The Tyrosine Phosphorylation Substrate p36 Is Developmentally Regulated in Embryonic Avian Limb and Is Induced in Cell Culture

V. Celeste Carter,* Anthony R. Howlett,‡ G. Steven Martin,* and Mina J. Bissell‡

*Department of Zoology, University of California, Berkeley, California 94720; ‡Laboratory of Cell Biology, Division of Biology and Medicine, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

Abstract. The 36-kD protein-tyrosine kinase substrate p36 has been variously postulated to be involved in membrane-cytoskeletal interactions, membrane traffic, and the regulation of phospholipase A_2, and its phosphorylation may play some role in malignant transformation by avian sarcoma viruses. Because embryonic tissues are resistant to transformation by avian sarcoma viruses, we have examined the expression of p36 in the developing avian embryonic limb. The level of p36 increased progressively from day 5 to day 14 of development. It was largely absent from day-5 mesenchyme, and was induced during the differentiation of mesenchymal cells into connective tissue and cartilage, but was not induced in differentiating muscle. In contrast, p36 was detected in ectodermal cells at all developmental stages examined. When day-5 limbs were dissociated and cultured, p36 was induced in all adherent cells, beginning at 2–4 h after plating, and reaching levels comparable to those observed with intact day-14 limb tissue within 48 h. The accumulation of p36 in culture was dependent on substratum adherence, suggesting that its stability is regulated by cell attachment or spreading. These findings are consistent with a structural or mechanical role for p36.

PHOSPHOTYROSINE-CONTAINING proteins are minor components of normal cells which in certain instances are associated with the regulation of cellular metabolism and growth. An increase in the phosphotyrosine content of cellular proteins is observed after oncogenic transformation by several avian sarcoma viruses (18, 26). One of the most extensively characterized cellular substrates is a 36,000-D protein, p36 (25). p36 is phosphorylated at tyrosine after transformation of cells by several retroviruses and after exposure of certain cell types to growth factors (18, 26). Biochemical fractionation of cells and immunofluorescence microscopy indicate that p36 is located on the cytoplasmic side of the plasma membrane in both normal and transformed cells, possibly functioning as a structural protein (1, 5, 6, 16, 23, 24).

p36 is differentially expressed in different tissues. In avian (15) or rodent (14) tissues, p36 was detected in fibroblasts, endothelial cells, epithelial cells, and cells of cartilage, but not in muscle or adult nervous tissue. In intestinal columnar epithelial cells, p36 is concentrated in the terminal web (14) confirming the cortical localization observed in cultured cells. Additionally, p36 isolated from intestinal epithelial cells binds F-actin and nonerythroid spectrin in vitro in a calcium-dependent fashion (10, 11, 13; Carter, V. C., J. Prior, and G. S. Martin, unpublished data), suggesting that it may play a structural role in the cell cortex. p36 binds Ca^{++} and anionic phospholipids and shows sequence homology to other Ca^{++}- and phospholipid-binding proteins such as lipocortin and calelectrin (reviewed in reference 20).

The normal cellular function of p36 and its possible role in transformation remain obscure. To investigate the factors regulating p36 expression, we have examined the synthesis of p36 in developing chick limb tissue using biochemical and immunocytochemical techniques. This tissue was selected for analysis because the embryonic limb has been shown to support replication of Rous sarcoma virus but to be resistant to transformation (7). The results indicate that p36 is developmentally regulated in avian limb, and is induced rapidly when limb bud cells are dissociated and plated in tissue culture. The stability of p36 appears to be dependent upon adhesion of the cells to a substratum.

Materials and Methods

Embryo Dissection and Culture

Chicken embryos (Spafas, Inc., Roanoake, IL) were dissected free of extraembryonic membranes on days 5, 9, and 14 of development. Limb buds (day 5) and the upper right limbs (days 9 and 14) were removed from embryos and pooled. For cryostat sectioning, limbs were embedded in OCT compound (Miles Laboratories, Inc., Naperville, IL) and frozen in 2-methylbutane (isopentane) cooled in a bath of liquid nitrogen. To generate single cell suspensions for cell culture or immunofluorescence analysis, the limbs were rinsed in phosphate-buffered saline (PBS) and mechanically minced. The limb tissue was then transferred to Hams F10 medium containing 1.5 mg/ml (316 U/mg) of Collagenase Type II (Sigma Chemical Co., St. Louis, MO), 1 mg/ml trypsin (Gibco, Grand Island, NY), 0.1 mg/ml EDTA, 1.3 mg/ml Hepes (Sigma Chemical Co.), and gently agitated at room tempera-

Table I. p36 Expression in Developing Avian Limb Tissue and in Dissociated Cells

	cpm [^{35}S]methionine in p36*		Level of expression of p36‡	
	Exp. 1	*Exp. 2*	*Exp. 1*	*Exp. 2*
Day-5 tissue	356	1,355	1.0	1.0
Day-5 cells (48 h)	3,557	15,310	10	11
Day-9 tissue	1,944	5,515	1.0	1.0
Day-9 cells (48 h)	4,064	12,301	2	2
Day-14 tissue	3,345	6,705	1.0	1.0
Day-14 cells (48 h)	2,430	17,453	0.7	3

* cpm in p36 immunoprecipitated from 0.1 mg total protein. Counts normalized to total [^{35}S]methionine incorporation.
‡ cpm [^{35}S]methionine in p36 in 48-h cultured cells/cpm [^{35}S]methionine in uncultured tissue.

ture for 45 min. The resultant slurry of cells and undigested material was passed through an 18-gauge needle, and washed twice in tissue culture medium. For immunofluorescence analysis, the cell suspensions were centrifuged in a Cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA) onto glass coverslips (No. 1; Thomas Scientific, Philadelphia, PA). For tissue culture, the cells were plated in 35-mm tissue culture dishes at a density of 2×10^6 per dish. The limb bud cells were maintained on plastic or planar type I collagen (22) using Medium 199 (Gibco), supplemented with 10% tryptose phosphate broth, 4% newborn calf serum, 1% chicken serum, 0.1% D-glucose, and 50 μg/ml Gentamycin at 37°C.

Suspension cultures were maintained in 1% methocel medium (Methylcellulose 4000 centipoise; Fisher Scientific, San Francisco, CA). This medium was prepared by mixing 2% methocel with an equal volume of 2× Dulbecco's modified Eagle's medium (DME) supplemented with 4% tryptose phosphate broth, 8% calf serum, and 2% chicken serum. Suspended cells were plated over a layer of 0.72% nutrient agar. Cells were harvested by dilution in several volumes of cold DME and centrifugation for 5 min at 1,000 *g*.

Radiolabeling of Limb Tissue and Cells, and Immunoprecipitation

Freshly isolated limb tissue and cultured limb bud cells were rinsed twice in PBS before radiolabeling. Radiolabeling medium was methionine-free DME supplemented with 2% tryptose phosphate broth, 4% calf serum, and 1% chicken serum. Minced tissue or cells scraped from the dishes were radiolabeled in suspension for 6 h at 37°C in 300 μl of radiolabeling medium containing 100 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL). The suspended cells and minced tissue were centrifuged at 1,000 *g* and the pelleted material was stored at −70°C until required for further processing. Attached cultured limb bud cells were radiolabeled at varying times post-plating in 1 ml of radiolabeling medium containing 300 μCi [^{35}S]methionine. Attached labeled cells were lysed by the addition of 500 μl RIPA lysis buffer (0.15 M NaCl, 0.01 M Tris-HCl [pH 7.4], 0.001 M EDTA, 1% [wt/vol] NP-40, 1% [wt/vol] Na deoxycholate, 0.1% [wt/vol] SDS, and 1% [vol/vol] Aprotinin), and the cell lysates were stored at −70°C.

Immunoprecipitation was carried out as previously described (24) with the following modifications. Radiolabeled samples of either limb tissue or cultured limb bud cells were resuspended in 1 ml RIPA lysis buffer. Limb bud tissue was further dissociated by homogenization in a ground glass Dounce homogenizer. After 15 min at 0°C, the lysates were clarified by centrifugation at 13,000 *g* for 10 min at 4°C. Protein concentrations were determined by the method of Bradford (3). Equivalent amounts (100 μg) of protein or equivalent amounts of TCA-precipitable counts were incubated with an excess of rabbit anti-p36 serum (24) for 30 min at 0°C. Antigen-antibody complexes were adsorbed for 10 min at 0°C with 20 vol of a 10% suspension of fixed *Staphylococcus aureus* per vol of antiserum. The adsorbed immune complexes were washed extensively, and solubilized in SDS gel sample buffer.

Immunoprecipitated proteins were separated by SDS PAGE (10% acrylamide, 0.13% *bis*-acrylamide). The gels were fixed, stained, dried, and fluorographed, and the band corresponding to p36 was excised and counted.

Western Blotting

Protein samples were resolved on SDS PAGE as described above and electrophoretically transferred to nitrocellulose filters (4, 28). After transfer, the blots were incubated in PBS containing 5% nonfat dry milk for 30 min at room temperature (19). The blots were incubated for 18 h with rabbit anti-p36 serum (1:100 dilution in PBS containing 1% nonfat dry milk) and then washed extensively with PBS. To detect antibody, the blots were incubated for 1 h with a 1:1,000 dilution of goat anti-rabbit peroxidase complex (Sigma Chemical Co.) in PBS, and developed in buffer (20 mM Tris, pH 7.5, 0.5 M NaCl) containing H_2O_2 and 4-chloro-1-napthol (Sigma Chemical Co.).

Immunofluorescence Microscopy

Cryostat sections (6 μm thick) mounted on glass coverslips (No. 1; Thomas Scientific), cytocentrifuge preparations of freshly dissociated limb cells, and limb bud cells cultured on glass coverslips were used for immunofluorescent analysis. The preparations were rinsed in PHEM buffer (0.06 M Pipes, 0.025 M Hepes, 0.01 M EGTA, 0.002 M $MgCl_2$, pH 6.9) at room temperature, and fixed with 2% paraformaldehyde in PHEM buffer for 20 min. They were then rinsed three times in 0.1 M glycine and postfixed with 0.1% Triton X-100 in PHEM for 20 min at room temperature. All preparations were washed in PBS containing 0.1% BSA (PBS/BSA) and incubated with rabbit anti-p36 serum or preimmune serum for 60 min at room temperature. Specimens were washed further in PBS/BSA containing 5% goat serum (Gibco), and incubated with fluorescein-conjugated goat anti-rabbit IgG (1:200) (Tago Inc., Burlingame, CA) for 60 min at room temperature. For dual-label immunofluorescence, p36-labeled cryostat sections were additionally incubated with a monoclonal anti-myosin (fast heavy chain) antibody (1:200), followed by rhodamine-conjugated goat anti-mouse $F(ab')_2$ (Tago Inc.)(1:100). After extensive washing, stained specimens were mounted in 9:1 (vol/vol) glycerol/50 mM Tris buffer containing 0.01% sodium azide and 2.5% DABCO (1.4 Diazabicyclo [2,2,2] octane), pH 8.9 (Sigma Chemical Co.), for viewing on a Zeiss inverted phase-contrast microscope equipped for epifluorescence.

Results

p36 Expression in Developing Avian Limb Tissue

To examine the expression of p36, chicken embryos were analyzed at days 5, 9, and 14 of development. Minced limb tissue was labeled with [^{35}S]methionine for 6 h in suspension and analyzed by immunoprecipitation. Limb buds excised from day-5 embryos synthesized low but detectable levels of p36. Between days 5 and 9 of development, a fivefold increase in the level of expression of p36 was observed, and a further twofold increase was observed from days 9 to 14 of development (Table I). To measure the accumulation of p36 in the developing limb tissue, cell lysates were resolved by SDS PAGE, and analyzed by immunoblotting. As shown in Fig. 1, p36 is present in day-5 limb bud tissue (lane *a*) at a low level, and at a significantly higher level in day-14 limb tissue (lane *c*). These results indicate that p36 is a developmentally regulated protein in the avian limb.

Immunolocalization of p36 in Frozen Limb Sections and Dissociated Limb Cell Cultures

To determine the sites of synthesis of p36 in the developing limbs, frozen sections were prepared and stained with anti-p36 serum. No appreciable staining of p36 was obtained in tissue preparations incubated with preimmune serum (data not shown).

Cryostat sections taken at day 5 showed p36 to be selectively localized in the ectodermal layers of the limb bud (Fig. 2 *A*). The mesenchymal cells of the limb interior were negative for p36 expression with the exception of rare foci of cells that may represent part of the endothelial lining of the limb vasculature (Fig. 2 *D*).

At day 9, the epidermal derivatives of the ectodermal

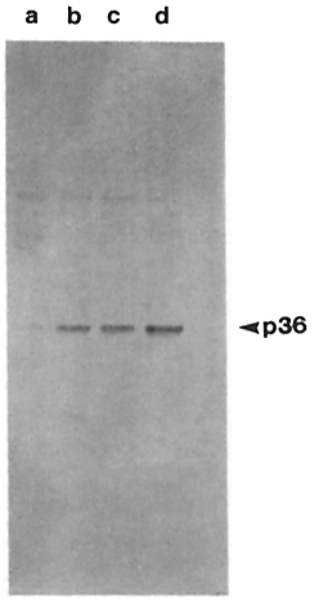

Figure 1. Immunoblot analysis of p36 expression in day-5 and day-14 avian limb tissue and dissociated limb cells. Limbs from day-5 and -14 embryos were either minced and analyzed directly or cultured for 48 h and analyzed for p36. Equivalent amounts of protein were resolved upon SDS PAGE and electrophoretically transferred to nitrocellulose filters. After transfer, the blots were incubated sequentially with 5% nonfat dry milk, rabbit anti-p36 serum, and goat anti-rabbit peroxidase complex. Peroxidase reaction product was developed with 4-chloro-1-napthol and H_2O_2. (*a*) 5-d limb tissue, (*b*) 5-d limb cells cultured for 48 h, (*c*) 14-d limb tissue, and (*d*) 14-d limb cells cultured for 48 h.

layers were positive for p36 (Fig. 2 *B*). p36 expression was also detected in certain mesenchymal-derived limb structures including cartilage (*C*) and the surrounding perichondrium (*P*) (Fig. 2 *E*) and also centers of blood vessel (*BV*) development (Fig. 2 *B*). The protein appeared to be absent from loose connective tissues including the dermis and from muscle tissue.

At day 14, the distribution of p36 was essentially similar to that at day 9 although the intensity of staining appeared greater for all structures that expressed p36. The epidermis (Fig. 2 *C*), cartilage (*C*) (Fig. 2 *F*), and perichondrium (*P*) (Fig. 2 *F*) were all positive for p36. At this time of development connective tissue structures including the dermis (Fig. 2 *C*) and the fascia (*F*) separating the large blocks of skeletal muscle (*M*) were also positive (Fig. 2 *G*). p36 remained conspicuously absent from the limb musculature which was identified morphologically in adjacent sections stained either by the Mallory-Heidenhain trichrome method (data not shown) or immunologically by dual-labeling with an anti-myosin monoclonal antibody (Fig. 2 *H*) and anti-p36 serum (Fig. 2 *I*).

The pattern of p36 expression in cytocentrifuge preparations broadly confirmed that observed in frozen sections. Values of <1, 14, and 21% p36-positive cells were obtained from cytocentrifuge preparations taken at days 5, 9, and 14, respectively (Fig. 3, *A–C*). It should be noted that these data from later embryos may be influenced by differential sensitivity of different tissues to digestion with the proteolytic enzymes used to prepare cell suspensions.

Induction of p36 in Day-5 Limb Bud Cells Placed in Culture

Limb tissue from embryos sacrificed at days 5, 9, and 14 of development was dissociated and cultured in 35-mm tissue culture dishes for 48 h at 37°C. Cultured limb bud cells were scraped from the dish, labeled in suspension with [^{35}S]methionine for 6 h at 37°C, and analyzed by immunoprecipitation and immunoblotting. The expression of p36 after 48 h in culture was roughly equivalent at the three developmental stages examined (Table I). Thus, the level of expression of p36 in day-5 limb bud cells cultured for 48 h was 10-fold higher than that in uncultured day-5 limb bud tissue. The induction of p36 was not as striking in day-9 or day-14 limb cells, but was still detectable: the level of expression of p36 was twofold and 1.6-fold higher than that in the corresponding day-9 and day-14 limb tissues.

Analysis of limb cell lysates by immunoblotting revealed an increased accumulation of p36 in cultured limb cells (Fig. 1). p36 was present in day-5 limb cells placed in culture for 48 h (Fig. 1, lane *b*) at a much higher level than in day-5 limb tissue (Fig. 1, lane *a*). A slight increase was also observed in day-14 limb cells when cultured for 48 h (Fig. 1, lane *d*).

These results indicate that p36 is induced when limb bud cells are placed in culture. Cultivation of limb cells leads to both an increased synthesis and an increased accumulation of p36. The amount of p36 in limb cells from all developmental stages examined after 48 h in culture is approximately equal, and it is slightly higher than that observed in day-14 limb tissue.

To determine the time course of induction of p36 in day-5 limb bud cells, day-5 limb bud tissue was dissociated, and the cells were plated in 35-mm tissue culture dishes. At varying times post-plating, the medium and floating cells were removed, and the attached cells were labeled with [^{35}S]methionine for 30 min. A marked increase in the rate of synthesis of p36 was observed by 4 h post-seeding (Fig. 4 *A*, lane *e*) with a slight increase in the rate of synthesis of p36 being observed as early as 2 h post-seeding (Fig. 4 *A*, lane *d*). Equivalent amounts of total cell protein were also analyzed by immunoblotting (Fig. 4 *B*). A low amount of p36 was detected by 6 h post-seeding (Fig. 4 *B*, lane *e*) and a continued accumulation of p36 was observed at 24 h (Fig. 4 *B*, lane *f*) and 48 h (Fig. 4 *B*, lane *g*) post-seeding. To confirm the results of these radiolabeling and immunoblotting experiments the expression of p36 in dissociated monolayer cultures was examined by immunofluorescence. Weak but significant immunofluorescence could be detected within 2 h in all cells at each time of development (Fig. 3, *D–F*). Examination of established cultures after 48 h showed that the intracellular localization of p36 had assumed the characteristic reticular distribution over the inner surface of the plasma membrane that has been reported previously (16, 24) (Fig. 3, *G–I*). Thus, results from radiolabeling, immunoblotting, and immunofluorescence analyses indicate that p36 is rapidly induced in attached cultured cells. However, substantial accumulation of p36 is observed only at later times in attached cultured cells.

Dependence of Induction of p36 on Attachment to a Substratum

To determine if the induction of p36 synthesis and/or accumulation in cultured day-5 limb bud cells was dependent upon anchorage of the cells to a substratum, day-5 limb bud cells were plated either on tissue culture dishes, or tissue culture dishes coated with collagen, or suspended in 1% methocel medium. After 48 h in culture the cells were scraped from the dishes and either lysed immediately for analysis by immunoblotting, or labeled with [^{35}S]methionine for 6 h in suspension for analysis by immunoprecipitation. p36 is easily detected by immunoblotting in cells plated

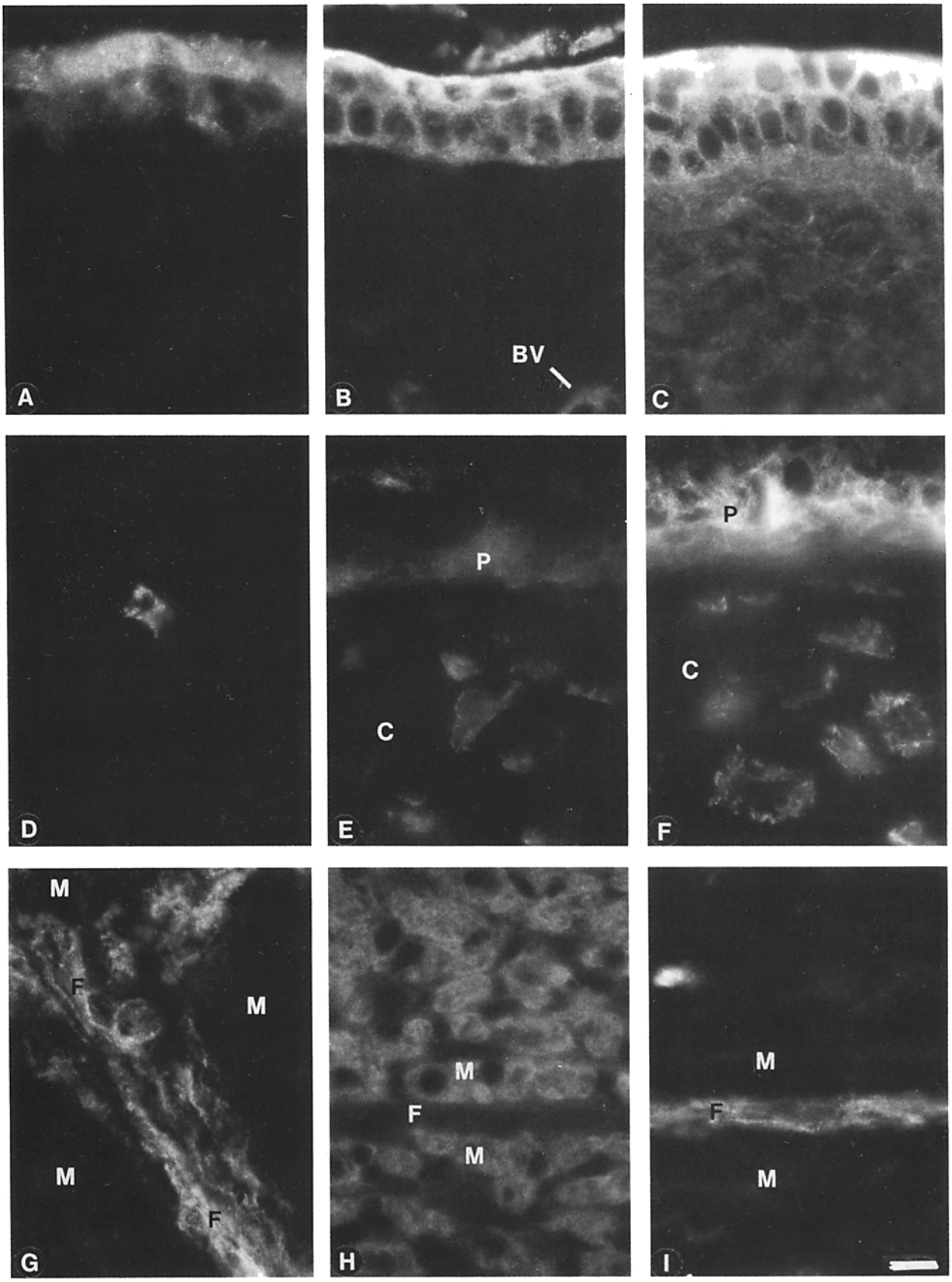

Figure 2. Immunofluorescent localization of p36 in frozen sections of day-5, -9, and -14 limbs. Limbs were embedded, frozen, and 6-μm transverse sections were cut at the approximate midpoint of the day-5 limb bud and at the level of the distal humerus-proximal radius/ulna at days 9 and 14. Sections were stained with anti-p36 serum (*A–G, I*) or anti-myosin antibody (*H*). *A–C* show the superficial regions and *D–F* show the internal regions of the developing limb at days 5 (*A* and *D*), 9 (*B* and *E*), and 14 (*C* and *F*). At day 5 of development,

on either plastic (Fig. 5, lane *a*) or collagen (Fig. 5, lane *b*), but it is not observed in cells suspended in methocel medium (Fig. 5, lane *c*). Cells recovered from 1% methocel medium and plated for an additional 48 h on plastic accumulate p36 to the same level as cells initially plated on a solid substratum (Fig. 5, lane *d*).

In contrast to the results obtained by immunoblotting, the synthesis of p36 was detectable in all day-5 limb cells after a 48-h culture regardless of the culture conditions (Table II). These results indicate that the accumulation of p36 is dependent upon anchorage of cells to a substratum but the protein is synthesized in culture irrespective of culture conditions.

Table II. The Level of Expression of p36 in Day-5 Limb Cells Cultured on Different Substrata

Cell culture conditions	cpm [^{35}S]methionine in p36*	
	Exp. 1	*Exp. 2*
Tissue culture plastic	3,302	7,097
Type I planar collagen	5,273	10,771
Methocel medium	2,770	8,546

* cpm in p36 immunoprecipitated from 0.1 mg of total protein. Counts normalized to total [^{35}S]methionine incorporation.

Discussion

The present study has established that p36 expression is developmentally regulated in the embryonic avian limb and that the expression of p36 is rapidly induced in cell culture. These observations pose several questions. First, does the pattern of p36 expression in vivo and in cell culture provide any clues about its function? Second, what are the mechanisms which regulate p36 expression? And finally, does the regulation of p36 expression in the embryo provide an explanation for the resistance of embryonic tissue to transformation by Rous sarcoma virus (7)?

The expression of p36 in avian limb tissue increases sevenfold between days 5 and 14 of development. This time period is one of considerable growth and morphogenetic activity. The major limb elements continue to differentiate and are laid down in a precise spatio-temporal pattern (17). The results of p36 immunolocalization in frozen sections demonstrate that p36 accumulates differentially in the various components of the developing avian limb. p36 expression was localized in the ectoderm at day 5 and later in the epidermis of day-9 and day-14 embryos. The protein was not present in the limb mesenchyme at day 5 with the exception of small foci of cells which probably form part of the limb vasculature. Of the three major mesenchymal tissue derivatives that differentiate within the limb, only two expressed p36 (connective tissue and cartilage) while the remaining major cell type (skeletal muscle) did not. The lack of p36 expression in the developing muscle was confirmed by dual-label immunofluorescence using myosin antibodies, and is in agreement with the findings of Greenberg et al. (15) and Gould et al. (14).

In the presence of Ca^{++}, p36 has been shown to bind to F-actin (10, 11, 13; Carter, V. C., J. Prior, and G. S. Martin, unpublished data) and anionic phospholipids (12; Carter, V. C., J. Guyden, and G. S. Martin, unpublished data). These observations have suggested a structural role for p36 in mediating membrane–cytoskeletal interactions. Thus, the appearance of p36 in skin, cartilage, connective tissue, and the endothelial linings of blood vessels in the developing avian limb may correlate with the structural and mechanical requirements of these differentiated tissues. In the later embryo and adult p36 is found in intestinal epithelial cells in the terminal web which also has a structural function (14). However, the function(s) of p36 may not be entirely mechanical. p36 exhibits sequence homology and antigenic cross-reactivity with other Ca^{++}- and lipid-binding proteins including *Torpedo* calelectrin and lipocortin (8, 9, 20). All members of this family contain a conserved amino acid sequence which is present in multiple copies and which may represent the Ca^{++}- and/or phospholipid-binding site (9). It has been postulated that members of this family may be involved in the Ca^{++}-dependent regulation of membrane traffic, the insertion of cytoskeletal elements in the membrane, or in the regulation of phospholipase A_2 activity (20, 27).

Our observations on the induction of p36 in culture confirm and extend the findings of Greenberg et al. (15) and Gould et al. (14). Biosynthetic analysis demonstrated that when cells from the day-5 limb were dissociated and cultured the induction of p36 occurred within 2 to 4 h post-seeding. The accumulation of p36 in cultured limb cells is due, at least in part, to an increased rate of synthesis. As detected by immunofluorescence microscopy, p36 appeared to be distributed uniformly among dissociated limb cells within 2 h in culture irrespective of embryo age. These data indicate that the induction of p36 expression is a rapid event following the disruption of limb structure and subsequent cell culture. When day-5 limb cells were plated on either plastic or collagen, or were suspended in methocel medium for 48 h, p36 was synthesized in all cells irrespective of culture conditions. However, the accumulation of p36 in cells held in suspension was not detectable by the immunoblot method. These results indicate that the accumulation of p36 is only observed when cells are allowed to adhere to an appropriate substratum. One can speculate that the accumulation of p36 may be dependent on the stabilization of the protein in a structural network associated with cytoskeletal and membrane elements, and that the formation of this cortical structure is dependent on cell

p36 is present in the covering layers of ectoderm at the surface of the limb but not in the underlying mesenchyme (*A*) with the exception of rare cells deep within the limb interior (*D*). At day 9 of development, p36 is expressed in differentiated epidermis and underlying blood vessels (*BV*) but not in the dermis (*B*). Towards the center of the limb p36 is now present in differentiated cartilage (*C*) and the surrounding perichondrium (*P*) (*E*). At day 14, p36 is present in the epidermis and now also in the dermis (*C*), and continues to be expressed with greater intensity in cartilage (*C*) and perichondrium (*P*) as shown in *F*. *G–I* show p36 expression in connective tissue associated with skeletal muscle at day 14. *G* shows p36 in fibrous connective fascia (*F*) separating three blocks of skeletal muscle (*M*). *H* and *I* show the lack of p36 expression in skeletal muscle by dual immunofluorescence. Two blocks of skeletal muscle (*M*) stained with anti-myosin/rhodamine (*H*) are separated by a band of connective tissue facia (*F*) stained with anti-p36/fluorescein (*I*). Bar, 10 μm.

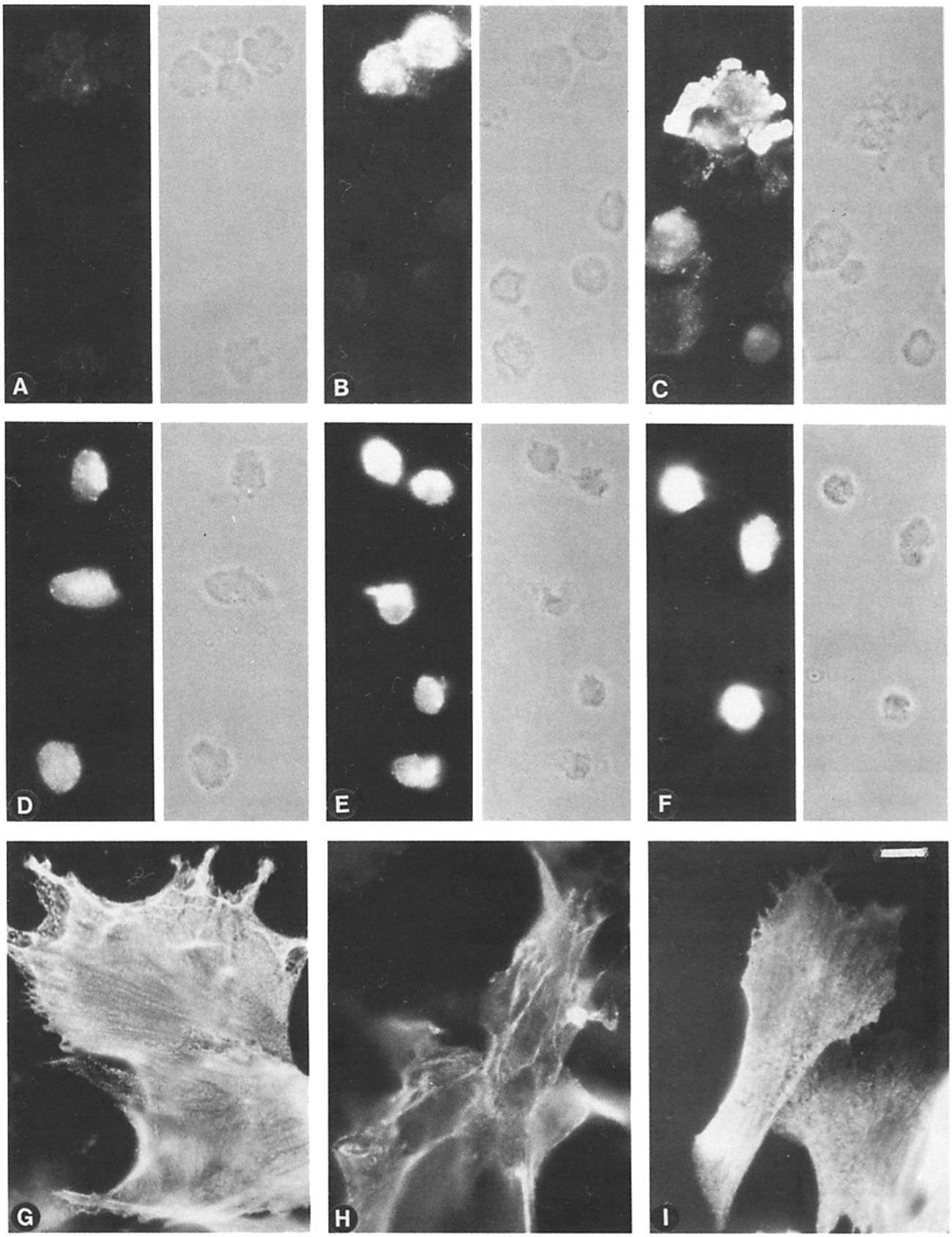

Figure 3. Immunofluorescent localization of p36 in dissociated limb bud cells. Cytocentrifuge preparations of limb cells dissociated from day-5 (*A*), day-9 (*B*), and day-14 (*C*) embryos were stained with anti-p36 serum and demonstrate differential expression of p36 and variation with embryo age. 2-h monolayer cultures of dissociated limb cells from day-5 (*D*), day-9 (*E*), and day-14 (*F*) embryos show the ubiquitous expression of p36. By 48-h in culture, limb cells from day 5 (*G*), day 9 (*H*), and day 14 (*I*) demonstrate the subcellular reticular distribution pattern of p36 characteristic of well-spread cells. Bar, 10 μm.

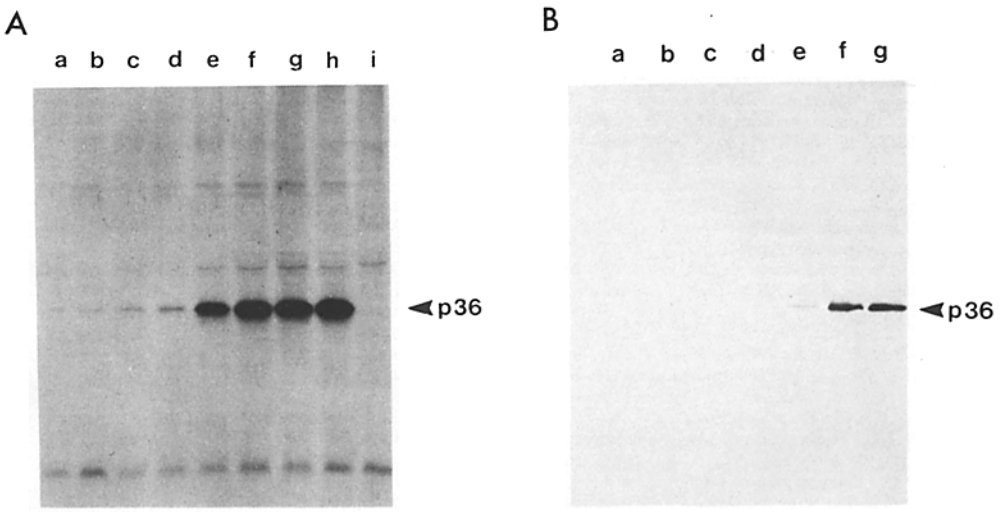

Figure 4. Time course of induction (*A*) and accumulation (*B*) of p36 in day-5 limb cells plated on tissue culture plastic. (*A*) Limb buds from day-5 embryos were dissociated, plated on tissue culture plastic and labeled with [^{35}S]methionine from 0 to 30 min (*a*), 30–60 min (*b*), 60–90 min (*c*), 1.5–2 h (*d*), 3.5–4 h (*e*), 5.5–6 h (*f*), 23.5–24 h (*g*), and 47.5–48 h (*h* and *i*). Cells were lysed and equivalent numbers of counts of total cell lysate were immunoprecipitated with equal amounts of preimmune (*i*) or anti-p36 serum (*a–h*). Immunoprecipitates were resolved upon SDS PAGE and the radioactive polypeptides detected by autoradiography. (*B*) Equivalent amounts of total cell protein (50 μg) were resolved upon SDS PAGE, transferred to nitrocellulose, and analyzed by the immunoblot method as described in Fig. 1. (*a*) 60-min, (*b*) 90-min, (*c*) 2-h, (*d*) 4-h, (*e*) 6-h, (*f*) 24-h, and (*g*) 48-h cell lysates.

spreading. Our findings are in some respects reminiscent of previous observations on the synthesis and accumulation of the membrane-associated cytoskeletal protein, spectrin, in the avian optic system. The segregation of the brain-specific and the erythrocyte-specific forms of spectrin is developmentally regulated and involves binding to specific membrane receptors (21). This binding appears to result in the stabilization and accumulation of the assembled spectrin complex on the membrane, while unassembled subunits are rapidly degraded in the cytosol (2). In the present study p36 accumulates in attached day-5 limb cells, but not in cells suspended in methocel. These results suggest that a p36 complex is formed and stabilized in attached cells, but p36 is degraded in the absence of complex formation in suspended cells.

Despite the fact that p36 was initially characterized as a major phosphotyrosine-containing protein in cells transformed by viruses encoding protein–tyrosine kinases, its role in neoplastic transformation is not clear. Injection of wild-type Rous sarcoma virus into the developing limb of chick embryos leads to virus integration, replication, and the expression of pp60$^{v\text{-}src}$ kinase activity in vitro, yet the limb remains sarcoma-free until the embryo's death at approximately day 13 (7). Analysis of viral expression and localization in day 10–injected embryo limbs (6 d post-injection) has shown that viral-specific proteins are expressed primarily in muscle tissue (Howlett, A. R., B. Cullen, and M. J. Bissell, manuscript in preparation). The absence of p36 in muscle tissue in vivo raises the possibility that the resistance of embryonic tissue to transformation by Rous sarcoma virus may result from developmental regulation of the cellular substrates for tyrosine phosphorylation. This possibility is currently under investigation.

We thank Dr. Richard Strohman (this institution) for providing monoclonal anti-myosin antibody and Drs. Ursula Abbot, Carol Erikson, and Kathryn Radke (University of California, Davis) for helpful discussion and suggestions.

This work was supported by National Institutes of Health grant CA17542 to G. S. Martin and by the Office of Health and Environmental Research, U. S. Department of Energy, Contract No. DE-AC03-76SF00098 to M. J. Bissell. V. C. Carter was supported by a Senior Fellowship from the American Cancer Society, California Division.

Received for publication 29 May 1986, and in revised form 14 July 1986.

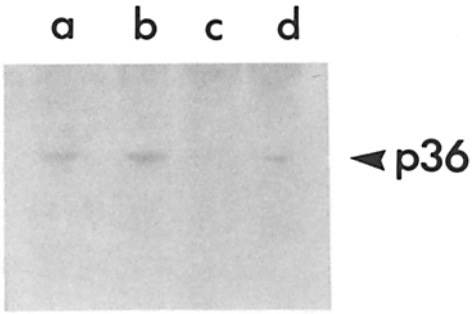

Figure 5. Induction of p36 in day-5 limb cells cultured on different substrata. Limb buds from day-5 embryos were dissociated and plated on tissue culture plastic (*a*), type I planar collagen (*b*), or suspended in methocel medium (*c*) for 48 h. A duplicate culture of 48-h cells suspended in methocel medium was further cultured on tissue culture plastic for an additional 48 h (*d*). Equivalent amounts of total cell protein were resolved upon SDS PAGE and analyzed by the immunoblot method as described in Fig. 1.

References

1. Amini, S., and A. Kaji. 1983. Association of pp36, a phosphorylated form of the presumed target protein for the src protein of Rous sarcoma virus, with the membrane of chicken cells transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 80:960–964.

2. Blikstad, I., W. J. Nelson, R. T. Moon, and E. Lazarides. 1984. Synthesis and assembly of spectrin during avian erythropoiesis: stochiometric assembly but unequal synthesis of α and β spectrin. *Cell.* 32:1081–1091.

3. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

4. Burnette, W. 1981. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated Protein A. *Anal. Biochem.* 112:195–203.

5. Cheng, Y.-S. E., and L. B. Chen. 1981. Detection of phosphotyrosine-containing 34,000-dalton protein in the framework of cells transformed with Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 78:2388–2392.

6. Courtneidge, S., R. Ralston, K. Alitalo, and J. M. Bishop. 1983. Subcel-

lular location of an abundant substrate (p36) for tyrosine-specific protein kinases. *Mol. Cell. Biol.* 3:340–350.

7. Dolberg, D. S., and M. J. Bissell. 1984. Inability of Rous sarcoma virus to cause sarcomas in the avian embryo. *Nature (Lond.).* 309:552–556.

8. Geisow, M., J. Childs, B. Dash, A. Harris, G. Panayotou, T. Südhof, and J. H. Walker. 1984. Cellular distribution of three mammalian Ca^{++}-binding proteins related to *Torpedo* calelectrin. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2969–2974.

9. Geisow, M., U. Fritsche, J. M. Hexham, B. Dash, and T. Johnson. 1986. A consensus amino-acid sequence repeat in *Torpedo* and mammalian Ca^{++}-dependent membrane-binding proteins. *Nature (Lond.).* 320:636–638.

10. Gerke, V., and K. Weber. 1984. Identity of p36 phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders: calcium-dependent binding to non-erythroid spectrin and F-actin. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:227–233.

11. Gerke, V., and K. Weber. 1985. Calcium-dependent conformational changes in the 36-kDa subunit of intestinal protein I related to the cellular 36-kDa target of Rous sarcoma virus tyrosine kinase. *J. Biol. Chem.* 260:1688–1695.

12. Glenney, J. R., Jr. 1985. Phosphorylation of p36 in vitro with $pp60^{src}$. Regulation by Ca^{++} and phospholipid. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 192:79–82.

13. Glenney, J. R., Jr., and P. Glenny. 1985. Comparison of Ca^{++}-regulated events in the intestinal brush border. *J. Cell Biol.* 100:754–763.

14. Gould, K. L., J. A. Cooper, and T. Hunter. 1984. 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. *J. Cell Biol.* 98:487–497.

15. Greenberg, M. E., R. Brackenbury, and G. M. Edelman. 1984. Changes in the distribution of the 34-kdalton tyrosine kinase substrate during differentiation and maturation of chicken tissues. *J. Cell Biol.* 98:473–486.

16. Greenberg, M. E., and G. M. Edelman. 1983. The 34kD-$pp60^{src}$ substrate is located at the plasma membrane in normal and RSV-transformed cells. *Cell.* 33:767–779.

17. Hinchliffe, J. R., and D. R. Johnson. 1980. The Development of the Vertebrate Limb. Clarendon Press, Oxford. 67–108.

18. Hunter, T., and J. A. Cooper. 1985. Protein-tyrosine kinases. *Annu. Rev. Biochem.* 54:897–930.

19. Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* 1:3–8.

20. Kretzinger, R. H., and C. E. Creutz. 1986. Consensus in exocytosis. *Nature (Lond.).* 320:573.

21. Lazarides, E., W. J. Nelson, and T. Kasamatsu. 1984. Segregation of two spectrin forms in the chicken optic system: a mechanism for establishing restricted membrane-cytoskeletal domains in neurons. *Cell.* 36:269–278.

22. Michalopoulos, G., and H. C. Pitot. 1975. Primary cultures of parenchymal liver cells on collagen membranes. *Exp. Cell Res.* 94:70–78.

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

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

25. Radke, K., and G. S. Martin. 1979. Transformation by Rous sarcoma virus: effects of src gene expression on the synthesis and phosphorylation of cellular polypeptides. *Proc. Natl. Acad. Sci. USA.* 76:5212–5216.

26. Sefton, B. M., and T. Hunter. 1984. Tyrosine protein kinases. *Adv. Cyclic Nucleotide Prot. Res.* 18:195–226.

27. Südhof, T. 1984. The calelectrins: new and ubiquitous Ca^{++}-regulated proteins with a possible role in cytotic processes. *Biochem. Soc. Trans.* 12:972–974.

28. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.