Expression of a Developmentally Regulated Antigen on the Surface of Skeletal and Cardiac Muscle Cells

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ABSTRACT H36 is a species-specific, cell-surface antigen on differentiating newborn rat skeletal myoblasts and myogenic lines. This membrane antigen has been defined by a monoclonal antibody raised by the fusion of SP 2/0-Ag14 myeloma cells with spleen cells from mice immunized with myotubes derived from the myogenic E63 line. H36 antigen, isolated by immunoaffinity chromatography, is comprised of two polypeptides with apparent molecular weights of 98,000 and 117,000. Fluorescence photometry and radioimmunoassays have been used to follow quantitative and topographic changes in the H36 determinant during myogenesis. H36 is present at a basal level on replicating myoblasts; it increases on prefusion myoblasts and persists on myotubes. At or near the time of prefusion, it becomes concentrated between adjacent aligned myoblasts and localized on membrane "blebs". H36 is present on both skeletal and cardiac cells but absent from a variety of cells that include fibroblasts, neuronal cells, and smooth muscle. There are $\sim 4 \times 10^5$ determinants per myoblast, and the K_a of the antibody is 3.8×10^8 liters/mol. The distributions of H36 on the top and attached surfaces of myoblasts and myotubes are distinct, which suggests localized specialization of these surfaces. H36 is an integral membrane component and upon cross-linking, it associates with the detergent-insoluble cytoskeletal framework. Inhibition of myogenesis by 5-bromodeoxyuridine or by calcium deprivation prevents the developmentally associated changes in the expression of H36. H36 is also absent or markedly reduced on the fu- and Ama102 developmentally defective mutant myoblast lines. We conclude that H36 is a muscle-specific, developmentally regulated cell-surface antigen that may have a role in myoblast differentiation and that can be used to determine the embryonic lineages of skeletal and cardiac muscle.

The fusion of mononucleate myoblasts to form multinucleate fibers is a striking morphologic event that is unique to the differentiation of skeletal muscle. The facility with which myoblasts can be isolated and grown has made this stage of muscle development readily amenable to experimental analysis, and the differentiation of both primary explants and cloned myogenic cell lines grown in vitro rather faithfully mimics in vivo development. As such, these cells have provided a convenient model system in which to examine developmental regulation, genetic expression, and biochemical differentiation, and, in particular, the synthesis and assembly of distinct isoforms of myofibrillar proteins and isozymes (for recent reviews, see references 10 and 37). Ironically, the characteristic fusion of myoblasts, which has provided the hallmark for defining myogenic cells, is one of the least understood events in this stage of development. Furthermore, attempts to study earlier stages of myogenesis have been stymied by the lack of distinct markers with which to identify and isolate those cells that develop into fusing myoblasts. One approach recently taken to resolve these gaps in our understanding of myogenesis has been to use monoclonal antibodies to define the events on the cell membrane that accompany myogenesis (17, 25, 26, 26a, 31, 48, 49), to identify specific molecular species with distinct functional capacities of the cell (12, 16, 35), and to explore cell surface markers of myogenic cells to identify the myogenic lineage.

We report here a cell surface antigen denoted H36 that is present on rat skeletal muscle and is defined by a monoclonal antibody raised in response to immunization with myotubes. We describe the quantitative and topographic stage-specific changes in H36 antigen that accompany normal development. Analyses of mutant myoblasts and other cell types, and mod-

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ulation of expression of H36 upon inhibition of myogenesis by 5-bromodeoxyuridine (BrdUrd)[1] and calcium deprivation, lead us to conclude that H36 is a muscle-specific cell-surface antigen that may be important to myoblast fusion and of use in defining the myogenic lineage.

MATERIALS AND METHODS

Cell Lines: E63, a myogenic clone of L8 rat skeletal myoblasts, and the developmentally defective fu- lines (fu-1, -3, -4, -5, and -15, mutants of L8) were isolated in this laboratory (22, 23). L6 myoblasts and the nonfusing amanitin-resistant mutant Ama102 (7, 45) were otained from Dr. M. Pearson (E. I. DuPont, Wilmington, DE). MM14 mouse myoblasts were obtained from Drs. T. Linkhart and S. Hauschka (University of Washington, Seattle, WA), Rat-1 fibroblasts from Dr. J. Wyke (Imperial Cancer Research Fund, London, England), and $FRECl_2$ rat fibroblasts and NRK rat kidney line from Dr. S. Rasheed (University of Southern California, Los Angeles, CA). With the exception of MM14, these cell lines were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% horse serum (HS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml kanamycin. MM14 myoblasts were grown on gelatin-coated dishes in DME supplemented with 15% HS, 3% chick embryo extract, 1% nonessential amino acids, and antibiotics as above. SP 2/0-Ag14 myeloma cells (42) were obtained from the Cell Distribution Center at the Salk Institute (La Jolla, CA) and were grown in DME with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. All cell lines were grown in a humidified atmosphere containing 10% CO_2. Growth media, sera, and other supplements were purchased from Gibco Laboratories (Grand Island, NY).

Primary Cell Cultures: Skeletal myoblasts from newborn Sprague Dawley rat hind limbs (Holtzman Co., Madison, WI) were prepared either by Dr. J. Thompson (University of Illinois, Urbana, IL), or in our lab as previously described (36). Fibroblast cultures were obtained from the same preparations by selective plating and subculturing. Kidney and liver cultures were prepared by trypsin dissociation of tissue from newborn rats; both cultures contained colonies of proliferating cuboidal, epithelial-like cells. Trypsin dissociation of preparturition rat uterus yielded cultures in which three morphologically distinct cell types were observed: epithelial-like colonies, fibroblast-like cells, and narrow bipolar cells which, on proliferation, formed parallel arrays. Additional smooth-muscle cultures were obtained from 3-d-old rats, from explants of aorta and from trypsin-treated stomach and duodenum. These primary cultures were maintained in DME with 5% HS, 5% FBS, and antibiotics.

Cardiac myoblasts were prepared from heart ventricles of 5-d-old rats by the method of Kasten (21) and were grown in DME supplemented with 10% FBS, 1% nonessential amino acids, and antibiotics. Human skeletal myoblasts were prepared by using the procedure of Blau and Webster (2) and were grown on collagen-coated dishes in Ham's F10 medium supplemented with 20% FBS, 0.5% chick embryo extract, and antibiotics. Clones were obtained by the method of limiting dilution using conditioned medium. All clones formed myotubes when the medium was replaced with DME that contained 10% HS. Chick embryo myoblasts were dissociated from thigh muscle of 12-d-old embryos by the vortex method of Bullaro and Brookman (3) and grown in medium 199 with 10% HS, 2% chick embryo extract, and antibiotics. Media and supplements were purchased from Gibco Laboratories.

Cultures of rat brain from preparturition fetuses were kindly provided by Dr. J. Weyhenmeyer (University of Illinois, Urbana, IL) (52).

Production of Monoclonal Antibody H36: 8-wk Balb/cV mice (19) were immunized at 7–8-d intervals with four intraperitoneal injections of E63 myotubes by using one 100-mm dish of cells per mouse for each injection. Cells were harvested with a rubber policeman and injected in 0.2 ml Dulbecco's phosphate-buffered saline, pH 7.5 (DPBS). 3 d after the fourth injection, spleens from two mice were excised, and a single-cell suspension was prepared as previously described (31). SP 2/0-Ag14 cells, grown for several generations in 20 μg/ml 8-azaguanine before use, were harvested in the log phase of growth. Fusion of spleen and myeloma cells was done by the method of Kennett et al. (27) using 35% polyethylene glycol-1,000 as previously described (31). Before cloning, hybridomas were maintained in HAT selective medium (DME with 18% FBS, 11% NCTC 135, 15 μg/ml hypoxanthine, 0.2 μg/ml aminopterin, 9 μg/ml thymidine, 150 μg/ml oxaloacetate, 50 μg/ml pyruvate, 8 μg/ml bovine insulin, nonessential amino acids, and antibiotics). Hybridoma culture fluids were screened by indirect immunofluorescence (IF) for antibody reactive with live E63 myotubes. Hybridoma H36 was cloned in 0.24% agarose (SeaKem, Marine Colloids, FCM Corp., Rockland, ME) that contained 50% conditioned medium. Antibody was collected from cells in log phase in HT.10 medium (HAT, without aminopterin and with only 10% FBS), supplemented with 0.02% sodium azide, and stored at −20°C. 8-Azaguanine and biochemicals used to supplement HAT and HT.10 were obtained from Sigma Chemical Co. (St. Louis, MO).

Ascites fluid was obtained by the injection of 10×10^6 hybridoma cells into the peritoneal cavity of Balb/cV mice primed with pristane (Aldrich Chemical Co., Milwaukee, WI). H36 antibody was purified from ascites fluid by ion exchange chromatography on DEAE-Sephacel (Bio-Rad Laboratories, Richmond, CA) and precipitation with ammonium sulfate (50% vol/vol final concentration). The antibody was >95% pure as determined by densitometric scanning of SDS 14% polyacrylamide gels (30). Transferrin was the only detectable contaminant in the preparation, and excess unlabeled transferrin added to iodinated H36 antibody had no effect on the binding of the immunoglobulin to E63 cells. H36 is an IgG1, kappa-chain immunoglobulin, as determined by radial immunodiffusion and IF using light-chain and subclass-specific antibodies (Litton Bionetics, Kensington, MD).

Indirect Immunofluorescence: Cells were grown on acetone-washed 18-mm No. 1 glass coverslips coated with collagen (for human and rat primary myoblasts) or gelatin in 60-mm dishes. E63 myoblasts were seeded at 2.75×10^5 cells/60-mm dish. After incubation, the cells were washed three times with serum-free medium, and 40 μl of hybridoma culture fluid buffered with 10 mM HEPES, pH 7.3, was placed on each coverslip. In some experiments, ascites fluid or purified antibody, diluted with DPBS, was used. SP 2/0-Ag14 culture medium or ascites fluid and purified normal mouse immunoglobulin were used as negative controls. After 30 min, cells were quickly rinsed, then washed four times, each for 10 min, with DPBS. The cells were then reacted with a 1:30 dilution of purified rabbit anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (FITC-RAMIg) in DPBS, then washed as above. RAMIg (Miles Scientific Div., Naperville, IL) was absorbed with 5 vol HS for 60 min at 5°C before the assay. For the initial characterization of H36 determinant on E63 cells and its quantitation by photometry, the antibody reactions were carried out at 5°C to minimize changes from the native distribution. In subsequent experiments, antibody reactions were conducted at room temperature.

After reaction with antibodies, the cells were fixed for 10 min with 95% ethanol, and the coverslips were mounted in glycerol/PBS (9:1) pH 8.5, and 10 mM *p*-phenylenediamine (Eastman Kodak Co., Rochester, NY) to retard photobleaching (20) and were sealed with Flo-Texx (Lerner Labs, New Haven, CT). Fluorescence was viewed by use of a Zeiss Universal microscope (Carl Zeiss, Inc., Thornwood, NY) with epiillumination optics, HBO 200-W mercury lamp, KP500 and KP490 excitation filters, and LP525 barrier filter. Photometry was done using a Jena photomultiplier and 110 EMI photometer. The response of this system to FITC is linear over the range of values obtained (26a). By using the Neofluar 40× objective, a field of 20–22 μm was measured. The photometer was zeroed on the negative control in each experiment. Kodak Ektachrome 400 film was used at an exposure index of 800.

Quantitation of Antibody Binding Sites: Antibody binding sites were quantitated by (*a*) increasing the concentration of radiolabeled antibody and (*b*) diluting a fixed concentration of radiolabeled antibody with increasing concentrations of unlabeled antibody to determine the amount of antibody required for 50% inhibition of binding of ^{125}I. In both procedures, purified immunoglobulin was radiolabeled with ^{125}I by a modification (31) of the chloramine T method (15), to a specific activity of 1–10 μCi/μg protein. Antibody incubations were done in triplicate, in 96-well microtiter plates, in 100 μl/well DME + 5 mg/ml BSA + 10 mM HEPES, pH 7.4, at 37°C for 4 h. After incubation, the cells were washed four times, each for 10 min, with DPBS, then extracted twice with 50 μl 0.3 N NaOH. Cell lysates were absorbed with cotton swabs and counted in a gamma counter.

Creatine Kinase Activity and Fusion: Creatine kinase specific activity was determined by the monitoring of the conversion of NADP to NADPH at 340 nm in the enzyme-coupled hexokinase-glucose-6-phosphate dehydrogenase reaction as previously described (22). Myoblast fusion was scored on cultures fixed in 70% methanol and stained with 4% giemsa. At least ten fields were scored per coverslip.

Anti-actin Antibody: Monoclonal anti-muscle actin antibody, B4, was kindly provided by Dr. J. Lessard (University of Cincinnati, Cincinnati, OH) (33).

[1] *Abbreviations used in this paper:* BrdUrd, 5-bromodeoxyuridine; DME, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; FITC–RAMIg, rabbit anti–mouse immunoglobulin conjugated with fluroescein isothiocyanate; HAT, a selective medium that contains DME with 18% FBS, 11% NCTC 135, 15 μg/ml hypoxanthine, 0.2 μg/ml aminopterin, 8 μg/ml thymidine, 150 μg/ml oxaloacetate, 50 μg/ml pyruvate, 8 μg/ml bovine insulin, nonessential amino acids, and antibiotics; HS, horse serum; HT.10, HAT medium without aminopterin and with only 10% FBS; IF, immunofluorescence.

Extraction of Cells with 1% Triton X-100: Cells were washed twice in DPBS and extracted for 4 min at 4°C with 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 300 mM sucrose, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM HEPES, pH 6.8 as previously reported (25).

Isolation of H36 Antigen: E63 cells grown to subconfluency on two 150-mm plates were labeled for 18 h with 8 ml methionine-free DME supplemented with 10% HS previously dialyzed against DPBS, 20 μM methionine, and 2.5 mCi [^{35}S]methionine (1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL). Cells were grown in two 150-mm plates and labeled with ^{125}I (2 mCi/mmol, Amersham Corp.) by the lactoperoxidase-glucose oxidase method (34) when an estimated 10% of nuclei were in myotubes. The cells were then washed three times with DPBS and incubated at 20°C for 45 min with 7 ml of DPBS that contained 0.02% NaN_3, 0.01% BSA, 0.01% gelatin, and 50 μg purified H36 antibody. The excess antibody was then removed and the H36 antigen–antibody complex was extracted with 250 mM KCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM HEPES pH 7.4, 200 mM sucrose, 10 mM CHAPS (Sigma Chemical Corp.) (41), 50 mM phenylmethylsulfonyl fluoride, 1% Trasylol (Sigma Chemical Corp.), 0.1% BSA, and 0.02% NaN_3 (buffer A) for 10 min at 4°C with two successive 7-ml aliquots. The extract was then centrifuged for 5 min at 4°C at 7,800 *g*. The supernatant was then sequentially passed through 1-ml columns of Affi-gel 10 (Bio-Rad Laboratories), Affi-gel 10 coupled with purified mouse immunoglobulin, and Affi-gel 10 coupled with RAMIg. The three columns were initially equilibrated with buffer A. After addition of the extract, the columns were eluted with buffer A, then with buffers B (0.5 M NaCl, 0.05 M HEPES pH 8.0, 1 mM EDTA, and 10 mM CHAPS) and C (0.15 M NaCl, 0.05 M HEPES pH 8.0, 10 mM CHAPS, and 0.1% SDS), as adapted from Schneider et al. (38). All washes were continued until no radioactivity could be detected in the effluents. Each column was then eluted with 0.05 M diethylamine, pH 11.3, and 10 mM CHAPS. These eluents were made pH 7.6 with 0.5 M NaH_2PO_4, and dialyzed against 10 mM HEPES pH 7.6 and re-run on fresh columns of the same Affi-gel 10 complex as that from which they were eluted. The eluents from the second set of columns were dialyzed against 5 mM Tris-HCl, pH 7.5, 0.01% SDS and concentrated 10-fold in a Savant speed vac concentrator (Savant Instruments, Inc., Hicksville, NY). Aliquots of the concentrates were electrophoresed in a 1.2-mm thick 7–20% gradient of polyacrylamide in SDS–Tris-glycine buffer (30) for 7 h at 20 mA. Molecular weight markers were included in additional aliquots of the samples. The gel with [^{35}S]methionine-labeled samples was treated with Enlightning (New England Nuclear, Boston, MA) for 15 min, dried, and exposed to Kodak XAR-5 film for 2 wk. The gel with ^{125}I-labeled samples was dried and exposed to Kodak XAR-5 in a cassette that contained an intensifying screen, for 1 wk.

RESULTS

We have used IF analysis and photometry on single cells as well as radioimmunoassays to study and quantitate the expression and topography of H36 antigen. Indirect IF of H36 was done by using cells of myogenic and nonmyogenic origins; both cell lines and primary cultures were screened. In this assay, live cells are reacted with the primary and fluorochrome-labeled antibodies at 5°C; under these conditions, the cell is impermeable to antibody, and only cell surface antigens are detected. H36 antibody bound to live cells at 5°C also reacts with RAMIg covalently bound to polyacrylamide beads (3–10 μm diameter), thus confirming the cell surface localization of the antigen.

Cell and Species Specificity of H36 Antigen

H36 is present on the L8E63 and L6 lines of rat myoblasts and on myoblasts from thigh muscle of newborn rats (Fig. 1; Table I). This antigenic determinant is not on skeletal myoblasts or myotubes of human or chick origin, and it is not on the MM14 line of myogenic mouse cells.

Expression of H36 appears to be highly restricted to myogenic cells. In mixed cultures of primary newborn rat myoblasts and fibroblasts, the fibroblasts do not stain (Fig. 1). Additional sources of rat fibroblasts, either cell lines (e.g., $FRECl_2$ and Rat-1 cells), or in explants of other tissues, or further passages of fibroblasts from myoblast explants, were uniformly negative for H36 (Table I). Of particular interest, five developmentally defective fu- mutants derived from the myogenic L8 line were also negative for H36 in IF analyses; the origins and properties of these mutants were previously reported (22, 23). In contrast, other antigens on E63 myoblasts that are identified by additional monoclonal antibodies are not uniformly absent on the fu- lines (26a). H36 is also markedly reduced on the Ama102 nonfusing mutant of rat L6 myoblasts and absent on early passage NRK cells, kidney, liver, and cardiac endothelial cells from newborn rat, and on adult rat uterine epithelium.

Cardiac muscle was the only other source of cells examined that was found uniformly to exhibit H36 antigen. Cultures of cardiac myocytes were prepared from the ventricles of 5-d-old rats by trypsinization and selective plating (21). These cultures were initially composed primarily of characteristic stellate-shaped myocardial cells and relatively few, but rapidly replicating, endothelial cells. The myocardial cells exhibited spontaneous contraction within 24 h of plating, and myofibrillar banding was detected on subsequent days. IF with H36 antibody was seen only on the myocardial cells: endothelial cells are clearly void of this antigen (Fig. 2). In contrast, primary explants of smooth muscle from newborn rat aorta, duodenum, and stomach did not react with H36 antibody. Secondary cultures were highly enriched for smooth muscle cells, as evidenced by their reactivity with purified muscle-specific B4 monoclonal anti-actin antibody (Fig. 2). Only a few cells exhibited faint IF with H36 antibody, and this was evident only with the high-resolution 1.4 numerical aperture 63× oil lens. Neurites grown from preparturition (day 20) rat brain were also generally negative for H36, even though some faint IF of H36 was detectable on glial cells with the 63 × lens. The uniform, intense reactivity characteristic of skeletal muscle was not evident on any of these cells. Thus, expression of H36 antigen apprears to be highly restricted to differentiating skeletal muscle and cardiac muscle cells.

Quantitation and Distribution of H36 Antigen

Both the amount and topographic distribution of H36 on the surface of differentiating myoblasts appear to be developmentally regulated. We have previously shown that the morphologic development of E63 myoblasts can be followed by phase contrast microscopy (26a). In this course of development, replicating, flat, rhomboid-shaped myoblasts elongate, align, and fuse to form multinucleate cells that rapidly develop into long fibers. Fusing myoblasts can be discerned in the phase image by the lack of distinct boundaries between adjacent cells. Prefusion myoblasts are elongate mononucleate cells distinguished by the integrity of their membrane and distinct immunofluorescent configuration on the cell surface. The relative amounts of H36 on single cells at distinct stages of differentiation were measured by photometric analysis of IF. The response of this photometer system is linear over the range of intensities measured (26a). Cells were seeded on sequential days and processed simultaneously to ensure that identical staining conditions were in effect. In these experiments, fusion began on day 5, and cells in culture from 2–8 d were scored. As seen in Fig. 3, relatively little H36 is present on replicating and aligned E63 myoblasts. There is a marked increase in H36 as the cells enter the period of rapid fusion and some decrease follows fusion, but abundant antigen does remain on fused myotubes. This quantitative profile of H36 on cells at different stages of development is one of several distinct patterns exhibited by different antigens on the cell surface during myogenesis (26, 26a).

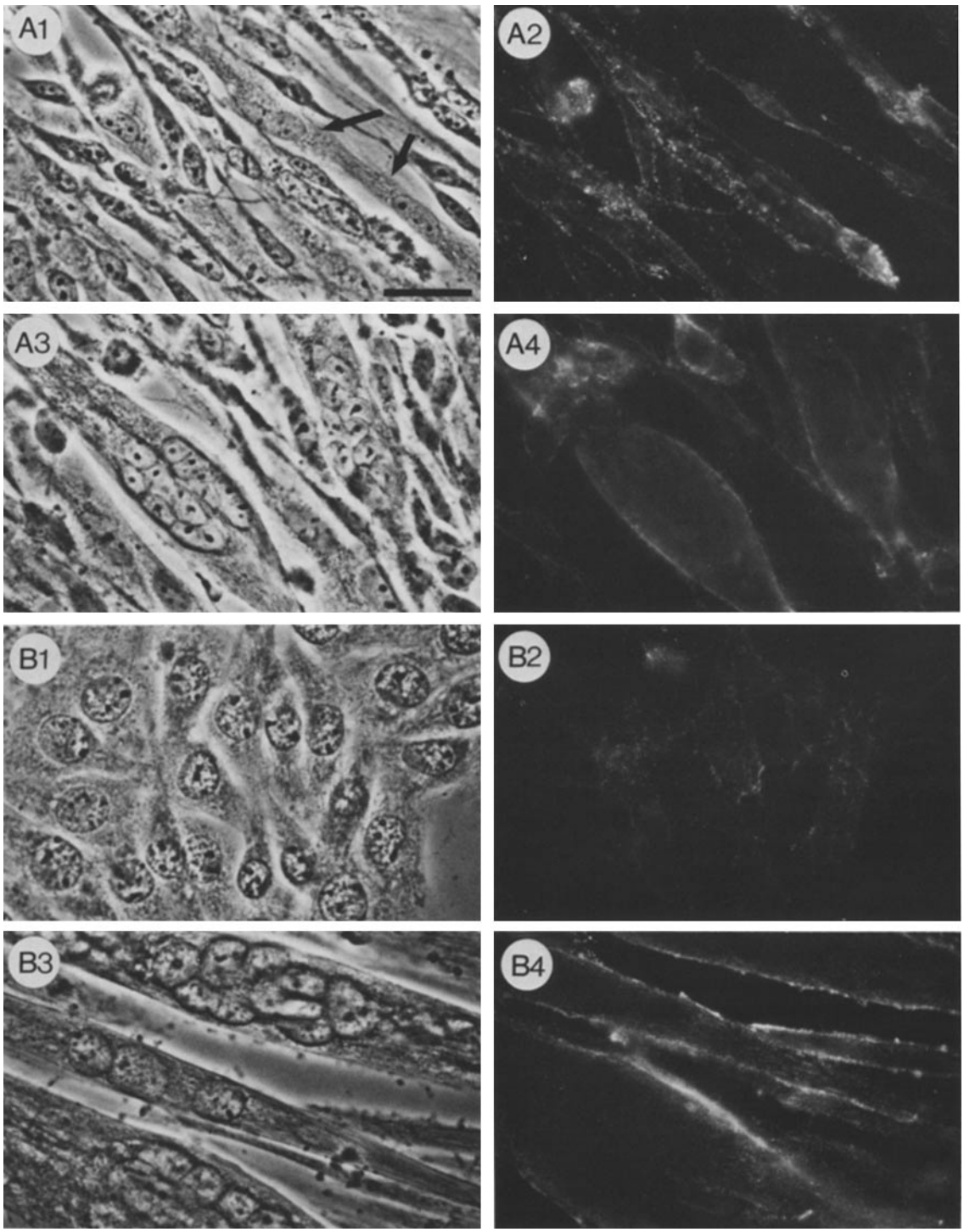

FIGURE 1 Expression of H36 antigen on myogenic rat cells. Live myoblasts were reacted at 20°C with monoclonal antibody H36 and then with FITC-RAMIg. (*A*) Primary myoblasts from newborn rat limb. H36 is abundant on myoblasts (*A1* and *A2*) and on myotubes (*A3* and *A4*) but is not detectable on fibroblasts in the same culture (*A1*, arrows). (*B*) E63 myoblasts. H36 is present on 2-d E63 cells (*B1* and *B2*), but it is more abundant on fusing cells and myotubes (*B3* and *B4*). Neofluar 40X objective. Bar, 30 μm.

H36 undergoes several interesting changes in its distribution as the cells develop. Although the amount of H36 is relatively constant on replicating and aligned myoblasts, it accumulates between confluent aligned cells (Fig. 4 *A*). Prefusion myoblasts have irregular cell margins that are frequently "blebbed", and this is evident in both phase and IF images (Fig. 4, *D–F*). IF on prefusion cells is distinct from the uniform distribution seen on myoblasts: it is very irregular on the upper surface and greatly increased in intensity (Fig. 4 *D*). On the attached surface of myoblasts, H36 is distributed in "stitch-like" aggregates in linear arrays parallel to the long axis of the cell (Fig. 4 *B*). This linear pattern is more pronounced on the attached surface of myotubes (Fig. 4 *G*), and some observations suggest that this pattern of organization may undergo transient modulation during fusion. H36 becomes relatively sparse on the upper surface of myotubes, but it is retained and abundant

TABLE I
Tissue and Species Specificity of H36 Antigen

	IF
Cell lines	
L8E63 rat skeletal muscle myoblasts	+
fu-1, -3, -4, -5, -15 (Developmentally defective, nonfusing mutants of L8)	−
L6 rat skeletal muscle myoblasts	+
Ama102 (nonfusing mutant of L6)	↓↓*
Rat-1 rat fibroblasts	−
$FRECl_2$ rat fibroblasts	−
NRK rat kidney cells	−
MM14 mouse myoblasts	−
Primary cultures	
Newborn rat:	
Skeletal muscle myoblasts	+
Skeletal muscle fibroblasts	−
Cardiac myoblasts	+
Cardiac endothelial cells	−
Liver cells	−
Kidney cells	−
Brain glial cells and neurites	−
Duodenum smooth muscle cells	−
Stomach smooth muscle cells	−
Aorta smooth muscle cells	−
Chick embryo skeletal muscle myoblasts	−
Human skeletal muscle myoblasts	−

* Greatly reduced in comparison with L6.

on the attached surface of these fibers (Fig. 4 *G*). It is not clear whether this asymmetry comes about by the selective loss of antigen from one surface or via redistribution from the upper to the attached surface of myotubes. Other antigens exhibit a variety of distinct surface polarizations (26a). Upon detachment of myotubes from coverslips either spontaneously or as a consequence of handling, H36 remains as "tracks" on the coverslip (Fig. 4, *I* and *J*), which suggests that it may function in the attachment of the cell to the substratum.

The relative amounts of H36 on E63 and primary skeletal myoblasts and myotubes, and on other cell types, were determined by IF photometry. Myoblasts explanted from newborn rats have approximately twice as much H36 as do E63 myoblasts and about threefold the amount on newborn cardiac cells (Table II). Whereas indirect IF assays can yield such relative quantitative data, the number of sites per cell cannot be derived since the molar ratio of fluorescein to antibody, the number of RAMIg molecules bound per H36 antibody, and the extinction coefficient of cell-bound FITC-RAMIg are not precisely known. To determine the number of H36 sites per cell, the monoclonal antibody was iodinated, and saturation and competition binding assays were done.

The number of H36 determinants on E63 myoblasts was calculated by reaction of confluent cultures of these cells with increasing amounts of ^{125}I-H36 antibody (Fig. 5). Specific binding of antibody was determined by correcting for nonspecific binding in the presence of excess unlabeled H36 antibody. Extrapolations from the linear portions of the curves indicate the saturation of the determinant on E63 cells was achieved at 600 ng antibody/ml. Assuming a 1:1 stoichiometry between antigen and antibody, we calculate there are 4.3×10^5 molecules of H36 determinant per E63 cell. Competition binding assays were done to confirm this determination. A constant amount of ^{125}I-H36 antibody and increasing amounts of unlabeled antibody were reacted with E63 cells (Fig. 5 *B*) and from the 50% inhibition values obtained, we calculate there are 3.7×10^5 H36 sites/cell. In an analogous experiment, the intrinsic association constant of H36 antibody, K_a, was calculated from the binding of ^{125}I-H36 antibody to confluent monolayers of E63 myoblasts to be 3.8×10^8 liters/mol. The antibody and determinant were assumed to bind in a 1:1 stoichiometry, and concentrations of free and bound antibody were determined at subsaturating amounts of antibody.

Chemical Characterization of H36 Antigen

In search of clues as to the chemical nature of H36 antigen, we treated cells with a variety of enzymes, or extracted with different reagents. None of the eight proteases (trypsin, chymotrypsin, pronase, papain, dispase, proteinase K, chlostripain, collagenase) used appeared to diminish the reaction of H36 with its homologous antibody. When these enzyme treatments were carried out to the extent that cells were removed from the coverslips, the suspended cells also specifically bound H36 antibody. Areas on the coverslip where cells were formerly attached were evident from the presence of H36 "tracks" detected by indirect IF. In most cases, these "tracks" were not in the normal linear "stitch-like" pattern, which indicates that the H36 antigen was integrally associated with the cell membrane and underwent a redistribution as the cell's morphology was altered in response to the proteases (Fig. 4). The anomalous finding that the proteases left much H36 on the cell surface, as well as leaving "tracks", suggests that the associations of H36 in the membrane on the top and bottom of the cell may be distinct.

Neither phospholipase C or D nor yeast lipase removed the H36 determinant, although its rearrangement on the membrane was noted. Neither the exoglycosidase (β-galactosidase, hyaluronidase, neuraminidase, β-glucuronidases, α-mannosidase, *N*-acetyl glucosaminidase) nor endoglycosidase F, either individually or in combinations, removed H36 from the cell surface; nor did alkaline or acid phosphatase, or arylsulfatase. Extraction of cells with 0.5 mM EDTA did not remove H36 antigen, although upon prolonged extraction the cells rounded and detached. Alterations in pH from 4.5 to 8.0 did not extract the determinant, nor did hypotonic (0.05 M) or hypertonic (2.0 M) saline. In contrast, cells extracted with 0.25–1.0% Triton X-100, 10 mM CHAPS, 1% octylglucoside, cold acetone, or chloroform/methanol (2:1) no longer reacted with H36 antibody, and no "tracks" were left as a consequence of these extractions. We conclude that the H36 determinant is an integral membrane component, and its association with the cell membrane is highly dependent on the integrity of membrane lipids.

We have used Western blots, immunoaffinity columns, and immunoprecipitation to try to identify H36 antigen in detergent extracts of cells. Although these procedures were successful in identifying known proteins added to cell extracts, none were effective in unambiguously resolving H36. The effective isolation and identification of H36 required the initial interaction of cells with the antibody, followed by solubilization in detergent and isolation of the antigen-antibody complex by immunoaffinity chromatography by use of a rabbit anti-mouse Affi-gel 10 column. As seen in Fig. 6, two polypeptides with apparent molecular weights of ~98,000 and 117,000 were isolated from cells labeled either with ^{125}I using lactoperoxidase, or by incorporation of [^{35}S]methionine.

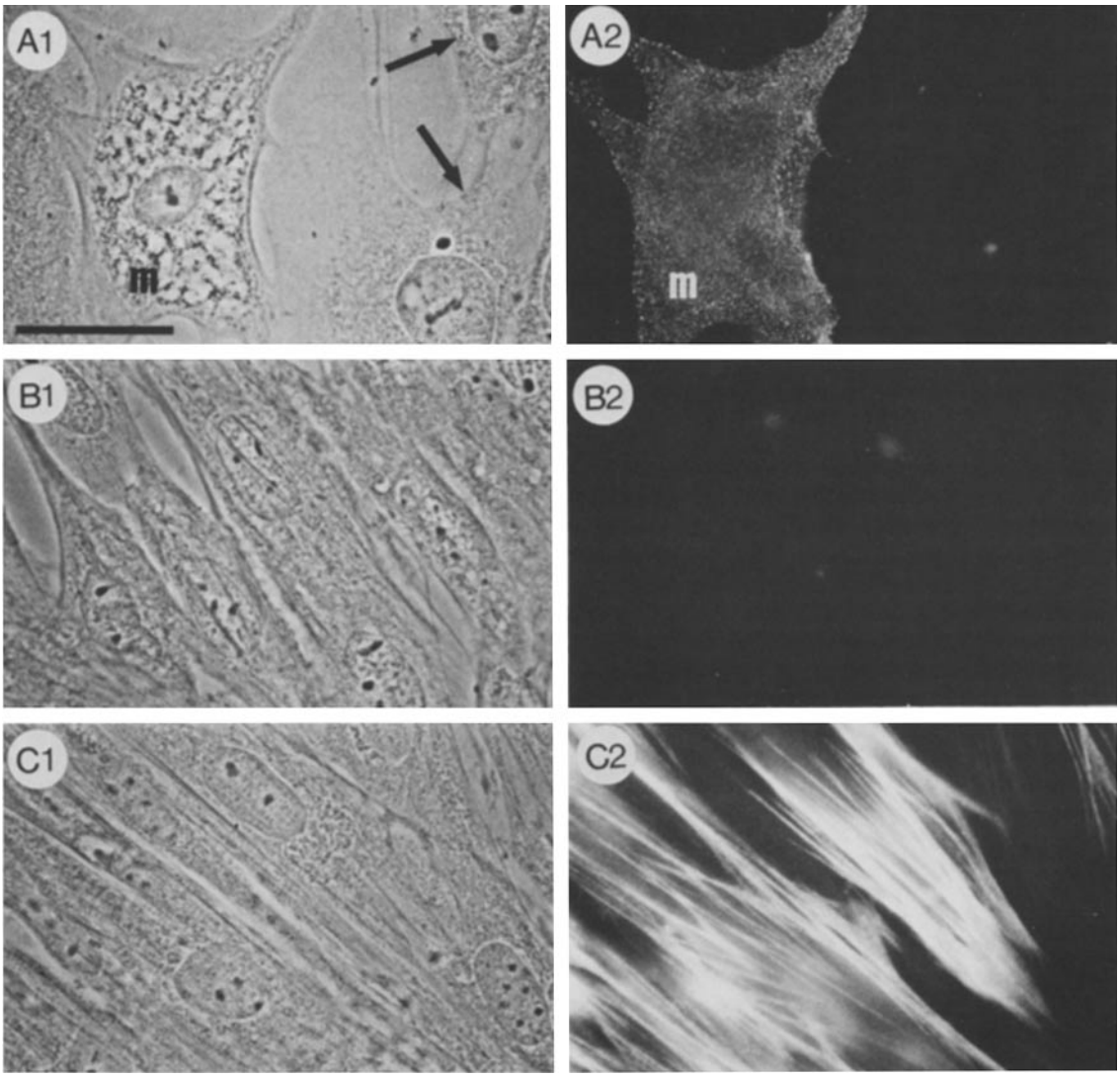

FIGURE 2 Specificity of H36 antibody. Live cells (*A* and *B*) were reacted at 20°C with monoclonal antibody H36 and then with FITC-RAMIg. (*A*) Cardiac cells from 5-d-old rats, cultured for 3 d. IF was observed on myocytes stained with H36 (*A1*; *A2*, *m*) but not on endothelial cells in the same preparation (*A1*, arrows). (*B*) Cells grown from 6-d-old rat duodenum, seen in phase contrast (*B1*), did not stain with H36 antibody (*B2*). (C) When fixed cells from the same culture of duodenum (*C1*) were reacted with B4 monoclonal antibody to smooth-muscle actin, most exhibited immunofluorescent cables (*C2*), confirming their smooth muscle cell origin. B4 antibody does not react with fibroblasts. Planapochromat 63X objective. Bar, 20 μm.

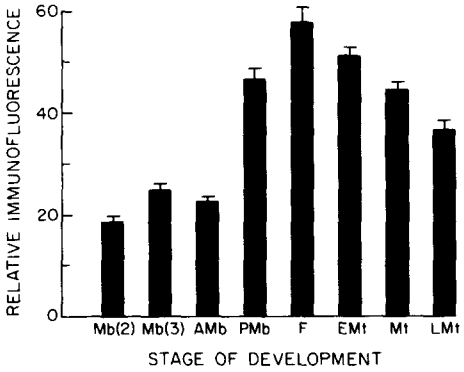

FIGURE 3 Quantitation of H36 antigen by IF photometry. E63 myoblasts were grown for 2–8 d on gelatin-coated coverslips and processed simultaneously for IF, by reaction at 5°C, with H36 antibody followed by FITC-RAMIg. Intensity was measured with a Jena microphotometer using a 40X Neofluar objective. Each point represents the mean of at least 30 measurements on different individual cells, ± standard error. A field within the cell boundary with a diameter of ~20–22 μm was measured. The photometer was zeroed against readings obtained from cells reacted with SP 2/0-Ag-14 culture fluid and RAMIg. 2 and 3 d myoblasts (*Mb[2]* and *Mb[3]*), aligned myoblasts (*AMb*), fusing cells (*F*), early myotubes (*EMt*), myotubes (*Mt*), and late myotubes (*LMt*) were identified by phase contrast microscopy. Prefusion myoblasts (*PMb*) were identified by characteristic fluorescence.

Association of H36 with the Cell Cytoskeleton

We have previously shown that the association of muscle membrane antigens with the Triton-insoluble cell framework can be modulated by cross-linking of cell surface antigens with antibodies (25). Although most H36 antigen is readily extracted with 1% Triton X-100, the determinant becomes refractory to extraction with detergent subsequent to reaction of the cells with primary and secondary antibodies (Table III).

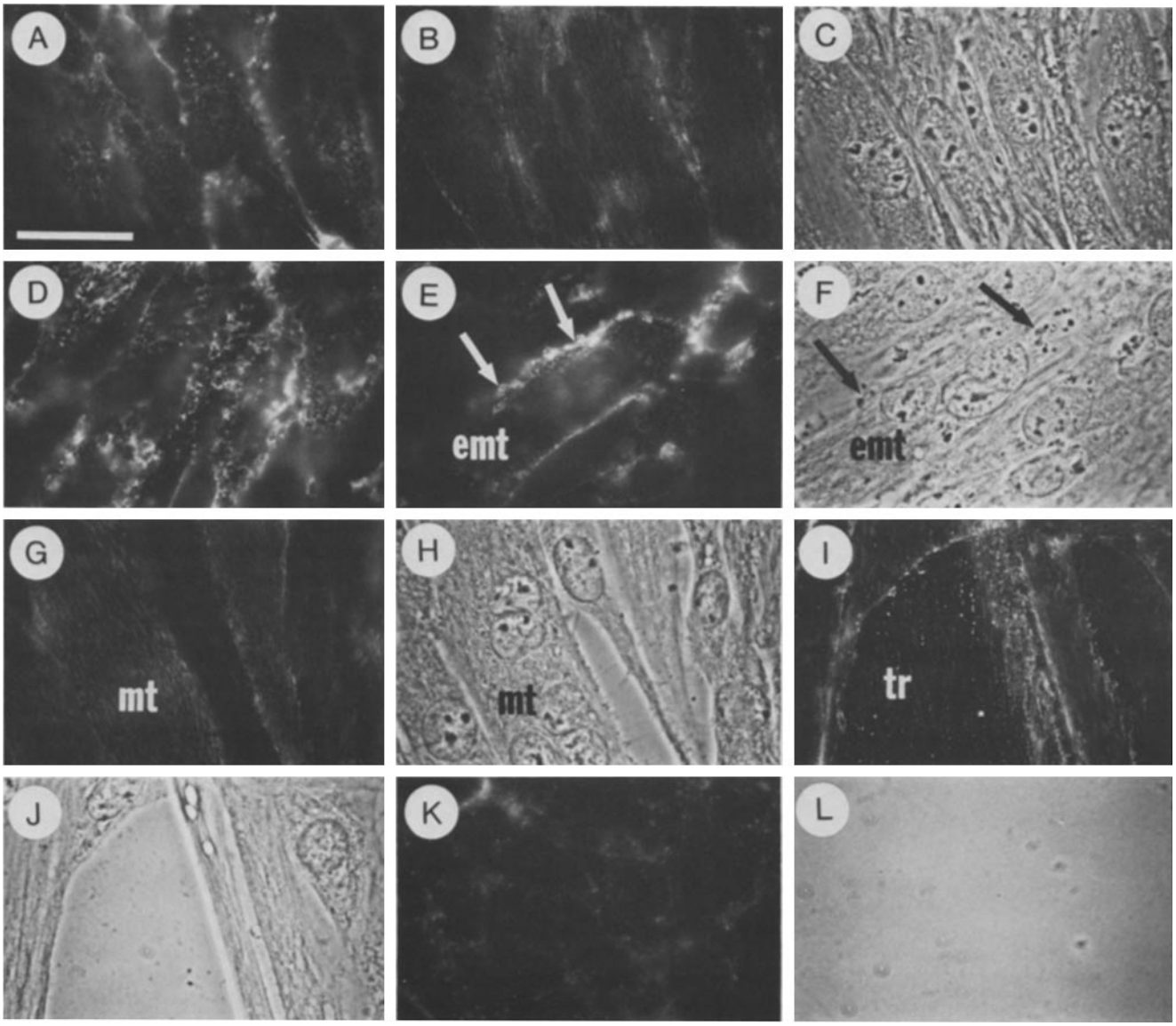

FIGURE 4 Distribution of H36 antigen on E63 myoblasts. Live E63 cells were reacted at 5°C with H36 antibody and then with FITC-RAMIg. The distributions of H36 on the top and attached surfaces of these cells are distinct. In contrast to the topography of H36 depicted in *A*, H36 on the attached surface of these cells appears in "stitch-like" parallel arrays of long aggregates (*B*). H36 accumulates between aligned confluent myoblasts (*A*). (*C*) Phase-contrast image of cells shown in *A* and *B*. Before fusion, H36 becomes more abundant on the upper surface of the cell and appears in a more irregular distribution (*D*), in part due to its presence on "blebs" extending from the cell surface (*D* and *E*), which persist on early myotubes (*emt*). Arrows indicate blebbed outline. Blebs are observed by phase contrast microscopy as phase-dense particles (*F*, arrows). Linear arrays of H36 persist on the attached surface of myotubes (*G*; *H*, *mt*) and tracks (*tr*) left by detached myotubes reflect this distribution (*I*). (*J*) Phase-contrast image of field shown in *I*. Trypsin treatment of myoblasts causes cell detachment but leaves residual tracks of H36 antigen (*K*). (*L*) Phase-contrast image of field shown in *K*. Planapochromat 63X objective. Bar, 20 μm.

H36 antibody alone is relatively ineffective in promoting cross-linking, presumably because of the distance between antigen molecules and/or the relative rigidity of this IgG1 antibody. The crosslinking achieved by subsequent reaction with RAMIg appears to be required to promote the association of H36 with the Triton-insoluble cytoplasmic framework. This supports the earlier stated premise that cell surface H36 and the monoclonal antibody bind with a 1:1 stoichiometry.

Expression of H36 is Effected by Inhibitors of Myogenesis

Incorporation of BrdUrd into DNA inhibits the morphologic and biochemical differentiation of myoblasts (6, 46). Similarly, calcium is required for myoblast fusion (29, 43). In rat cells, calcium is also required for the expression of other parameters of the differentiated phenotype (29, 44), although conflicting results have been reported (47). We therefore reasoned that if the increased expression of H36 is part of the overall unfolding of myogenic development, then inhibition of myogenesis with BrdUrd or calcium deprivation should alter expression of H36. Alternatively, if the H36 determinant is unrelated to myogenic development, neither BrdUrd nor lowered calcium would affect the expression of H36. To ensure uniformity in the IF assays, cells were plated on consecutive days and simultaneously processed for IF and determination of fusion and creatine kinase activity. As seen

TABLE II

Quantitation of H36 Antigen by Fluorescence Photometry

	Photometry units	
	N	$\bar{x} \pm SD$
Primary newborn myoblasts	30	42.93 ± 8.42
Primary newborn myotubes	30	69.83 ± 13.42
Primary newborn fibroblasts	30	0.40 ± 0.53
E63 myoblasts	30	21.23 ± 1.88
E63 myotubes	24	44.30 ± 14.80
Cardiac myocytes	30	13.70 ± 3.96
Cardiac endothelial cells	24	0.15 ± 0.80

Cells were prepared and assayed for H36 antigen by IF as indicated in Materials and Methods. N, number of cells scored. $\bar{x} \pm SD$, the mean ± standard deviation. The photometer was zeroed against readings obtained from each cell type reacted with SP 2/0-Ag14 culture fluid and FITC-RAMIg.

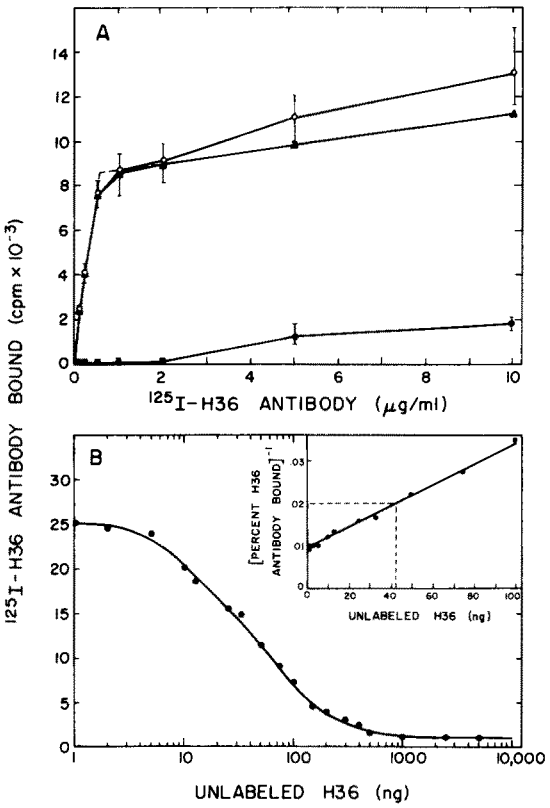

FIGURE 5 Quantitation of H36 antigen by radioimmunoassay. (*A*) E63 myoblasts were reacted with increasing concentrations of ^{125}I-H36 antibody. The specific binding of labeled antibody (▲——▲) was determined by subtracting the nonspecific binding that occurred in the presence of 100 μg/ml unlabeled H36 antibody (●——●) from the total ^{125}I-H36 bound in the absence of unlabeled antibody (○——○). The linear increase and plateau portions of the specific binding curve were extrapolated (dashed line); the intersection of these lines indicates that saturation was achieved at 600 ng H36 antibody/ml. (*B*) E63 myoblasts were reacted with increasing amounts of unlabeled H36 antibody and a fixed concentration of ^{125}I-H36 (50 ng antibody/100 μl per well). The number of H36 determinants per cell was calculated from the amount of unlabeled H36 antibody needed to inhibit binding of 50% of the ^{125}I-H36. This can be readily determined from a plot of the inverse of percent of maximum counts bound versus unlabeled H36 added (*inset*). A value of 0.02 corresponds to 50% inhibition, which occurred at 42 ng H36 antibody/well. By determining the amount of H36 antibody bound to the cells under saturating conditions and the number of cells per well, the number of H36 determinants per cell was calculated to be 4.3 × 10^5 in *A* and 3.7 × 10^5 in *B*.

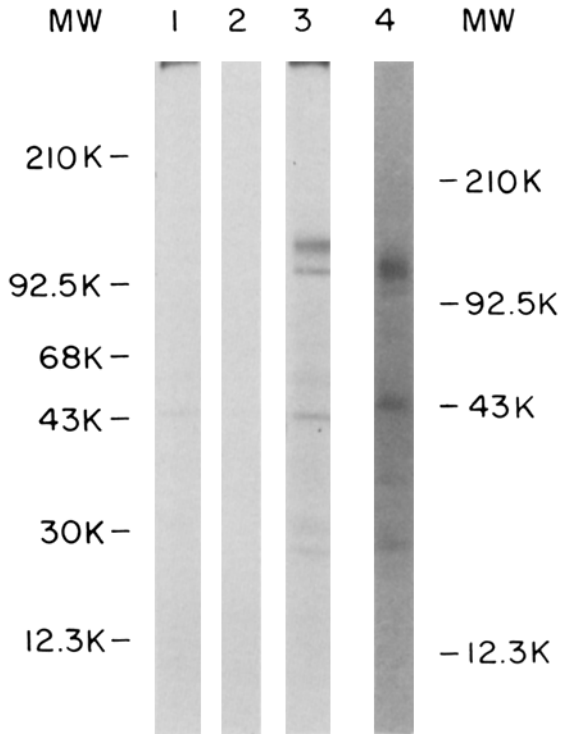

FIGURE 6 SDS polyacrylamide gel electrophoresis of H36 antigen. E63 cells were labeled with [^{35}S]methionine or ^{125}I, reacted with H36 antibody, and extracted in CHAPS. The extract was serially passed through Affi-gel 10, Affi-gel 10-mouse IgG, and Affi-gel 10-RAMIg columns. The bound materials were eluted and rebound to the respective columns of origin, as indicated in Materials and Methods. Samples were concentrated, electrophoresed in 7–20% polyacrylamide gradient gels in SDS–Tris-glycine buffer (30), and autoradiographed. ^{14}C-labeled myosin heavy chain (*210 K*), phosphorylase B (*92.5 K*), BSA (*68 K*), actin (*43 K*), carbonic anhydrase (*30 K*), and cytochrome C (*12.3 K*) were included in aliquots of samples run in parallel lanes (*K*, kilodaltons). [^{35}S]methionine-labeled proteins which bound to Affi-gel 10 and Affi-gel 10–IgG are visualized in lanes *1* and *2*, respectively. The ^{35}S (lane *3*) and ^{125}I (lane *4*)-labeled proteins that repeatedly bound to the RAMIg columns contained proteins with apparent molecular weights of 117,000 and 98,000, and these are specifically absent from the eluates of the control columns.

TABLE III

Effect of Antibody on Extraction of H36 with Triton X-100

Protocol	Percent IF	Radioimmunoassay		
		Soluble	Insoluble	Percent insoluble
		cpm		
Tx-1°-2°	9	—	8,786	7
1°-Tx-2°	10	122,694	14,131	10
1°-2°-Tx	79	16,717	105,725	86

IF photometry and radioimmunoassay were done in myoblasts grown on coverslips for 2 d. The percent IF values given are referenced to unextracted controls and background values obtained with SP 2/0 culture fluid have been subtracted. The mean cpm from three coverslips are indicated; 126,575 cpm bound to unextracted cells and was the value upon which the percent insoluble cpm was determined in the first protocol. Tx, extraction with Triton; 1°, H36 monoclonal antibody; 2°, RAMIg.

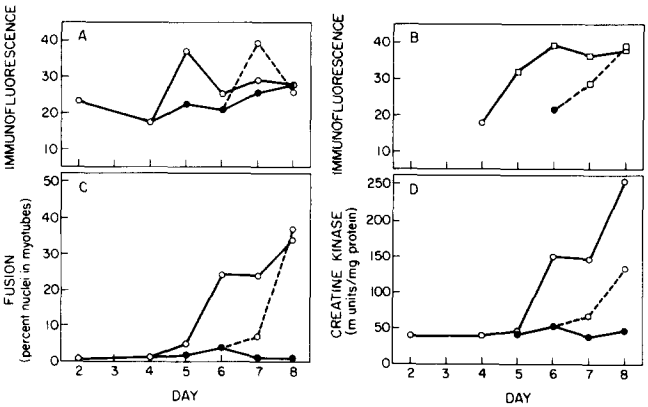

FIGURE 7 Inhibiton of myogenesis and expression of H36 by low calcium. 2.75 × 10^5 E63 cells/60-mm dish were seeded on gelatin-coated 18-mm coverslips. On day 4, the cells were washed twice with calcium-free PBS and then grown in DME containing 90 μM Ca^{+2}, or in the same medium supplemented with 1,800 μM Ca^{+2}. The same media were replaced on day 6, or, where indicated by the dashed line, cells previously grown in 90 μM Ca^{+2} were switched to medium containing 1,800 μM Ca^{+2}. To ensure uniformity of the assays, cells were plated on sequential days so that all analyses could be done on cells processed at the same time. (*A*) Fluorescence photometry of H36 on myoblasts grown in 1,800 μM Ca^{+2} (○——○), 90 μM Ca^{+2} (●——●), and in 90 μM, then 1,800 μM Ca^{+2} (○——○). The mean values of at least 30 determinations are given. (*B*) Fluorescence photometry of H36 on myotubes (□——□) that developed from myoblasts (○) grown in 1,800 μM Ca^{+2}, or on myotubes (□----□) that developed upon addition of 1,800 μM Ca^{+2} to myoblasts (●) previously grown in low calcium. (*C*) Fusion and (*D*) creatine kinase activity in cultures grown in 1,800 μM Ca^{+2} (○——○), 90 μM Ca^{+2} (●——●), and in 90 μM Ca^{+2}, then 1,800 μM Ca^{+2} (○---○).

in Figs. 7 and 8, both 3 × 10^{-5} M BrdUrd and the lowering of the calcium concentration to 90 μM inhibited fusion, creatine kinase activity, and expression of H36. The inhibition of myogenesis effected by lowering the calcium concentration was reversible. Within 24 h after addition of calcium to the medium, the transient increase in H36 on myoblasts and its concomitant increase on myotubes, as well as fusion and creatine kinase activity, paralleled that seen earlier in control cultures (Fig. 7). In contrast, removal of BrdUrd did not lead to increased expression of H36 nor the other characteristics of myogenic development within 48 h. The lack of reversal upon removal of BrdUrd is likely due to the fact that the analogue was incorporated over several generations, and when the drug was removed, no further cell replication took place. Expression of H36 on myoblasts grown in BrdUrd was consistently less than that on control cells. Interestingly, although fusion was drastically inhibited by growth in BrdUrd, the level of H36 on the few myotubes that did form under these conditions was equivalent to that on normal prefusion myoblasts (Fig. 8 *A*).

DISCUSSION

The use of IF photometry with monoclonal antibodies allows the detection of single molecular species on individual cells at distinct stages of development. A monoclonal antibody has been raised and used to define a species-specific, muscle-specific cell-surface antigen. IF photometry of H36 on rat skeletal myoblasts indicates that this antigen is present at a basal level on replicating and aligned myoblasts, and its expression is increased just before fusion. H36 is not extracted from the cell membrane by either high or low salt, nor by low pH, nor by treatment with a variety of enzymes: we therefore conclude that it is an integral membrane component. Approximately 4 × 10^5 determinants are present per cell in subconfluent cultures. The reactivity of H36 antibody with cells is not reduced by prior extensive absorption with HS, and therefore the reactivity of this antibody with the cell is not due to the association with any component of serum. Isolation of [^{35}S]methionine-labeled H36 antigen confirms that H36 is a protein synthesized by the cell.

H36 antigen is extracted, or denatured, by organic solvents and detergents; its isolation therefore necessitated reaction in situ with the monoclonal antibody, followed by solubilization and isolation of the antigen–antibody complex. Two polypeptides with apparent molecular weights of 117,000 and 98,000 were thereby isolated using a rabbit anti-mouse immunoglobulin affinity column and identified by SDS polyacrylamide gel electrophoresis. This is the first report of a developmentally regulated protein of this size on the surface of myogenic cells. A 105,000-mol-wt protein has been reported to undergo an increase in phosphorylation of L6 myoblasts (39, 40), and two polypeptides with apparent molecular weights of 110,000 and 98,000 are phosphorylated on the membrane of mutant Ama102 cells, but not on wild type L6 myoblasts (40).

Several lines of evidence suggest that expression of H36 antigen is part of the muscle-specific developmental phenotype. H36 is not expressed on any of the developmentally defective fu- mutants of the L8 line that we previously isolated (22, 23), and its expression on the Ama102 developmentally defective mutant of L6 myoblasts is also markedly reduced. In addition to not fusing, the fu- lines are, in contrast with

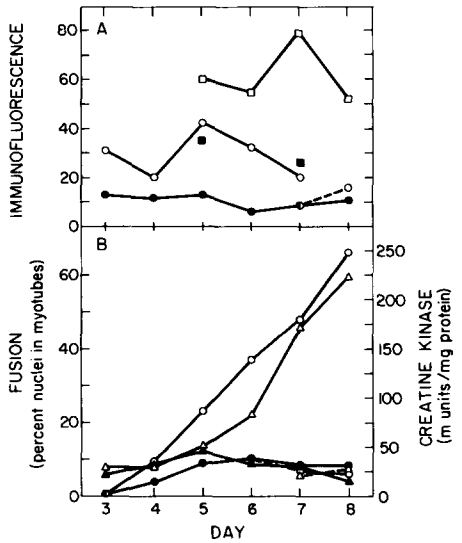

FIGURE 8 Inhibition of myogenesis and expression of H36 by BrdUrd. E63 cells were plated and processed as in Fig. 7. On days 2 and 4, the medium was supplemented with 3 × 10^{-5} M BrdUrd where indicated. Some cultures grown in BrdUrd were switched to normal medium on day 6. (*A*) Fluorescence photometry of H36 antigen on control myoblasts (○——○) and myotubes (□——□), and on myoblasts grown in BrdUrd (●——●). The few myotubes that developed in BrdUrd were scored on days 5 and 7 (■). (*B*) Fusion (○, ●) and creatine kinase activity (△, ▲) in control cultures (○——○, △——△); cells grown in BrdUrd (●——●, ▲——▲); cells grown in BrdUrd and then in control medium (○---○, △---△).

E63 cells, transformed, tumorigenic, and do not express other characteristics of the differentiated phenotype (23). Treatment of fu- cells with trypsin did not reveal "cryptic" H36. The Ama102 mutant has both wildtype and an α-amanitin resistant, structurally altered RNA polymerase II (7, 45). The mutant polymerase functions normally with regard to housekeeping transcription, however it appears that it cannot transcribe those genes that are developmentally regulated (8, 45). The biochemical and morphologic differentiation of myoblasts are also inhibited by growth in low concentrations of BrdUrd. Such treatment likewise inhibited the normal increased expression of H36 on myoblasts as well as fusion and creatine kinase activity. E63 cells express only the muscle-specific MM isoform of creatine kinase (29). Similarly, low calcium reversibly inhibits development of the myogenic phenotype and the developmentally regulated expression of H36. These experiments with developmentally defective mutants and inhibitors of myogenesis indicate that expression of H36 is a developmentally regulated characteristic of skeletal muscle differentiation.

H36 is almost completely extracted from the cell membrane by a variety of detergents. A small quantity of detergent-insoluble H36 has been determined by electron microscopy to be associated with microfilaments (Goff, J., and S. Kaufman, unpublished observations). Furthermore, binding of H36 antibody to the cell surface increases the amount of antigen that is insoluble in Triton, in a time and temperature-dependent fashion (Lowrey, A., and S. Kaufman, unpublished observations). The inextractability of the antigen in detergent and its association with cytoskeletal filaments is enhanced by reaction with secondary antibody. Previous studies have shown that cross-linking by bivalent antibody is required to promote such membrane–cytoskeletal associations (13, 25, 28). This inducible association of cell surface H36 with the cell cytoskeleton substantiates the conclusion that H36 is an integral membrane component. Cross-linking of H36 also promotes its aggregation and concomitant localized clearing from the cell surface. Such localized clearings may also come about through cell–cell interactions, and the potential consequences of this to cell fusion have been previously discussed (24).

The accumulation of H36 antigen between confluent, aligned myoblasts and its presence on membrane "blebs" suggests that the alteration in the spatial distribution of H36 is part of the membrane remodeling that accompanies development. Purified membrane blebs isolated from differentiating myoblasts have recently been reported to promote the fusion of a nonfusing mutant (9), and thus these structures appear to be significant in myoblast fusion.

The distributions of H36 on the top and attached surfaces of the cell are distinct. The relevance of this asymmetry, and the mechanisms by which distinct distributions and selective retention are established, are unknown. Although the cell culture dish is far removed from the in vivo environment, it is likely that in vivo associations of cells, with each other or with extracellular components, may promote or require localized specializations on the cell membrane. Thus, the asymmetry of H36 in the membrane of cells in culture may be indicative of the capacity of cells to establish subcellular specializations in vivo.

Monoclonal antibodies have recently been used to describe a variety of events on the surface of differentiating myoblasts (17, 25, 31, 49). Our combined applications of radioimmunoassays and microscopic fluorescence photometry of antigens on single cells have demonstrated that both topographic and quantitative alterations occur on the surface of differentiating myoblasts, that the membrane is in a dynamic state, and that a dramatic remodeling of the myoblast membrane accompanies development (25, 26a). Several components of the extracellular matrix associated with skeletal muscle have also been defined by monoclonal antibodies (1, 4, 5, 11, 50) as have antigens that are present on both myoblasts and satellite cells (18, 48). For the most part, the structure and function of these antibody-defined components have not been determined. Two antibodies that disrupt the attachment of myoblasts to their substratum have been reported (16, 35), and an antibody reactive with the sodium and potassium stimulated ATPase has been used to quantitate this membrane enzyme (12).

The expression of several molecular species common to muscle and many other cell types appears to be developmentally regulated during myogenesis, e.g., thy-1 (32) OKTK-9 (51), fibronectin (14, 31), and others (31, 51). The expression of yet other antigens is restricted to muscle and to one or more additional cell types (12, 35, 48, 51). Although some antigens on myoblasts have been reported to be less abundant or absent on one type of nonmuscle cell, the degree to which expression of these antigens is restricted to muscle has not been determined (17, 18, 51). In those cases when more extensive testing for cell specificity has been done, developmentally regulated components on myoblasts have also been found on other cell types (12, 31, 45). With the exception of the acetylcholine receptor, H36 is the only membrane antigen reported to date that has undergone extensive testing and that has been shown to be muscle-specific. Analysis of H36 on a variety of cell types demonstrated that expression of H36 is restricted to cardiac and skeletal muscle. Cells that have at least 100-fold less H36 on their surface fall below our limit of detection by fluorescence photometry and have been cited as negative for H36 (Table II). Radioimmunoassays indicate that there are $\sim 4 \times 10^5$ H36 determinants per myoblast in subconfluent cultures (Fig. 5), and analogous experiments (data not shown) indicate that there are <200 H36 determinants per developmentally defective fu-1 cell. H36 is present on fetal rat myoblasts and on myogenic lines derived from these, and on juvenile (4 wk) myotubes (Cameron, J., and S. Kaufman, unpublished observations). The restriction of H36 antigen to cardiac and skeletal muscle should prove useful in identifying and isolating cells of both the cardiac and skeletal myogenic lineages.

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