteins were detected by fluorography (Fig. 2C). Alignment of the fluorographs with silver-stained gels demonstrated that the 81-kD protein recognized by anti-ezrin serum co-migrated with p81. In the second experiment, total [³⁵S]methionine-labeled A431 cell proteins were separated by two-dimensional gel electrophoresis and electrophoretically transferred to a nitrocellulose sheet. The blot was probed with anti-ezrin serum followed by ¹²⁵I-labeled Staphylococcal protein A. ¹²⁵I-Protein A bound specifically to only one spot, which co-migrated with the major unphosphorylated form of

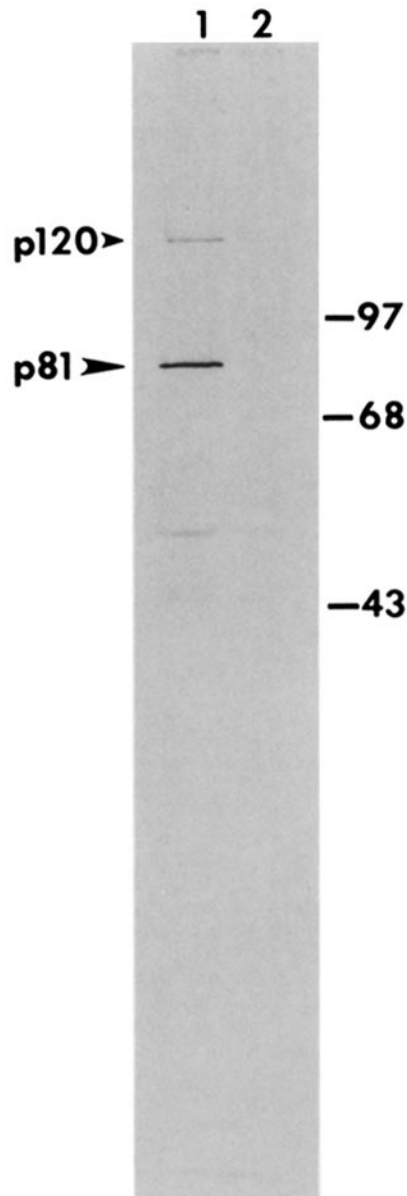


Figure 1. Immunoprecipitation from A431 cells using anti-ezrin serum. A431 cells were labeled for 18 h with [³⁵S]methionine, lysed in RIPA-VF buffer, and subjected to immunoprecipitation with either anti-ezrin serum (lane 1) or with nonimmune serum (lane 2) as described in Materials and Methods. Immunoprecipitates were analyzed by electrophoresis on a 12.5% SDS polyacrylamide gel followed by autoradiography. Arrowheads point to the specifically immunoprecipitated proteins. Molecular weight markers are phosphorylase b (97 kD), BSA (68 kD), and ovalbumin (43 kD). Exposure time was 18 h.

p81 (data not shown).

To establish further the relatedness of the microvillar core protein and p81, the tryptic peptides of [³⁵S]methionine-labeled p81 immunoprecipitated from LIM1215 cells, a human colonic tumor cell line that possesses microvilli (55), primary chicken embryo cells, and human A431 cells were compared. The large number of tryptic peptides shared by p81 from chicken and human cells (>50%) demonstrated that this protein has been highly conserved throughout evolution (Fig. 3, A-C). The identical pattern of tryptic peptides gen-

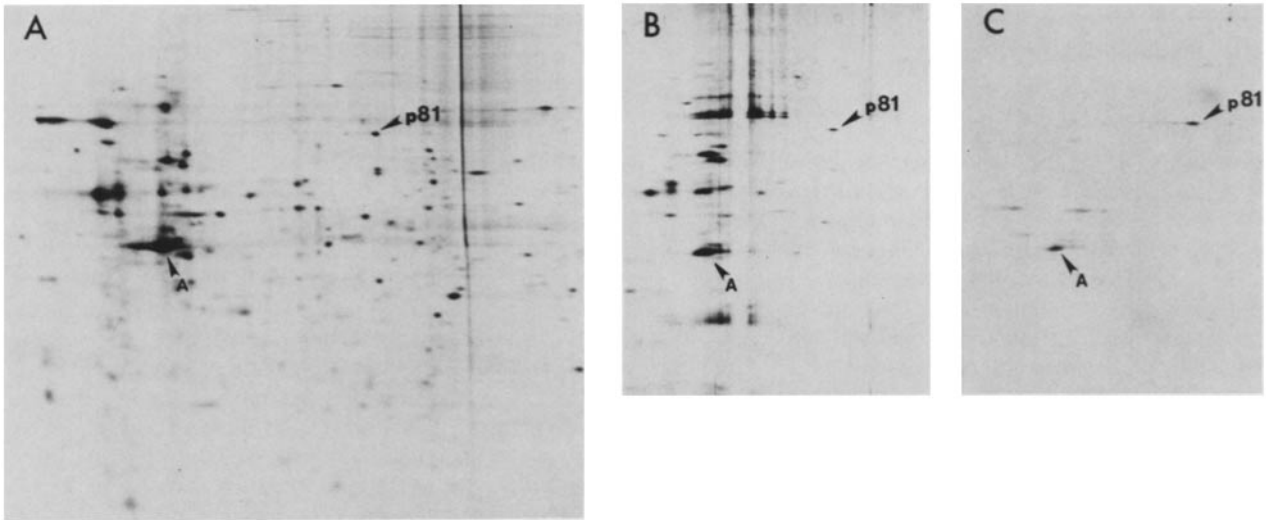


Figure 2. Migration of A431 cell p81 on two-dimensional gels. A431 cells were labeled with [³⁵S]methionine and solubilized in two-dimensional gel sample buffer. Proteins were resolved by two-dimensional gel electrophoresis as described in Materials and Methods with pH 6–8 range ampholytes and visualized by fluorography (A). In a second experiment, ~10 μg of protein from unlabeled A431 cells was mixed with a [³⁵S]methionine-labeled anti-ezrin immunoprecipitate from A431 cells and resolved by two-dimensional gel electrophoresis with pH 6–8/10 range ampholytes. The gel was silver stained (B) and then subjected to fluorography (C). Relevant portions of the gels are shown. Note that silver staining decreases the efficiency of fluorography. Acidic proteins are on the left. Arrowheads point to actin (A) and to p81. Exposure times were 18 h (A) and 5 d (C).

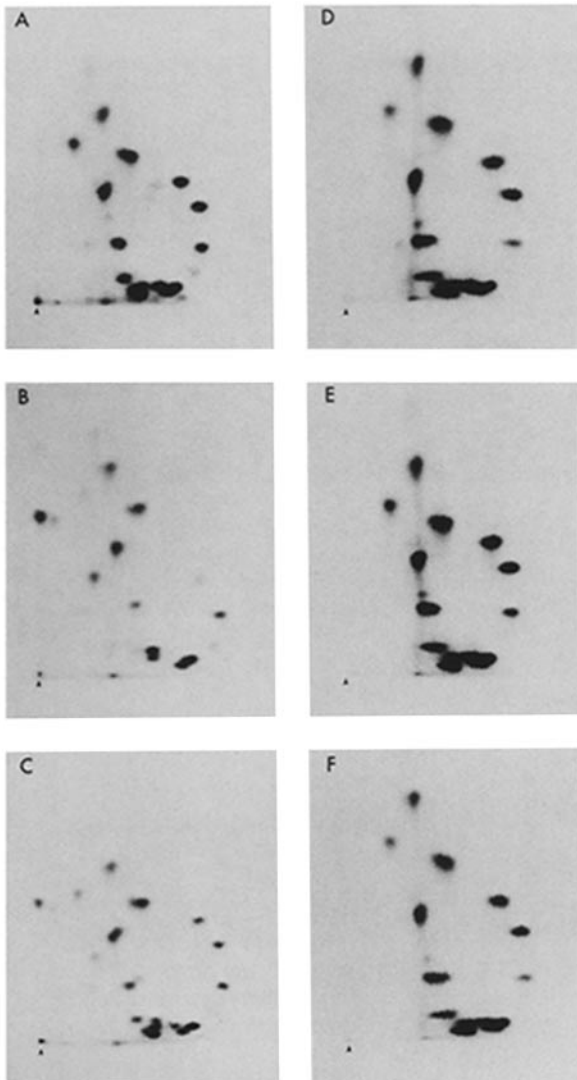


Figure 3. Two-dimensional tryptic peptide maps of p81 from A431, chicken embryo, and LIM1215 cells. p81 was immunoprecipitated with anti-ezrin serum from [³⁵S]methionine-labeled A431 cells, LIM1215 cells, and chicken embryo cells and subjected to tryptic cleavage. The resultant peptides were resolved in two dimensions as described in Materials and Methods. p81 from: (A) A431 cells; (B) chicken embryo cells; (C) A431 and chicken embryo cells mixed; (D) A431 cells; (E) LIM1215 cells; (F) A431 and LIM1215 cells mixed. A–C and D–F are from different experiments. In each case the origin is marked with an arrowhead. Electrophoresis was performed at pH 4.7 in the horizontal dimension, with the anode on the left. Plates were impregnated with 2-methyl-naphthalene and exposed at –70°C. cpm loaded onto thin layer plates and exposure times are as follows: (A) 18,000 cpm, 5 d; (B) 3,500 cpm, 2 wk; (C) 3,500 cpm of each, 10 d; (D) 31,000 cpm, 3 d; (E) 39,000 cpm, 3 d; (F) 13,000 cpm of each, 3 d.

erated from A431 p81 and LIM1215 p81 (Fig. 3, *D-F*) indicated that no unusual modification of p81 occurs in intestinal cells.

Intracellular Distribution of p81 in A431 Cells

Since ezrin was purified as a component of the microvillar core (3), we anticipated that p81 would be found in microvilli of A431 cells and would behave in biochemical fractionations as if it were a component of the A431 cytoskeleton. We attempted to confirm these predictions with both indirect immunofluorescence and biochemical fractionation. In addition, we asked whether or not EGF treatment and/or phosphorylation altered the distribution of p81.

Because of the unwanted specificity in the anti-ezrin serum towards the unrelated 120-kD protein, affinity-purified Ig which does not recognize this protein was used for immunofluorescence staining. p81 in A431 cells was concentrated in surface projections such as microvilli and retraction fibers (Fig. 4). This pattern was more pronounced when cells were grown on poly-D-lysine-coated coverslips (Fig. 4*C*). It was not detectable unless the cell membrane was permeabilized (data not shown). This pattern of p81 staining in A431 cells is similar to that observed in other tissue culture cells (3).

To determine the degree of association between p81 and the cytoskeleton, three different detergent extraction conditions reported to leave the cytoskeleton intact were applied to

A431 cells (6, 11, 54). [³⁵S]Methionine-labeled or ³²P-labeled cells treated with or without EGF were extracted under these three conditions. In addition, we determined whether or not p81 was in the soluble or particulate fractions of cells lysed by homogenization after swelling in hypotonic buffers containing either MgCl₂ or EDTA. After all the fractions had been adjusted to RIPA-VF conditions, p81 was immunoprecipitated with excess anti-ezrin serum, and the percentages of p81 in each fraction were estimated by counting gel bands and scanning autoradiographs (Table I). Between 37 and 61% of p81 was insoluble depending on the procedure. The distribution of the phosphorylated form of the protein (pp81) paralleled that of the total p81 population. In addition, treatment of the cells with EGF did not appear to alter the distribution of p81 (Table I). The greatest percentage of p81 was found associated with the cytoskeleton prepared using procedure *c* (see Materials and Methods). This insoluble material was further fractionated by incubation with DNase I, followed by a Ca²⁺ extraction, and then a 2 M NaCl extraction. SDS polyacrylamide gel analysis of proteins from each extract and the residual material illustrated that each extract contained a significantly different population of proteins (data not shown). This suggested that nonspecific leaching out of proteins during this long procedure was minimal. As additional controls, the fractionation properties of fodrin, a cortical cytoskeletal protein (27), and another protein-tyrosine

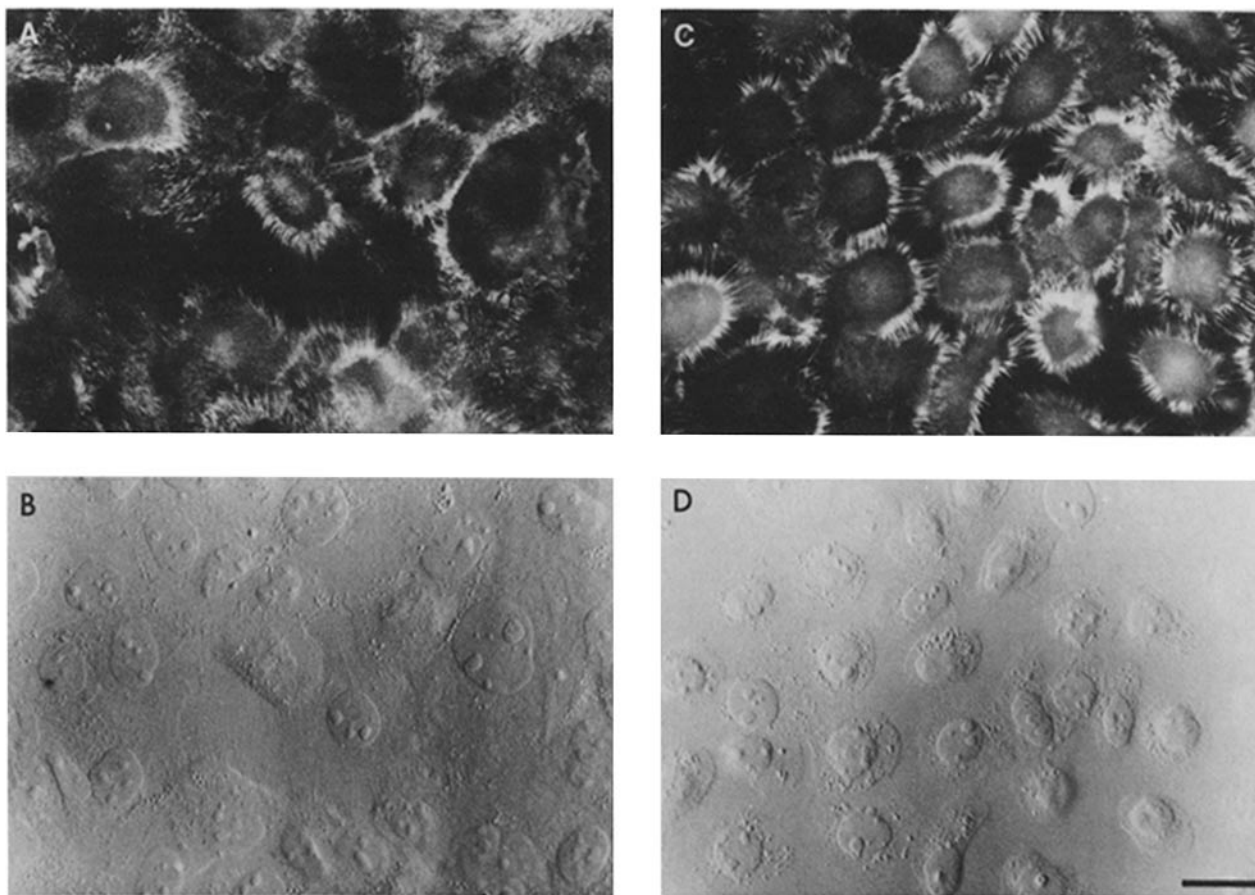


Figure 4. Indirect immunofluorescent staining with affinity-purified anti-ezrin Ig in A431 cells. A431 cells were grown on glass coverslips uncoated (*A*) or coated (*C*) with poly-D-lysine. They were incubated with affinity-purified anti-ezrin Ig followed by affinity-purified fluorescein-conjugated goat anti-rabbit IgG as described in Materials and Methods. Nomarski images of *A* and *C* are shown in *B* and *D*, respectively. Bar, 12 μ m.

Table I. Distribution of p81 and pp81 between A431 Cell Fractions

	p81		pp81	
	-EGF	+EGF	-EGF	+EGF
	% particulate			
Detergent lysis				
Procedure a	42	45	57	60
Procedure b	37	43	52	56
Procedure c	61	68	69	71
Hypotonic lysis				
Mg ²⁺	48	54	49	46
EDTA	56	59	52	48

A431 cells were labeled with [³⁵S]methionine or ³²P-orthophosphate and were either untreated or treated with EGF at 60 ng/ml for 10 min. The cells were lysed according to one of five procedures detailed in Materials and Methods and separated into soluble and particulate fractions. All resultant fractions were adjusted to RIPA-VF conditions. ³⁵S-labeled p81 and ³²P-labeled pp81 were immunoprecipitated from each fraction and resolved on 12.5% polyacrylamide gels. After fluorography or autoradiography, the percentages of ³⁵S-labeled p81 and ³²P-labeled pp81 in each fraction were determined by counting solubilized gel bands and by scanning autoradiographs. Only values for the particulate fractions are shown. There is about five times more pp81 in EGF-treated A431 cells than in control cells (see Fig. 8). The data for distribution of p81 include only a small (~10%) contribution from pp81. The numbers provided for ³²P-labeled pp81 are the average of results obtained by the two methods of data analysis described from one experiment. This experiment was repeated with visually similar results several times but was quantitated only once. The numbers given for ³⁵S-labeled p81 represent the average of two separate quantitated experiments. The error in the calculated percentages is ±5%.

Table II. Distribution of Fodrin, p81, and p36 in Sequential Extractions of A431 Cells

Extractions	Fodrin	p81	p36
1% Nonidet P-40	27	21	29
50 μg/ml DNase I	9	18	20
1 mM Ca ²⁺	5	19	4
2 M NaCl	13	19	22
Residual material	46	23	25

A431 monolayers were labeled with [³⁵S]methionine and extracted according to procedure c detailed in Materials and Methods. The particulate material was extracted further with 50 μg/ml DNase I, then 1 mM Ca²⁺, and finally 2 M NaCl as described in Materials and Methods. Residual material was scraped off the dish, and all fractions were adjusted to RIPA-VF conditions. Fodrin, p81, and p36 were immunoprecipitated together from each fraction, and the immunoprecipitates were resolved by SDS polyacrylamide gel electrophoresis. After fluorography, bands corresponding to each protein were cut out, solubilized, and counted. The values in each column represent the fraction of the protein recovered at each step expressed as a percentage of the total. The error in the calculated percentages is ±5%.

kinase substrate, p36, were monitored as well. p36 is known to associate with particulate fractions of cells (7, 11) in a Ca²⁺-dependent manner (25, 29) and to be located at the inner surface of the plasma membrane (20, 31, 44, 48). The percentages of fodrin, p81, and p36 found in each secondary fraction are listed in Table II. Some p81 was found in all fractions. Like fodrin and actin, a high percentage was soluble under all fractionation conditions used. p36 was found in all but the Ca²⁺ extract. Relatively few proteins were released during the Ca²⁺ and DNase I extractions but fodrin and actin were. Thus, p81 shared some fractionation characteristics with bona fide cytoskeletal proteins. At least 37% of p81 was resistant to detergent extraction and could be released only when cytoskeletal elements were disrupted.

As p81 was released in procedure c by agents that disrupt cytoskeletal elements, we tested whether p81 might be released in association with another protein during extraction. Samples of each extract were separated on glycerol gradients in the

appropriate buffers. Portions of each gradient fraction were run directly on SDS polyacrylamide gels, while the remainder was adjusted to RIPA-VF conditions and subjected to immunoprecipitation with anti-ezrin serum. As an example, immunoprecipitations of the gradient fractions from the DNase I extract are shown in Fig. 5. p81 behaved as an unassociated monomer in all extracts. If it were complexed with another protein, that protein would have to be small enough so as not to cause a detectable shift in the sedimentation of p81. Alternatively, its association with another protein might be sensitive to the hydrodynamic forces encountered during the centrifugation.

p81 Shows a Wide Tissue Distribution

Since ezrin was purified from the apical region of intestinal epithelial cells where p36 appears to be concentrated (25, 30, 32), we looked to see if the overall tissue distribution of p81 was identical to that of p36. To determine which mouse tissues expressed p81, Western blots containing equal amounts of proteins from several mouse tissues were probed with anti-ezrin serum followed by ¹²⁵I-protein A (Fig. 6). Small intestine, thymus, kidney, and lung expressed the highest levels of p81; bone marrow, spleen, and lymph nodes had lower levels; p81 was scarce in brain, heart, testis, and fascia; and p81 was undetectable (<2% of the level in small intestine) in muscle and liver. This distribution was similar, though not identical to that of p36 (30).

In the small intestine and kidney, ezrin expression is restricted to the microvilli of epithelial cells (3; Allen, R.,

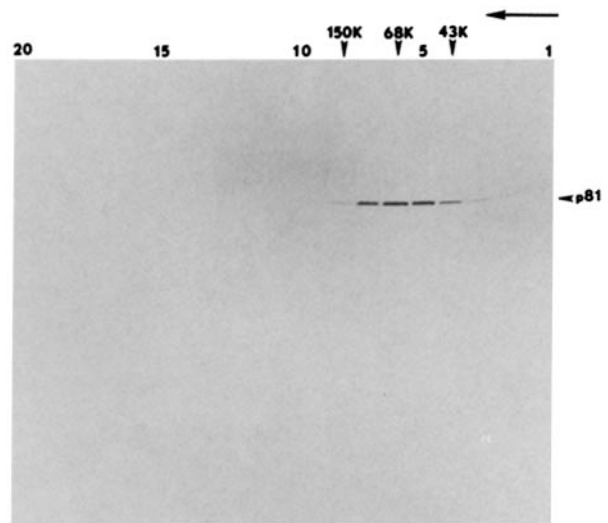


Figure 5. Glycerol gradient analysis of p81 released by DNase I treatment of A431 particulate proteins. A431 cells were labeled with [³⁵S]methionine, extracted according to procedure c (see Materials and Methods), and the residue extracted further as detailed in Materials and Methods. Each extract was separated on a 5 ml 10–30% glycerol gradient in parallel with the molecular weight standards, ovalbumin (43 kD), BSA (68 kD), and rat immunoglobulin (150 kD). 20 fractions were collected from each gradient, and p81 was immunoprecipitated from each after they were adjusted to RIPA-VF conditions. Immunoprecipitates were resolved on a 12.5% polyacrylamide gel, and the gel was subjected to fluorography. The results of one gradient, the material released by 50 μg/ml DNase I treatment as shown here. p81 was found in fractions where a monomeric 81-kD protein should sediment. The arrow indicates the direction of sedimentation. Exposure time was 18 h.

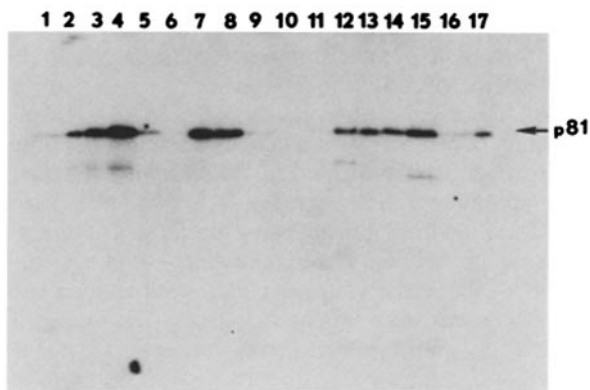


Figure 6. Western blot of adult mouse tissues. Protein from each tissue was resolved on a 15% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose sheets, incubated with anti-ezrin serum, and then probed with ^{125}I -protein A. Lane 1, 2 μg small intestine; lane 2, 5 μg small intestine; lane 3, 10 μg small intestine; lane 4, 20 μg small intestine. Lanes 5–17 were loaded with 20 μg of protein from: lane 5, brain; lane 6, liver; lane 7, kidney; lane 8, lung; lane 9, heart; lane 10, thigh muscle; lane 11, fascia; lane 12, bone marrow cells; lane 13, spleen; lane 14, lymph node; lane 15, thymus; lane 16, testes; lane 17, urinary tract. Exposure time was 18 h with an intensifying screen at -70°C .

personal communication). To determine whether there is a subpopulation of cells that accounts for the high expression of p81 in thymus and spleen, frozen tissue sections of each organ from rat were prepared for indirect immunoperoxidase staining using affinity-purified anti-ezrin Ig. Stained sections of each organ are shown in Fig. 7. In the spleen, periarteriolar lymphocytes stain strongly as do small B cells and large B cell blasts. Some lymphocytes in the red pulp are also positive. Structural cells and megakaryocytes were weakly positive. Red cell and white cell precursors, red cells, and endothelial cells do not stain (Fig. 7A). In the thymus, p81 is most prevalent in subcapsular lymphocytes (Fig. 7B). Lymphocytes from the medulla stain more strongly than those from the cortex. The epithelial reticular cells are weakly reactive. These data suggest that the expression of p81 is not restricted to cell types with highly organized microvilli.

p81 Can Be Phosphorylated on Serine, Threonine, and Tyrosine Residues

Using the anti-ezrin serum, we investigated the phosphorylation state of p81 in A431 cells. Subconfluent A431 cells were labeled with ^{32}P -orthophosphate at 37°C for 18 h and then treated with EGF for 10 min. After lysis of the cells in

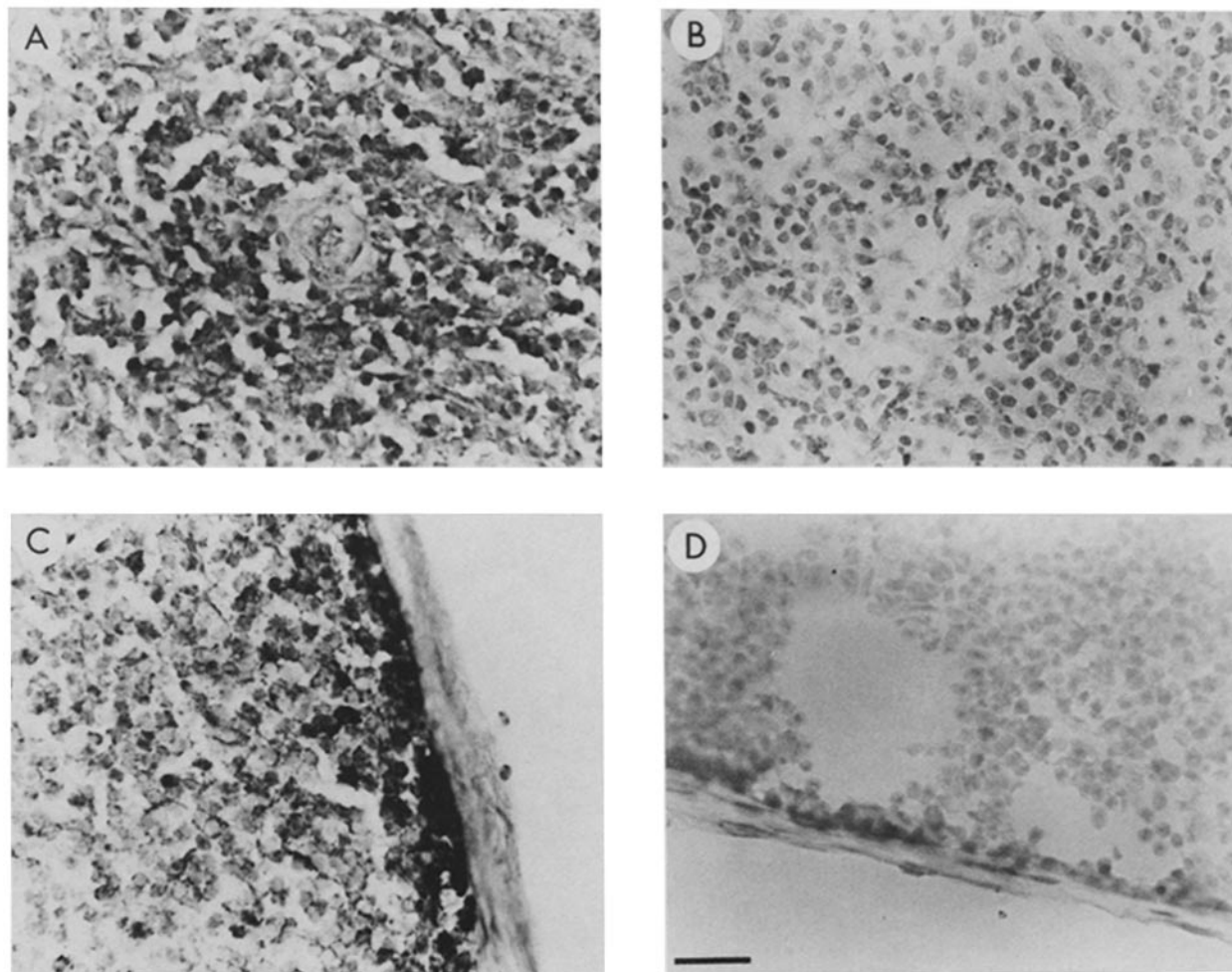


Figure 7. Indirect immunoperoxidase staining of p81 in rat spleen and thymus. Frozen tissue sections of rat spleen (A and B) and rat thymus (C and D) were incubated with affinity-purified anti-ezrin Ig followed by affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG and diaminobenzidine as described in Materials and Methods (A and C). B and D show photographs of similar sections stained only with the second layer antibodies and diaminobenzidine. Bar, 12 μm .

RIPA-VF, pp81 was immunoprecipitated with anti-ezrin serum (Fig. 8A). Untreated cells contained detectable levels of phosphorylated pp81. EGF treatment increased the phosphate labeling of pp81 about fivefold (Fig. 8A, lanes 1 and 2). When the gel shown in Fig. 8A was then incubated in alkali to enhance detection of phosphothreonine and phosphotyrosine residues (Fig. 8B), alkali-stable radioactivity was detectable in pp81 from untreated cells. pp81 from unstimulated cells, isolated in the presence of phosphatase inhibitors, was found to contain phosphoserine and phosphothreonine, whereas pp81 from EGF-stimulated cells contained phosphoserine, an increased amount of phosphothreonine, and phosphotyrosine (Fig. 8C, a and b). Alkali-stable radioactivity, however, was only detectable in pp81 from untreated cells if phosphatase inhibitors were included in the lysis buffer. This would explain our earlier results where pp81 was not

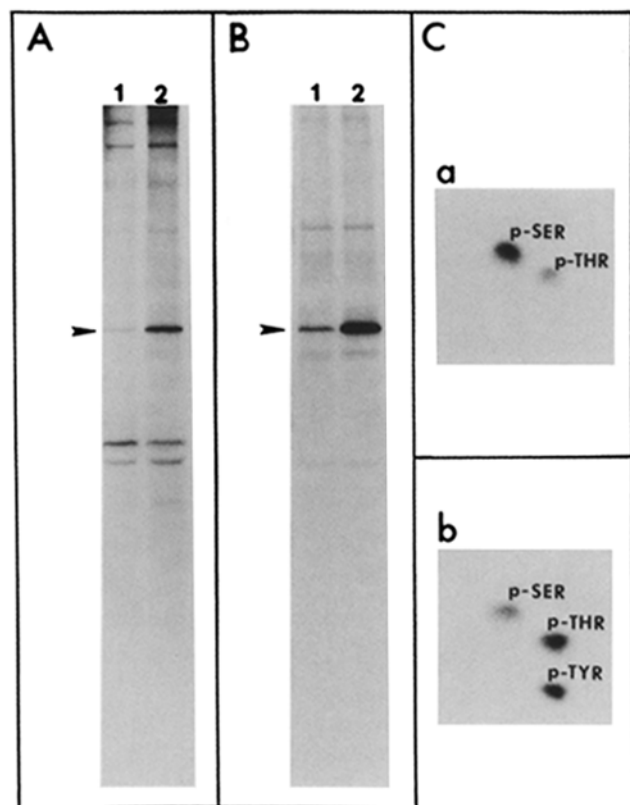


Figure 8. Immunoprecipitations of pp81 from ^{32}P -labeled A431 cells and phosphoamino acid analysis of the immunoprecipitates. A431 cells were labeled with ^{32}P -orthophosphate, lysed in RIPA-VF buffer, and pp81 was immunoprecipitated from untreated cells (lane 1 in A and B) and EGF-treated cells (lane 2 in A and B) as described in Materials and Methods. The immunoprecipitates were resolved on a 12.5% SDS polyacrylamide gel, and pp81 was detected by autoradiography (panel A). After alkali treatment of the gel shown in A, alkali-stable phosphoproteins were detected by autoradiography (B). Arrowheads indicate pp81. Gel bands containing pp81 from untreated and EGF-treated cells comparable to those shown in A were analyzed for their phosphoamino acid content as described in Materials and Methods. C, insert a, shows the phosphoamino acids derived from pp81 from untreated A431 cells. C, insert b, shows the phosphoamino acids derived from pp81 from EGF-treated cells. Cerenkov cpm applied to thin layer plates were in C, insert a, 100 cpm, and 180 cpm in C, insert b. p-SER, phosphoserine; p-THR, phosphothreonine; p-TYR, phosphotyrosine. Exposure times were as follows: A, 18 h; B, 3 d; and C, at -70°C with an intensifying screen, 3.5 d.

detected on alkali-treated two-dimensional gels of untreated cells (34). Apparently a phosphothreonine-specific phosphatase in A431 cells is active on pp81 during two-dimensional gel sample preparation and also in unmodified RIPA buffer.

The fraction of pp81 newly modified at tyrosine in response to EGF treatment was estimated in the following manner. Anti-ezrin immunoprecipitates of [^{35}S]methionine-labeled control and EGF-stimulated A431 cells (prepared in unmodified RIPA buffer and thus lacking detectable levels of phosphothreonine) were resolved on two-dimensional gels. The fluorographs of these gels were quantified by two-dimensional computer-assisted densitometry. The percentage of phosphorylated pp81, which migrates to a more acidic position than the majority of the protein, was calculated to be 10% in EGF-treated cells. This percentage represents a minimum estimate since it is likely that some phosphotyrosine was lost in addition to the phosphothreonine due to protein phosphatase action in unmodified RIPA buffer.

The phosphorylation sites of pp81 were investigated by two-dimensional tryptic peptide analysis of pp81 immunoprecipitated in the presence of phosphatase inhibitors. pp81 isolated from control cells contained three major phosphopeptides termed 1, 2, and 3 (Fig. 9A). Phosphopeptides 1 and 2 contained only phosphothreonine, whereas phosphopeptide 3 contained phosphothreonine and phosphoserine (data not shown). pp81 from stimulated cells contained phosphopeptides 1–3 and one major new phosphopeptide termed phosphopeptide 4 (Fig. 9B). Phosphopeptide 4 contained phosphotyrosine exclusively (data not shown).

Discussion

In this study, we have characterized a protein-tyrosine kinase substrate in A431 cells that has a molecular mass of 81,000. pp81 is homologous to the intestinal protein ezrin, and may indeed be identical. Like ezrin in intestinal epithelial cells, pp81 is localized in the submembranous cortical skeleton where it is concentrated in microvilli. We obtained no evidence that pp81 is specifically associated with any other cellular protein.

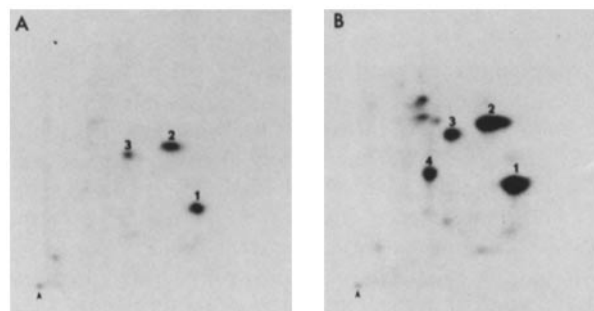


Figure 9. Two-dimensional tryptic phosphopeptide maps of pp81 from untreated and EGF-treated A431 cells. pp81 was isolated from equal numbers of untreated or EGF-treated ^{32}P -orthophosphate-labeled A431 cells, subjected to tryptic digestion, and separated in two dimensions as described in Materials and Methods. In each case the origin is marked (arrowhead). Electrophoresis was performed in the horizontal dimension at pH 1.9, with the anode on the left. A shows the tryptic phosphopeptides of pp81 from untreated cells, and B shows the tryptic phosphopeptides of pp81 from EGF-treated cells. Cerenkov cpm loaded onto thin layer plates was 400 cpm in A and 1,400 cpm in B. Exposure time at -70°C with an intensifying screen was 2 d.

The protein has been highly conserved throughout evolution. In the mouse, p81 is found in a wide variety of tissues.

In many ways our findings with p81 parallel what is known about another protein tyrosine kinase substrate, p36. First, both are concentrated in the apical cytoskeletal region of intestinal brush border cells (25, 30, 32), indicating that they are associated with structural components. p81, and under some conditions p36 (7, 11, 48), fractionate with cytoskeletal elements when tissue culture cells are extracted with detergent. These properties are consistent with the notion that p81 and p36 are either part of the cytoskeletal framework or associated with cytoskeletal proteins in detergent-insoluble fractions. However, we have been unable to demonstrate binding of soluble p81 to other proteins in sedimentation studies under a variety of conditions. Similarly, reconstitution studies of microvillar core proteins have not provided evidence for specific association with other proteins (Bretscher, A., unpublished results). p81 binding to detergent-insoluble fractions is not divalent cation dependent. This is in contrast to p36, which has been found in dimeric form in association with a 10-kD protein (22, 25) and has also been shown to bind actin and fodrin in a Ca^{2+} -dependent manner (25, 29). The fractionation properties of p81 do not appear to change significantly when the protein is phosphorylated in response to EGF. However, without a more complete understanding of the function of p81, subtle changes in distribution or associations cannot be ruled out.

As well as exhibiting similar intracellular distributions, p81 and p36 appear to have similar anatomical distributions in the mouse. Both proteins are expressed at very low levels in brain, muscle, and liver with high levels in intestine, thymus, and lung (30). Neither protein co-distributes in tissues with any known protein-tyrosine kinase (1, 19, 37, 53, 56), although in A431 cells examined by indirect immunofluorescence, the distributions of p81 and p36 overlap that of the EGF receptor (Gould, K. L., unpublished results). This would be consistent with p81 being a primary substrate for the EGF receptor protein-tyrosine kinase. We do not yet know if there is an active protein-tyrosine kinase located in the small intestine or if p81 or p36 is phosphorylated on tyrosine there.

The results of the gross anatomical survey concealed some differences between p81 and p36 that were found when the cell types expressing each protein were identified. In the thymus, for example, p36 is located predominantly in the structural framework of the organ (30), whereas p81 is found in the lymphocytes and only at low levels in the epithelial cells. In addition the level of p81 in kidney is considerably higher than that of p36. This may be of relevance in the microvillar structures of kidney proximal tubules.

p81 is phosphorylated in A431 cells on serine and threonine residues. In response to EGF, ~10% of the protein is additionally phosphorylated on tyrosine residues, and the extent of threonine phosphorylation increases. Tryptic digestion of pp81 from EGF-treated cells reveals only one major phosphotyrosine-containing peptide, suggesting a single site of tyrosine phosphorylation. This study of the phosphorylation sites in p81 demonstrated the need for specific phosphatase inhibitors in lysis buffers. In the absence of sodium fluoride and sodium orthovanadate, pp81 from untreated cells was barely detectable and contained only phosphoserine. In response to EGF, p81 was additionally phosphorylated on tyrosine. However, when isolated in the presence of phosphatase inhibitors, pp81

from untreated cells was readily detectable and contained phosphothreonine and phosphoserine. Its phosphorylation on threonine as well as on tyrosine was stimulated by EGF treatment. Phosphorylation of the threonine site(s) is probably not due to activation of protein kinase C in EGF-treated A431 cells as purified p81 is neither phosphorylated by protein kinase C *in vitro* nor phosphorylated to a greater extent *in vivo* in response to tumor promoters (Gould, K. L., and J. Meisenhelder, unpublished results). It is also worth noting here that despite their similarity in size and subcellular location, p81 is unlikely to be protein kinase C. Indirect evidence for this includes their very different tissue distributions (41), their different pIs (38), their distinct intracellular distributions (45), and the inability of purified p81 to act as a protein kinase *in vitro*. More direct evidence is the disparate two-dimensional phosphopeptide maps of the two proteins (our unpublished results).

p81 appears to be the product of a single gene. *In vitro* translation of poly A-containing RNA from a number of rodent cell and tissue sources followed by immunoprecipitation yielded a single protein that in every case co-migrated on one- and two-dimensional gels with p81 from metabolically labeled rodent cells (Saris, C., personal communication). Thus, the protein does not appear to be synthesized as a precursor. p81 is also a very stable protein with a half-life of ~17 h. Although it is abundant, comprising ~0.03% of the total cell protein in A431 cells, it is 5–10-fold less abundant than p36 (17, 47). Due to this lower abundance, pp81 may have been overlooked in other studies searching for substrates of tyrosine phosphorylation. Indeed, by immunoprecipitation, but not by two-dimensional gel analysis, we have been able to identify a phosphotyrosine-containing form of p81 in Rous sarcoma virus-transformed chicken cells (Gould, K. L., unpublished results). We are currently investigating under what other conditions p81 is phosphorylated on tyrosine.

Without a functional assay for p81 we cannot assess whether phosphorylation of p81 is likely to be of consequence in the response of cells to EGF or in viral transformation. The relatively low stoichiometry of p81 tyrosine phosphorylation could be of significance if a critical subpopulation of the protein were phosphorylated. Alternatively p81 might be phosphorylated gratuitously as a consequence of its proximity to a variety of membrane-associated protein-tyrosine kinases. The answer to this question must await further studies on the structure and function of p81.

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