The 46,000-dalton Tyrosine Protein Kinase Substrate Is Widespread, Whereas the 36,000-dalton Substrate Is Only Expressed at High Levels in Certain Rodent Tissues

KATHLEEN L. GOULD,*‡ JONATHAN A. COOPER,‡ and TONY HUNTER‡
*Department of Biology, University of California at San Diego, La Jolla, California 92093; and ‡Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138

ABSTRACT   Proteins of molecular mass 46,000 (p46) and 34,000–39,000 (p36) daltons are phosphorylated at tyrosine in Rous sarcoma virus-transformed chicken and mouse fibroblasts. p46 has recently been identified as an isozyme of enolase but the function of p36 is unknown. The expression of these proteins in various mouse and rat tissues has been examined. In most tissues, except muscle, p46 is found at relatively constant levels. In muscle, a more basic, related protein is present. In contrast, the abundance of p36 varies more widely from tissue to tissue, suggesting that it has a function in some but not all differentiated cells. By SDS gel electrophoresis and immunoblotting, high levels of p36 (60–120% of its relative abundance in fibroblasts) were found in small intestine, lung, and thymus, and intermediate levels (20–50%) were found in spleen, lymph nodes, and testes. No p36 was detectable in brain and muscle. Where studied, p36 mRNA expression paralleled protein levels. The cell types within each tissue expressing p36 were identified by immunofluorescence and immunoperoxidase staining. These cell types include all endothelial cells and fibroblastic cells examined, as well as various epithelial cells, cardiac muscle cells, macrophages, and testicular interstitial cells. We were unable to detect p36 in skeletal or smooth muscle cells, erythrocytes, nerve cells, or lymphocytes in any of the examined tissues. p36 appears to be concentrated in the terminal web region of intestinal columnar epithelial cells.

Several of the acutely oncogenic RNA viruses encode transforming proteins exhibiting tyrosine protein kinase activity (36). A rapid increase in phosphotyrosine content of cellular proteins accompanies transformation by these viruses (5, 15, 47, 59, 61). A similar increase in total cell phosphotyrosine occurs in response to two growth factors, epidermal growth factor (35) and platelet-derived growth factor (14). Modification of cellular proteins by phosphorylation on tyrosine induced by these agents is implicated in the transformation and growth stimulation of the target cells. As putative participants in the pathway(s) leading to altered growth states of the cells, the identity and normal function of the substrates of tyrosine protein kinases are of interest.

The identities of a few cellular targets of tyrosine protein kinases are now known (20, 58). However, the function of the first described substrate (52), a protein whose molecular mass has been estimated from 34,000–39,000 daltons (15, 25, 52) remains a mystery. We will refer to this protein as p36.

p36 is a highly conserved protein and has been recognized as a substrate for tyrosine phosphorylation in avian, murine, rat, human, and vole cells transformed by a variety of tumor viruses encoding tyrosine protein kinases (11, 16, 17, 24, 25, 32, 42, 45, 49, 51), as well as in human A431 cells treated with epidermal growth factor (26, 35) and in certain 3T3 cells treated with platelet-derived growth factor (14). p36 is insignificantly phosphorylated in untransformed cells (16, 23, 25, 45, 51) or in untreated A431 (17, 26) and 3T3 cells (14). The phosphorylation induced by growth factors is particularly interesting, since it shows that p36 can be phosphorylated by tyrosine protein kinases in cells not transformed by acutely oncogenic retroviruses. p36 can serve as a substrate for purified pp60$^{v\text{-}src}$ in vitro (25), indicating that it may be a direct target of the virally coded kinase. Both in vivo and in vitro, the phosphorylation of the polypeptide occurs at an identical tyrosine residue (25). In vivo, p36 is also phosphorylated at a serine residue(s) in transformed cells (19, 25, 51).

As a first step in ascertaining the function of p36, previous studies sought to define its intracellular location in cultured fibroblasts (2, 11, 18, 22, 33, 46, 50) and epithelial cells (46). Cell fractionation experiments and indirect immunofluorescence microscopy have established an association of p36 with the cytoplasmic face of the plasma membrane (2, 18, 22, 33, 46, 50). This location does not appear to change as a result of phosphorylation in transformed cells (22, 33, 46). It has also been reported that p36 is absent from some B cell lines (60). This raises the possibility that p36 is expressed in only certain cell types and that the identification of these cell types might enable us to eliminate some candidate functions of p36.

Another cellular target of tyrosine protein kinases is a 46,000-dalton protein (p46) (15) that has recently been identified as an isozyme of the enzyme, enolase (20). The phosphorylated form of p46 has been detected in chicken embryo fibroblasts transformed by Rous sarcoma virus, Fujinami sarcoma virus, PRCII, Y73, and Esh sarcoma virus, and in mouse 3T3 cells transformed by Rous sarcoma virus, Abelson murine leukemia virus (A-MuLV)[1] and Snyder-Theilen feline sarcoma virus (reference 16, and our unpublished data). p46 is not phosphorylated at tyrosine when 3T3 cells are treated with growth factors, or in uninfected 3T3 or CEF, although a charge variant of this enolase isozyme is constitutively phosphorylated, predominantly at serine (16). Prior to the identification of p46, information regarding its tissue distribution was of interest.

In this study, we used antibodies raised against p36 and p46 (19) to define the cell types that express the two proteins in mice and rats. By examining tissues, rather than cultured cell lines, we were able in most cases to examine terminally differentiated cells. Our results indicate that p36 is detectable in fibroblastic cells of connective tissue and in endothelial cells in all the tissues examined. It is present at far lower levels in, or absent from, all skeletal and smooth muscle cells, nerve cells, erythrocytes, and most lymphocyte populations analyzed. On the other hand, proteins reacting with antibodies to p46 were ubiquitous, as expected of a protein with an indispensable function in cellular metabolism.

MATERIALS AND METHODS

Cells: The A-MuLV-transformed mouse NIH 3T3 fibroblast cell line, ANN-1 (54), the rat skeletal muscle line, L_6 (66), the mouse smooth muscle cell line, BC$_3$H1 (55), the RAW 264 and RAW 309 Cr.1 mouse macrophage lines (53) transformed by A-MuLV (from W. Raschke, La Jolla Cancer Research Foundation), the thymic epithelial line, TEPI 1 (4), and the mouse T cell lines, BW 5147 (40), T1M1.4 (37), S49.1TB (37), SAKRTLS (38), AKR1.G.1 (38), and EL 4.G.1 (39) were grown in DME supplemented with 10% calf serum. L_6 cells were obtained from D. Schubert (The Salk Institute) and plated at 5 × 10^4 cells/60-mm dish. They fused spontaneously after 1 wk in culture. Unfused, dividing cells were killed by the addition of 0.5 μg/ml cytosine arabinoside. Myotubes were collected 2 wk later. BC$_3$H1 cells were kindly provided in both their undifferentiated and differentiated state by J. Patrick (The Salk Institute). The 18.81 line of A-MuLV-transformed mouse pre-B cells (62) and the WEHI 231 line of mouse B lymphoma cells (from W. Raschke) (64) were maintained in DME supplemented with 10% fetal calf serum and 10^{-5} M 2-mercaptoethanol. The T cell lines and TEPI 1 cells were from J. Lesley and C. MacLeod respectively (The Salk Institute). C. MacLeod also provided cell extracts from the T cell lines SL12-1, SL12-4, SL12-9, SL2-2, SL2-4, SL3-2, and SL3-4 (C. MacLeod, unpublished results), as well as from SAK8 (29) and W7TB (6). The rat pheochromocytoma cell line, PC12 (34) (from D. Schubert), was grown in DME supplemented with 10% newborn calf serum and 5% horse serum. They differentiated after addition of the β-subunit

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[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; EN, enolase; CEF, chick embryo fibroblast; EGF, epidermal growth factor.

of nerve growth factor, as described previously (34). Differentiated cells were collected 6, 11, and 14 d after initial nerve growth factor treatment.

Antisera: Anti-p36 and anti-p46 sera were prepared against partially purified chicken fibroblast proteins as previously reported (19). Antisera were used at 1:200 dilutions for Western blots and immunoprecipitations. For use in indirect immunofluorescence and immunoperoxidase staining, anti-p36 serum and preimmune serum were preabsorbed on 18.81 cells as already described (46). Briefly, 10^8 18.81 lymphoma cells were fixed, permeabilized, and resuspended in 0.5 ml PBS. 20 μl of either anti-p36 serum or preimmune serum was added to the cells and allowed to incubate for 30–60 min. The preabsorbed antisera were used at this 1:25 dilution.

Western Blotting: Tissues were removed from mice, rinsed in Tris-(hydroxymethyl)aminomethane (Tris)-buffered saline, and quickly homogenized in ice-cold 20 mM Tris-HCl pH 8.8, 1 mM dithiothreitol, 1 mM EDTA, and 1% Trasylol (Mobay Chemical Corp., New York, NY) and frozen at −70°C until their protein concentration was determined. Appropriate volumes were then adjusted to contain 2% SDS, 20% 2-mercaptoethanol, 0.1 M Tris-HCl, pH 6.8, 10% glycerol, (SDS gel sample buffer) and boiled for 2 min. Lymphocytes were isolated from thymus and spleen using Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). Tissue culture cells were rinsed in TBS and dissolved in SDS gel sample buffer. Protein concentrations were determined by the method of Lowry (44), after precipitation with trichloroacetic acid, or by the method of Bradford (7). Nerve-specific enolase (EN) was from P. J. Marangos (National Institute of Mental Health, Bethesda, MD). Protein samples of 20 or 30 μg were resolved on SDS polyacrylamide gels (15% acrylamide, 0.087% bis-acrylamide) and electrophoretically transferred to nitrocellulose filters as previously described (10, 63), except that the transfer buffer included 0.1% SDS. After transfer, the blots were rinsed in buffer A (10 mM Tris-HCl pH 7.4, 0.9% NaCl, and 0.01% NaN$_3$). All the following treatments were performed with gentle shaking at room temperature in the bottom half of a tissue culture flask. To block remaining protein binding sites, blots were incubated sequentially for 2 h each with (a) 5% bovine serum albumin, (b) 1% ovalbumin, and (c) 0.05% gelatin, all in buffer A. Antiserum was diluted 1:200 in buffer A containing 0.25% gelatin and filtered. After 4 h in antibody solution (20 ml for a blot 130 cm^2) the blots were washed five times in buffer A, two times in buffer A with 0.05% Nonidet P-40 (Bethesda Research Laboratories, Bethesda, MD), and five times again in buffer A over a period of 1 h. To detect specifically bound antibody, the blots were incubated for 2 h with ^{125}I-*Staphylococcus aureus* protein A, specific activity 7 μCi/μg, (New England Nuclear, Boston, MA) at a concentration of 0.01 μCi/ml in buffer A containing 0.25% gelatin. Blots were washed five times in buffer A, two times in buffer A containing 0.05% Nonidet P-40, two times in buffer A containing 0.4% sodium N-lauroylsarcosine, and five times again in buffer A over a 1-h period. Protein samples of 20 μg were resolved on two-dimensional gels as previously described (15, 28). Western blots of two-dimensional gels were made and treated as described above. The blots were exposed to Kodak XAR5 x-ray film, pre-exposed to 0.1–0.2 OD, with an intensifying screen, for up to 24 h at −70°C.

RNA and In Vitro Translations: Mouse embryo mRNAs and several mouse tissue mRNAs were a generous gift of R. Müller and J. Tremblay (The Salk Institute) (12). mRNA from tissue culture cells was prepared in the following manner. Cells were lysed in 20 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 10 mM NaCl, 5 mM vanadyl ribonucleoside complex, and 0.28% Nonidet P-40 and their nuclei were removed by low speed centrifugation. The supernatant was adjusted to 0.5% SDS, 10 mM EDTA, 0.1 M NaCl, and 1 mg/ml proteinase K, incubated at 37°C for 1 h, and extracted with phenol/chloroform (1:1, vol/vol). The aqueous phase was precipitated with ethanol. Poly-A-containing RNA was selected by passage through an oligo-dT cellulose column. Fetal and adult muscle mRNAs were prepared from rat thigh muscle that was homogenized in 50 mM Tris-HCl pH 7.4, 0.01 M MgCl$_2$, 0.1 M NaCl, and 0.25 M sucrose. Debris was pelleted at 12,000 rpm for 10 min. The supernatant was extracted several times with warm phenol/chloroform and the aqueous phase was precipitated with ethanol. Poly-A-containing RNA was purified by oligo-dT cellulose chromatography. In vitro translations were performed in a mRNA-dependent reticulocyte lysate system as reported previously (48, 57). 1 μg of mRNA was used in each translation reaction. [^{35}S]Methionine was present at ~1 mCi/ml in a final reaction volume of 11 μl.

Immunoprecipitation: Immunoprecipitations were performed as previously described (57) in "RIPA buffer" (57) with 2 mM EDTA included. ~5 × 10^5 cpm of in vitro translation products and 2 × 10^6 cpm of biosynthetically labeled polypeptides were immunoprecipitated in each reaction.

Biosynthetic Labeling: Cells were labeled by incubation with 50 μCi/ml [^{35}S]methionine (>1,000 Ci/mmol, Amersham, Arlington Heights, IL) at 37°C for 18 h in methionine-free DME supplemented with 5% fetal calf serum and 5% DME. Cells were washed three times in Tris-buffered saline and proteins were solubilized in either cold RIPA buffer or two-dimensional gel lysis buffer (15, 28).

Quantitation of p46 and p36 in ANN-1 Cells: [^{35}S]Methionine-labeled ANN-1 cell extracts were quantitatively immunoprecipitated using excess antibodies raised against p46 and p36 proteins. Immunoprecipitates were resolved in SDS polyacrylamide gels, the gels impregnated with 2,5-diphenyloxazole and p46 and p36 proteins detected by fluorography. Gel bands corresponding to p46 and p36 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 immunoprecipitated provided an estimate of the abundances of p36 and p46.

Immunofluorescence and Immunoperoxidase Staining: Tissues were removed from rats and immediately frozen on dry ice in O.C.T. Compound (Miles Laboratories, Elkhart, IN). 4-μm thick sections were cut on a cryostat at −20°C and mounted on glass slides. All subsequent treatments were carried out at room temperature. Following each treatment, the mounted sections were washed three times for 5 min in PBS. Sections were fixed in 2% formaldehyde, 130 mM KCl, 16.9 mM K_2HPO_4, 3.1 mM KH_2PO_4, 5 mM NaCl, 1 mM NaN_3, 5 mM $MgCl_2$, and 1 mM EGTA pH 7.5 for 30 min. For immunoperoxidase staining, sections were incubated in 0.05% phenylhydrazine for 30 min before addition of antibody. Sections were incubated with 20–30 μl of preabsorbed anti-p36 serum or preabsorbed preimmune serum (both at 1:25 dilutions) for 30 min and either 30 μl of a 1:15 dilution of affinity purified fluorescein-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) for 30 min or 30 μl of a 1:50 dilution of affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) for 90 min. Sections stained for fluorescence were mounted under glass coverslips in 90% glycerol, 0.2 M NaN_3, and 10 mM Tris-HCl, pH 7.4. They were viewed with a Nikon Optiphot microscope with episcopic fluorescence attachment through a Plan Apo 40X oil objective. Photographs were taken with Kodak Tri-X film. Sections incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG were treated with diaminobenzidine (0.05% with 1 μl/ml 3% H_2O_2 included) for up to 10 min and washed. Nuclei were then stained with hematoxylin for 1–2 min, rinsed twice with water, and dehydrated in ethanol and xylenes. The sections were then mounted under glass coverslips with Permount (Fisher Scientific Co., Fairlawn, NJ) and inspected with a Nikon Optiphot microscope. Photographs were taken through a Plan Apo 40X oil objective with appropriate filters with Kodak Ektachrome 50 (tungsten) film.

Immunofluorescence of Intestinal Epithelial Cells: Intestinal epithelial cells were prepared as described previously (8), except that cells were allowed to attach to poly-lysine–coated coverslips at 37°C for 30–45 min before fixation in ice-cold acetone for 5 min. The cells were fluorescently stained, viewed, and photographed as described above.

RESULTS

Tissue Distribution of p46 and p36

Antibodies raised to p46 and p36 proteins, partially purified from chicken fibroblasts, were used to analyze the distribution of homologous proteins in the mouse and rat. The sera used were highly specific when used to immunoprecipitate [^{35}S] methionine-labeled mouse fibroblast proteins. In addition, the sera recognize the appropriate proteins in the mouse and rat tissues examined, as judged by their SDS gel mobilities. The contaminating proteins noted previously in immunoprecipitates prepared from chick cells (19) were much less evident; presumably p36 and p46 are more highly conserved between species than the contaminating proteins.

Immunoblotting was chosen to study the expression of p46 and p36 in various mouse tissues. The alternative approaches of immunoprecipitation of biosynthetically labeled tissues, or competition radioimmunoassay, are both subject to errors if the proteins studied are not soluble under the conditions for antibody binding. Additionally, competition radioimmunoassay is inaccurate if there are any cross-reactive antibodies. Animal tissues were homogenized and their proteins dissolved by boiling in a buffer containing SDS and mercaptoethanol (see Materials and Methods). Equal amounts of protein from each tissue were subjected to SDS PAGE. The proteins were electrophoretically transferred to nitrocellulose filters and the blots were probed with anti-p46 or anti-p36 serum followed by [^{125}I]-labeled Staphylococcal protein A. Bound [^{125}I]-pro-

tein A was detected by indirect autoradiography. Representative blots are shown in Fig. 1.

Quantitation of protein levels in the tissues was permitted by the inclusion on each gel of samples containing different amounts of protein from the mouse fibroblast cell line, ANN-1. The levels of p36 and p46 in these cells were arbitrarily set at 100%. In fact, p46 and p36 comprise about 0.12% and 0.05%, respectively of total protein in ANN-1 cells (see Materials and Methods). Densitometry of fluorographs exposed for different lengths of time, as well as gamma counting of ^{125}I-containing bands cut from the Western blots, indicated that the amount of anti-p46 serum bound was linearly proportional to the amount of protein blotted in the range from 6% to 100% of the standard. Such a linear relationship did not pertain in the case of the anti-p36 serum, but a calibration curve was plotted for each experiment enabling estimation of levels as low as 5% of the standard. Proteins from the murine pre-B cell line, 18.81, previously shown to lack detectable levels of p36 (60), were included on each gel as a negative control for p36 expression.
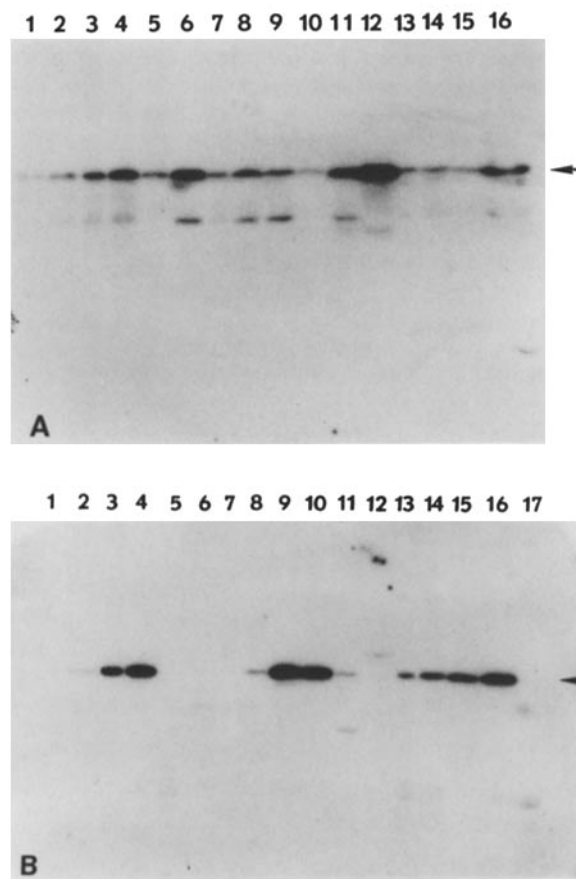

FIGURE 1 Western blots of adult mouse tissues. 20–30 μg of protein from each tissue and from the 18.81 cell line were resolved on 15% polyacrylamide gels along with various amounts of protein from the ANN-1 cell line. Proteins were electrophoretically transferred to nitrocellulose sheets, incubated with anti-serum raised against p46 (A) or p36 (B) and then probed with ^{125}I-protein A. Arrows point to p46 in A and p36 in B. Lanes 1, 1.2 μg ANN-1; lanes 2, 5 μg ANN-1; lanes 3, 10 μg ANN-1; lanes 4, 20 μg ANN-1; lanes 5, 18.81; lanes 6, brain; lanes 7, liver; lanes 8, kidney; lanes 9, small intestine; lanes 10, lung; lanes 11, heart; lanes 12, thigh muscle; lanes 13, spleen; lanes 14, lymph nodes; lanes 15, testes; lanes 16, thymus; lane 17, thymus cells teased from the organ. Exposure times were 7 h with an intensifying screen at −70°C.

46,000-dalton protein(s) reacting with anti-p46 serum was found in all tissues examined, in accord with the identification of p46 as an isozyme of the glycolytic enzyme, EN (20) (Fig. 1A). A lower molecular weight protein, also recognized by anti-p46 serum, appears to have been a contaminant of the immunogen (19) and is unrelated to p46 by tryptic peptide analysis (unpublished data). In tissues with the highest rates of glucose utilization (brain, heart, and skeletal muscle), the 46,000-dalton antigen was particularly abundant. The lowest levels of p46 detected were in lung and spleen, where it was present at 15–20% of the level in ANN-1 cells. In contrast, Western blots probed with anti-p36 serum indicated that p36 expression varied more widely from tissue to tissue (Fig. 1B). Brain, erythrocytes, and muscle lacked detectable levels of the protein (<5% of the level in ANN-1 cells) while gut, lung, and thymus had levels comparable to ANN-1 cells. The average concentrations of both p46 and p36 relative to ANN-1 cells are listed in Table I.

A common feature of those tissues containing abundant p36 is their considerable lymphocyte populations. Since some lymphocyte cell lines have high levels of p36 (60), a crude fractionation of thymus and spleen was undertaken to determine whether the presence of p36 in those tissues could be explained by high levels of p36 in lymphocytes. Thymus and spleen were removed from a mouse and bisected. Blood cells were teased from one half, freed of stroma by filtration, and fractionated by gradient centrifugation. Equal amounts of protein from the teased cells and the remaining tissue halves were analyzed for the presence of p36 by immunoblotting. In each case p36 was found in the control half of the organ, but at a much reduced level in the teased thymic cells (Fig. 2).

p36 was undetectable in the lymphocyte fraction or the other blood cells (chiefly erythrocytes and granulocytes, data not shown). By elimination, we conclude that p36 is in the cells of the stroma of spleen and thymus.

p46-related Proteins Found in Specific Tissues

Our anti-p46 serum was raised against a protein exhibiting a pI of 7.4 purified from fibroblasts (19). Minor charge variants of this protein exist in fibroblasts, possibly due to posttranslational modifications (19). Analysis of mouse tissues by two-dimensional gel electrophoresis and visualization of the proteins by silver staining showed that a protein with the same mobilities as p46 was abundant in kidney, gut, brain, liver, and thymus, less abundant in bone marrow and spleen, and rare in skeletal muscle and heart (data not shown). Enolase (EN) is a dimer, with acidic brain-specific (γ), basic muscle-specific (β), and intermediate (α) subunits (3). Type α-EN subunits have been found in many tissues, including liver and brain, by gel electrophoresis under nondenaturing conditions, but are absent from muscle (3). Two-dimensional gel analysis of in vitro translation products of muscle and fibroblast mRNAs suggest that α- and β-EN may be different gene products (data not shown). Presumably type α-EN is our "p46." Our Western blot data indicated, however, that there are high levels of a 46,000-dalton protein reactive with anti-p46 serum in skeletal muscle and heart. It therefore seemed probable that our anti-p46 serum cross-reacted with the muscle-specific β form of EN and perhaps the brain-specific γ form as well. We investigated the cross-reactivity of the antiserum in the following way.

[^{35}S]Methionine-labeled ANN-1 proteins were mixed with unlabeled protein obtained from mouse muscle tissue. This mix was resolved on a two-dimensional gel and immunoblotted with anti-p46 serum. The major polypeptide identified by

TABLE I

p36 and p46 Expression in Mouse Tissues

	Levels		Levels	
	p36*‡	p36 mRNA§	p46*‖	p46 mRNA§
Controls				
ANN-1	100%	++	100%	++
18.81	<5%	−	10–30%	++
Tissues				
Brain	<5%	±	120–140%	++
Liver	<10%	±	20–35%	+
Kidney	10–20%	+	50–60%	++
Muscle	<5%	−	200%	ND¶
Fetal muscle	ND	+	ND	ND
Heart	10–25%	ND	85–105%	ND
Spleen	20–25%	+	15–20%	+
Testes	30–50%	+	45–55%	+
Thymus	60–80%	++	50–80%	+
Lymph nodes	60–70%	ND	ND	ND
Urinary tract	50–70%	ND	ND	ND
Lung	90–110%	ND	15–20%	ND
Small intestine	100–120%	++	30–50%	+
Bone marrow	10–20%‖	ND	20–40%	−
Erythrocytes	<5%‖	ND	ND	ND

* The levels of p36 and p46 in ANN-1 cells were arbitrarily set at 100%. The actual amounts, determined as described in the Materials and Methods, are 0.05% and 0.12%, respectively.

‡ The range of four experiments.

§ p36 and p46 mRNA abundance assessed on a scale of − (undetectable) through ++ (abundance equal to ANN-1 mRNA), from two separate experiments.
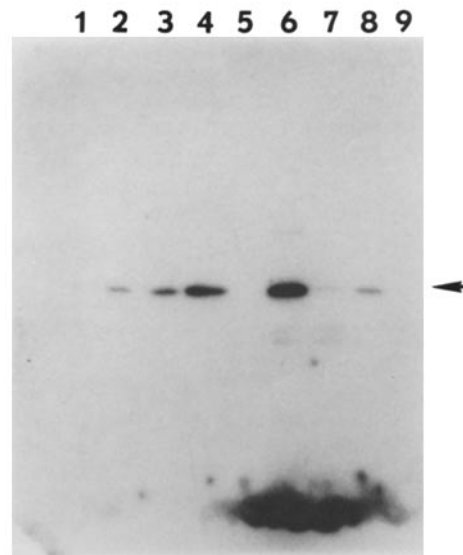
‖ The range of two experiments.

¶ ND, not done.

FIGURE 2 Western blot of mouse thymus and spleen fractions. Mouse thymus and spleen were bisected and teased to release nonstromal cells. These cells were collected and freed of debris by filtration. Equal amounts of protein from the intact tissues and from the teased cells were analyzed for the presence of p36 by immunoblotting. An arrow indicates p36. Lane 1, 3 µg ANN-1; lane 2, 5 µg ANN-1; lane 3, 10 µg ANN-1; lane 4, 20 µg ANN-1; lane 5, 18.81; lane 6, thymus; lane 7, thymus cells; lane 8, spleen; lane 9, spleen cells. Exposure time was 16 h with an intensifying screen at −70°C.

the immunoblot exhibited the same molecular weight as the fibroblast form but had a significantly more basic pI (data not shown). This indicated that the antiserum cross-reacted with the muscle specific form of EN. The antiserum also reacted with purified nerve-specific γ-EN subunits in an immunoblot (data not shown).

Tissue Distribution of p46 and p36 mRNAs

To examine whether the abundances of p46 and p36 reflected the level of mRNA coding for each, poly-A-containing RNA was purified from various mouse tissues. The RNA was translated in a mRNA-dependent reticulocyte lysate system. Only undegraded RNA preparations, judged by their ability to direct synthesis of high molecular weight proteins, were used. Equal amounts of acid-precipitable [^{35}S]methionine-labeled products from each mRNA were immunoprecipitated with either anti-p46 or anti-p36 serum. Immunoprecipitates were resolved on SDS polyacrylamide gels (Fig. 3). The mRNAs are classified as abundant (++), moderate (+), scarce (±), or undetectable (−) (Table I). There appears to be a good correlation between the amount of p46 and the level of p46-specific mRNA. One exception to this relationship is the 18.81 cell line. In this case, the mRNA level was abundant while the level of p46 was only 30% of that in ANN-1 cells. Nevertheless, our results suggest that the level of p46 mRNA is an important regulator of EN abundance.

In general, the level of mRNA for p36 correlates well with the abundance of p36 itself. Brain, muscle, and liver exhibited the lowest levels of both p36 and p36-specific mRNA. Thus, p36 expression also appears to be regulated largely by the level of its mRNA.

p36 Expression in Cell Lines

Several cell lines were examined for the expression of p36 either by immunoblotting, by biosynthetic [^{35}S]methionine labeling followed by immunoprecipitation, or both. The specific method used in each case is listed in Table II. Some T cell and B cell lines lacked the protein, consistent with the low level of p36 in lymphocytes from thymus and spleen. The only B cell line known to contain p36 is the pre-B cell line,
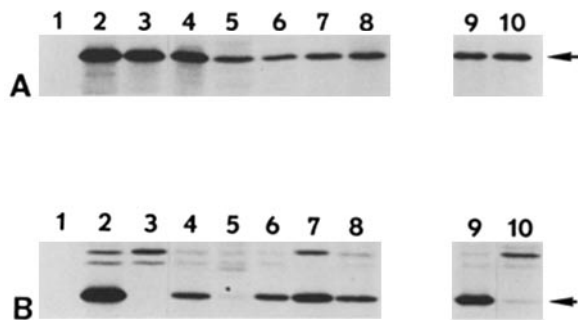

A

B

FIGURE 3 Estimation of p36- and p46-mRNA abundance by in vitro translation. Poly-A containing RNAs, obtained from various mouse tissues and from the cell lines, ANN-1 and 18.81, were translated in a mRNA-dependent reticulocyte lysate system. Equal amounts of acid precipitable cpm were immunoprecipitated with anti-p46 serum (A) or anti-p36 serum (B). The arrows point to p46 in A and p36 in B. In each case lanes 9 and 10 are from a separate gel. Lanes 1, no added mRNA; lanes 2, ANN-1; lanes 3, 18.81; lanes 4, kidney; lanes 5, liver; lanes 6, small intestine; lanes 7, thymus; lanes 8, spleen; lane 9 (A), brain; lane 10 (A), 18.81; lane 9 (B), ANN-1; lane 10 (B), brain. Exposure times were 15 d at −70°C.

TABLE II

Expression of p36 in Cell Lines

Cell type	Cell line	p36 expression	Method of detection*‡
Fibroblast	ANN-1	+	WB and IP
Pre-B lymphoma	18.81	−	WB and IP
Muscle lines	L6 myoblast	+	WB and IP
	L6 fused (2 wks)	+	
	BC3H1	+	
	BC3H1 differentiated	+	
Pheochromocytoma (nerve cell)	PC12	+	WB and IP
	PC12 + NGF	Transient increase	
Macrophage lines	RAW 264.7	+	WB and IP
	RAW 309 Cr.1	+	
T cell lines	SL12-1	+	WB
	SL12-4	−	
	SL12-9	+	
	SL2-2	−	
	SL2-4	−	
	SL3-2	−	
	SL3-4	−	
	W7TB	−	
	SAK.8	−	
T cell lines	SAKR TL S12.1.G.1	−	IP
	AKR1.G.1	−	
	S49.1TB.2.3	−	
	EL4.G.1.4	+	
	BW5147G.1.4	+	
	T1M1.4.G.1.3	+	
Thymic epithelium	TEPI I	+	IP
B cell line	WEHI 231	−	WB

* WB, Western blot.
‡ IP, immunoprecipitation.

RAW 309.1.1 (60). Several T cell lines, differing in many features including their stage of differentiation and mouse strain of derivation, were positive for p36. The thymic epithelial cell line, TEPI 1, contained p36, as expected of a cell line derived from the thymic stroma. p36 was also detected in two macrophage lines, RAW 309Cr.1 and RAW 264.

The two myoblasts lines examined, BC3H1 and L6, contained p36 at approximately equal levels in both their differentiated and undifferentiated stages. The differentiated myotubes contained 10% myoblast contamination. The PC12 line contained p36 and upon treatment with nerve growth factor, this level increased eightfold within 6 d. By day 14, the amount had dropped to its original level. This suggests that developmental regulation of p36 expression could be studied in this cell line.

Developmental Regulation of p36 Expression

Our detection of p36 in myoblasts and myotubes but not in adult muscle suggested that p36 expression might be developmentally regulated. In vitro translation of poly-A–containing RNA from rat fetal and adult muscle, followed by immunoprecipitation with anti-p36 serum indicated that p36 mRNA was present in fetal muscle, while it was undetectable in adult muscle (Fig. 4A, lanes 8 and 9). The decrease of p36 in muscle may thus occur at a late developmental stage that is not reached within 2 wk of myotube formation in culture.

To investigate the question of developmental regulation on a broader scale in the whole embryo, we obtained poly-A–

containing RNA from different stages of mouse embryogenesis and from placenta. p36 was immunoprecipitated from in vitro translation products and resolved on SDS polyacrylamide gels (Fig. 4A, lanes 1–7). In embryos at day 11 (Fig. 4A) and day 12 (data not shown), the level of p36 mRNA expression was considerably less than from day 13 on. Unfortunately, embryos isolated at days 7 and 9 were contaminated with placenta. Since placenta contains p36 mRNA, we could not determine the level of p36 mRNA in embryos before day 11. Is is possible, however, that p36 expression is relatively low until day 13 of mouse embryogenesis. Translation products were also immunoprecipitated with antiserum to p46

FIGURE 4 Immunoprecipitates of [³⁵S]methionine-labeled polypeptides synthesized in vitro from mouse embryo mRNAs. Poly-A containing RNA was obtained from mouse embryos at various stages of embryogenesis and from placenta and translated in a mRNA-dependent reticulocyte lysate system. Equal numbers of acid precipitable cpm were immunoprecipitated with anti-p36 serum (A) or anti-p46 serum (B) and the immunoprecipitates were analyzed by SDS PAGE and fluorography. Arrows indicate p36 (A) and p46 (B). Lanes 1, placenta; lanes 2, 7-d-old embryos; lanes 3, 9-d-old embryos; lanes 4, 11-d-old embryos; lanes 5, 13-d-old embryos; lanes 6, 15-d-old embryos; lanes 7, 17-d-old embryos. Lanes 8 and 9 are from a separate gel in which immunoprecipitates from translations containing adult rat muscle mRNA (lane 8) and fetal rat muscle mRNA (lane 9) were analyzed for the presence of p36. Exposure times were 18 d for lanes 1–7 and 10 d for lanes 8 and 9 at −70°C.

(Fig. 4B, lanes 1–7). In this case, there was little variation in expression with time and in contrast to p36 no difference was found in the expression of p46 between days 11, 12, and 13.

Cell Types Expressing p36

Having identified the tissues with highest levels of p36, we wished to determine whether specific cell types within each tissue expressed the protein and if so, identify them. Frozen tissue sections 4 μm thick were prepared from adult rats for indirect immunofluorescence and immunoperoxidase staining. The results of this survey are summarized in Table III. Examples of tissues stained by immunoperoxidase and immunofluorescence are shown in Figs. 5 and 6, respectively. Specificity of staining was demonstrated by staining consecutive tissue sections in parallel with anti-p36 serum, preimmune serum, and second antibody alone. The sera were absorbed first on fixed, permeabilized 18.81 lymphoid cells (46). Since these cells do not express p36, the absorption does not remove antibodies recognizing p36, but does reduce nuclear fluorescence (46). This nuclear fluorescence may indicate the presence in the nonabsorbed sera of antibodies not evident in immunoprecipitation experiments.

In all tissues analyzed, endothelial cells (note especially Fig. 5E) and fibroblastic cells of connective tissue stained specifically. In addition, epithelial cells from various tissues (e.g., small intestine [Fig. 5, A and B, Fig. 6, A and B], thymus, bladder [Fig. 5, G and H], and mammary gland), macrophages present in sections of lung, testicular interstitial cells, and cardiac muscle cells (Fig. 5, C and D) gave positive staining. Other epithelial cells (e.g., liver parenchymal cells, seminiferous epithelium, columnar bronchiolar epithelium, cuboidal epithelium of bile ducts, cuboidal and squamous epithelia of kidney tubules, and skin epidermal cells), and fat cells of the mammary gland appeared negative. Smooth and skeletal muscle cells (Fig. 5, E and F), nerve cells, lymphocytes, and erythrocytes in all tissues analyzed stained weakly or not at all, supporting the results of the immunoblotting experiments.
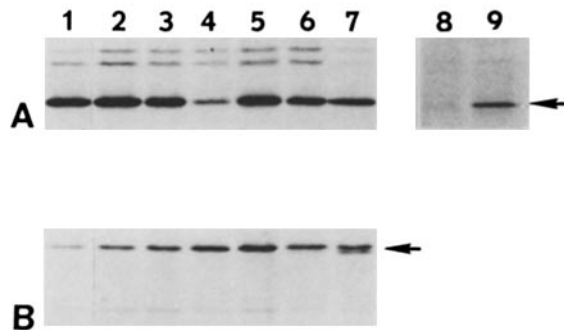
TABLE III

Distribution of p36 in Rat Tissue Cell Types by Immunofluorescence and Immunoperoxidase Staining

Positive cell types	Negative cell types
Epithelial cells	Epithelial cells
Columnar epithelial cells of small intestine*	Seminiferous epithelial cells§
Thymic epithelial cells*	Cuboidal epithelial cells of liver bile ducts§
Transitional epithelial cells of bladder*	Squamous and cuboidal epithelial cells of kidney tubules§
Cuboidal epithelial cells of mammary gland ducts‡	Columnar bronchiolar epithelial cells*
Amnion cells‡	Epidermal cells of skin‡
All examined endothelial cells§	Liver parenchymal cells§
Mesenchymal cells	Mesenchymal cells
Fibroblasts of all tissues examined§	Fat cells of mammary gland§
Hematopoietic cells	Hematopoietic cells
Macrophages in lung§	Erythrocytes§
Muscle cells	Lymphocytes of spleen and thymus§
Cardiac muscle cells§	Muscle cells
Other cells	Smooth muscle cells in all examined tissues§
Testis interstitial cells§	Skeletal muscle cells§
	Other cells
	Neurons in all examined tissues‡

* Derived from endoderm.
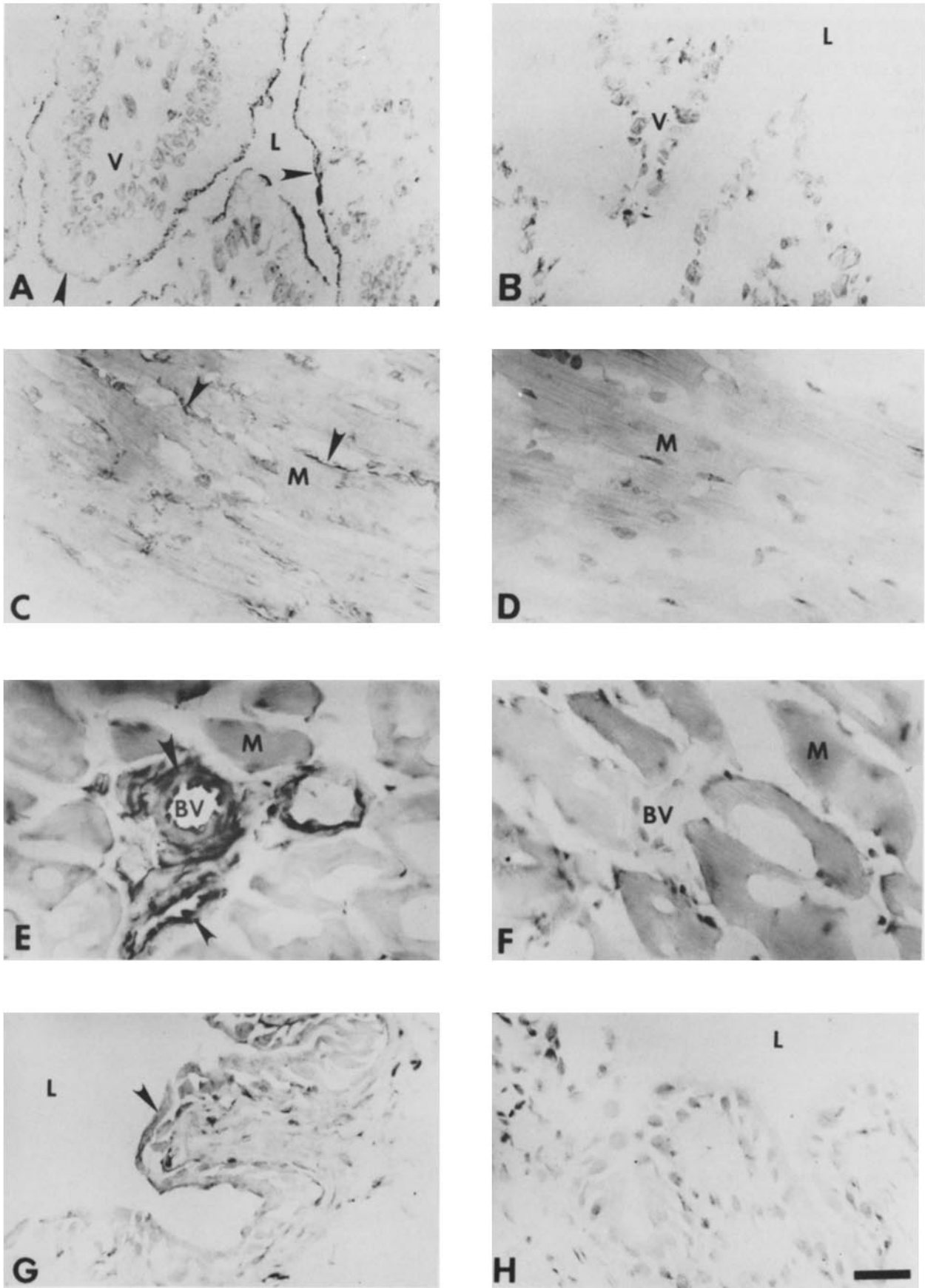‡ Derived from ectoderm.
§ Derived from mesoderm.

FIGURE 5 Indirect immunoperoxidase staining produced by anti-p36 serum in various rat tissues. Anti-p36 serum and preimmune serum were preabsorbed on 18.81 lymphoma cells as described in Materials and Methods. Second antibody was affinity purified horseradish peroxidase-conjugated goat anti-rabbit IgG. Nuclei were stained by hematoxylin and also appear black in the figure. A, C, E, and G were stained with preabsorbed anti-p36 serum. B, D, F, and H were stained with preabsorbed preimmune serum. A and B are sections through the small intestine. Arrowheads indicate staining at the luminal border of the villi (V) in A; (L) lumen. C and D are sections of cardiac muscle. Arrowheads point to the stained region along the muscle fiber in C. Note that the muscle fiber (M) itself does not stain. E and F are thigh muscle sections. Note the strongly staining endothelial cells (indicated by the arrows) lining blood vessels (BV) in E. G and H are sections of urinary bladder. The arrowhead in G points to a fold of transitional epithelium along the lumen (L) staining specifically. Bar, 25 μm. × 200.
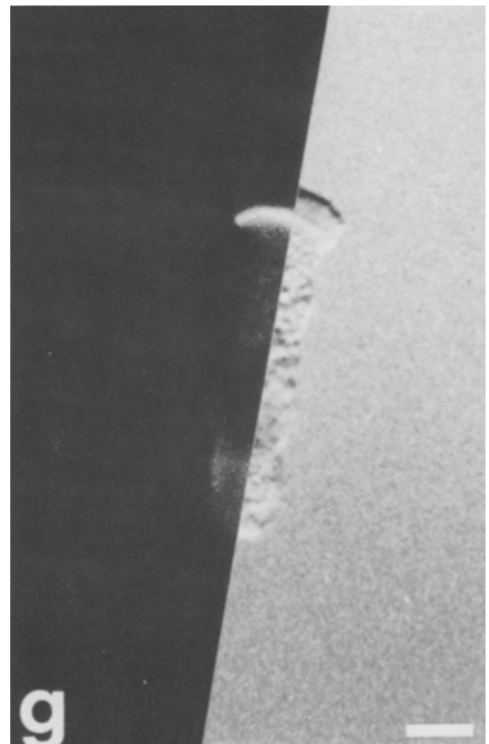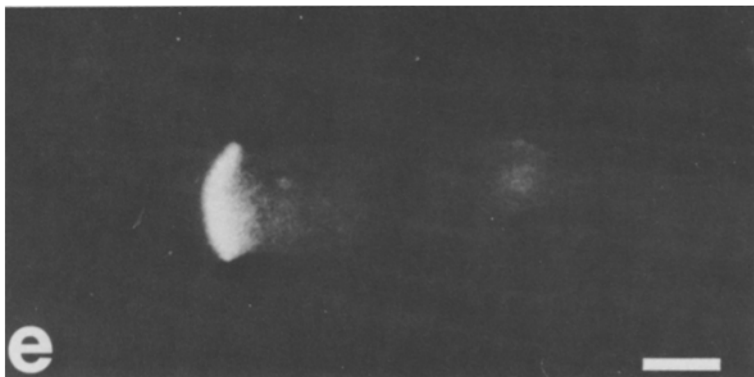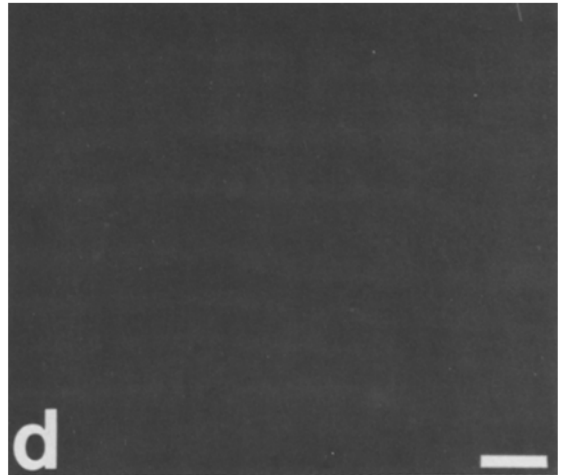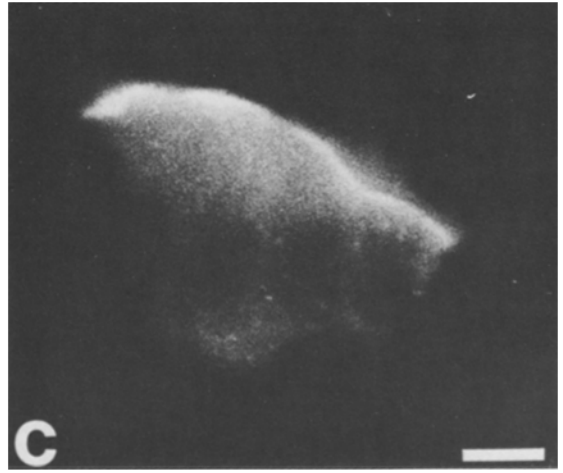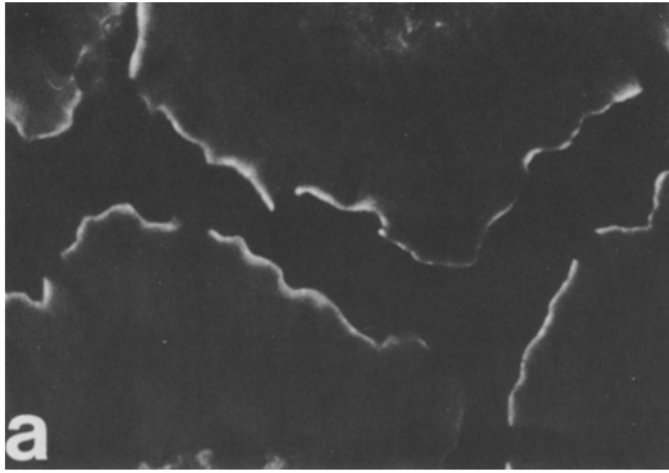
In most of these differentiated cell types, staining could not be localized precisely within the cell due to technical difficulties. In cardiac muscle cells, however, staining appeared to be concentrated at or below the plasma membrane (Fig. 5C), that is, in the region of the sarcoplasmic reticulum. An interesting staining pattern was seen in the small intestine. p36 appeared to be concentrated at the luminal surface of the columnar epithelial cells lining the villi (Fig. 5A and Fig. 6A). Two specialized structures of these cells are located in this same region, the microvilli and the terminal web. The terminal web lies directly underneath the microvilli and is composed of a dense cytoskeletal network. To distinguish between staining of the microvilli and the terminal web, single cell suspensions of the epithelial cells were prepared and subjected to indirect immunofluorescence staining (Fig. 6, C–E, and G). These images were compared with those obtained using Nomarski optics (Fig. 6G). We cannot differentiate between staining of membrane or staining of cytoskeletal structures in this region.

DISCUSSION

The function of a major substrate of tyrosine protein kinases, p36, remains unknown although an association with the inner surface of the plasma membrane of some cultured cell lines has been established (2, 18, 22, 33, 43, 46, 50). To gain additional information as to the possible functions of the protein, we have determined the tissue distrubution of p36 in the mouse and rat by biochemical and immunocytochemical techniques. Simultaneously, we examined the tissue distribution of p46, another substrate of tyrosine protein kinases, which has recently been identified as a form of the glycolytic enzyme, EN (20). Knowledge of the tissue distribution of a protein might provide valuable insight as to its possible function. One would predict that a glycolytic enzyme would be ubiquitous, with highest expression in skeletal muscle, heart muscle, and brain, tissues in which glucose is metabolized at a high rate. Indeed, the pattern of p46 expression we obtained from Western blotting correlated with that prediction. In contrast, it is difficult to perceive a theme underlying p36 expression. It was detected in endothelial cells, in fibroblastic cells, macrophages, heart muscle cells, testicular interstitial cells, and certain epithelial cells. It was present at very low levels or absent from skeletal and smooth muscle cells, nerve cells, erythrocytes, and lymphocytes.

A potential complication emerged from our analysis of the tissue distribution of p46. Examination of tissue proteins by two-dimensional gel electrophoresis followed by silver staining had indicated that a protein corresponding to fibroblast p46 was rare in skeletal muscle and heart. Analysis of one- and two-dimensional immunoblots of muscle protein revealed that our antiserum reacted with the muscle isozyme of EN, as well as the form found in fibroblasts and other tissues (p46). If the antiserum were specific for p46, and had not recognized the muscle isozyme, a less uniform distribution of p46 would have been apparent. With regard to p36, this example emphasizes the possibility that there might be proteins related to p36 in structure and function but not recognized by our anti-p36 serum, in those cells where p36 is undetectable.

The wide range in the levels of p36 expression between tissues found by Western blotting suggested that p36 was probably not present in all cell types in the animal and confirmed a prediction of previous work (27, 60) that p36 distribution would not be ubiquitous. In cultured fibroblasts, p36 is a relaively abundant protein, comprising 0.2–0.25% of the protein in primary chicken fibroblasts (19, 51) and ~0.05% in the mouse fibroblast line, ANN-1. The level in certain differentiated cell types of the mouse and rat must exceed this because certain tissues surpass this level, while only specific cells within the tissue gave a strong immunohistochemical reaction with anti-p36 serum. For example, whole thymus contained high levels of p36, but both immunofluorescence and physical separation of cells showed that most of the p36 was present in the quantitatively fewer stromal cells rather than the lymphocytes. p36 is therefore a major protein component of certain cells.

The expression of p36 and p46 may be controlled primarily by the concentrations of their respective mRNAs. For both proteins, there was good correlation between the level of protein in a given tissue and the level of its corresponding mRNA, determined by in vitro translation and immunoprecipitation. It is interesting to note that the tissue distribution of p36 does not coincide with the tissue distributions of known tyrosine protein kinases for which p36 is a substrate. When the level of pp60^{c-src} in various tissues was determined by measuring its enzymatic activity in an immunoglobulin protein kinase assay, brain exhibited the highest activity (21, 41) followed by kidney, lung, muscle, and connective tissue. Immunoprecipitation shows that the c-abl gene product is abundant in thymocytes (65). Poly-A–containing RNA corresponding to the c-yes and c-ros genes is expressed at high levels in kidney and RNA corresponding to the c-fps gene is most abundant in bone marrow (56). The distribution of the EGF receptor, reported to possess tyrosine protein kinase activity (9, 13), also differs from that of p36. By ^{125}I-EFG binding studies on mouse embryo tissues (days 11–18), EFG receptor was present in decreasing order in amnion, lung,

FIGURE 6   Indirect immunofluorescent staining produced by anti-p36 serum in the small intestine. Anti-p36 serum and preimmune serum were preabsorbed on 18.81 lymphoma cells and incubated with frozen sections of rat small intestine as described in Materials and Methods. Second antibody was affinity purified fluorescein-conjugated goat anti-rabbit IgG. (a) Villi of small intestine stained with anti-p36 serum; (b) villi of small intestine stained with preabsorbed preimmune serum. Note the staining along the luminal edge of the villi in a. Bar, 12 μm. × 200. Columnar epithelial cells were dissociated from the small intestine, fixed, permeabilized, and stained as described in Materials and Methods. (c) A small clump of cells stained with preabsorbed anti-p36 serum; (d) a similar group of cells stained with preabsorbed preimmune serum. The luminal side of the cells is up in each case. Again, note the staining along the luminal edges of the cells in c. (e) A single columnar epithelial cell stained with preabsorbed anti-p36 serum; (f) same field as shown in d viewed by Nomarski optics. Note the microvillar region at the left edge of the cells. (g) This panel shows a composite of another single columnar epithelial cell. The left side of the panel shows the cell stained with preabsorbed anti-p36 serum. The right side of the panel is the Nomarski image of the cell. Note that the microvilli extend beyond the stained crescent which is in the area of the terminal web. Bars in C–G, 4 μm. × 200.

liver, brain, and heart (1). Adult heart contains higher levels of p36 than does either liver or brain, although it may not be pertinent to compare the adult situation with that of the embryo. Also, one can imagine that regulation of some aspect of cell physiology by p36 could occur at two levels—expression of p36 and, potentially, modification of p36 function by phosphorylation.

The presence of p36 and p36 mRNA in some tissues but not others implies developmental regulation. p36-specific mRNA was not detected in adult muscle, in contrast with the abundance of p36-specific mRNA in fetal muscle, and of p36 in primary cultures of chicken myoblasts (42) and rat myoblast cell lines. Even after 2 wk of fusion L_6 cells continue to express p36, suggesting that it is not simply fusion that triggers the decline in p36-specific mRNA but rather a later event, such as contact by nerves. It is also possible that the in vitro model is not an accurate reflection of the in vivo situation. Many, but not all, lymphoid cell lines express p36, but p36 was not detected in freshly-prepared thymic or splenic lymphocytes. All other cultured cells analyzed express p36. Thus, p36 expression may be turned on when cells are put into culture. In any case, the decrease of p36 in muscle is contrary to the apparent overall pattern of p36 developmental regulation. In the whole mouse embryo, p36 mRNA expression is low on days 11 and 12 and significantly higher in later stages. Because of placental contamination, p36 mRNA expression could not be determined in embryos isolated before day 11, but it is possible that, in general, p36 expression is low throughout early development.

Interpretation of immunofluorescence and immunoperoxidase staining data relies on the specificity of the antiserum. Our anti-p36 serum is not monospecific, although when used against mouse and rat tissues, p36 is the major immunoreactive protein. Thus, serum used for immunofluorescence and immunoperoxidase staining was preabsorbed with fixed and permeabilized cells that do not contain detectable p36. Staining exhibited with this preabsorbed serum is blocked by the addition of excess purified p36 (46). An additional argument for the specificity of the preabsorbed serum is the coincidence of its staining pattern in fibroblasts with that of a monoclonal antibody prepared to p36 (33) and with other polyclonal antibodies raised against purified p36 (43, 50). In most respects, our data on the expression of p36 in rodent tissues resemble the data of Greenberg et al. (31) on the expression of the homologous protein in chicken tissues.

It was not possible to ascertain the precise intracellular location for p36 by immunofluorescence or immunoperoxidase staining of frozen tissue sections in most of the positively staining cells. With fluorescent stained sections, it is often difficult to distinguish the cell type staining positively, let alone the intracellular distribution of a protein. Immunoperoxidase staining has the advantage of allowing one to distinguish cell types easily but the brown precipitate, indicative of positive staining, often covers the entire cell. Both procedures are limited to detecting only populations of antigen which are accessible to antibody. Immunoelectron microscopy would be needed to gain a more detailed picture. Two instances where p36 could be localized within the cell were heart muscle cells and brush border cells of the small intestine.

In heart, the staining appeared along the edge of the cell by both immunofluorescence and immunoperoxidase staining. Staining was not evident within the muscle fibers. The thickness and ruffled characteristic of the stained region suggested

that it may represent the sarcoplasmic reticulum. This localization within cardiac muscle cells may thus be analogous to the plasma membrane localization within fibroblasts.

In small intestine, the luminal edges of the columnar epithelial cells lining the villi stained exclusively with both immunofluorescence and immunoperoxidase procedures. Purified intestinal columnar epithelial cells do have an increased amount of p36 per microgram of protein by Western blot analysis in comparison to whole gut (data not shown). It is quite possible that the bulk of p36 in the gut is present in the columnar epithelial cells. Within these cells, immunofluorescence staining shows that p36 is localized primarily in the terminal web region. In part, this apparent localization may merely indicate better accessibility of the antigen to the antibody. The crescent-shaped staining pattern given by p36 in columnar epithelial cells is shared with the spectrin-like protein found in chicken intestine, TW 260/240 (30). This colocalization is especially intriguing as other investigators have recently noted similar staining patterns by anti-p36 and anti-spectrin sera in cultured fibroblasts (33, 43, 50). Although p36 may not precisely co-localize with the spectrinlike protein, it is seen in the same cortical region and stains in the same type of reticular pattern in fibroblasts (33, 43, 50). Our finding that p36 localizes like the spectrin related protein TW 260/240 in small intestine, provides more evidence for a cortical localization of p36. The brush border is an amenable system for studying the possible associations of p36 with the plasma membrane or with other proteins closely apposed to the membrane since it is easily separated from the rest of the cell. Studies on the interactions of p36 in brush borders are underway.

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