An Integral Glycoprotein Associated with the Membrane Attachment Sites of Actin Microfilaments

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ABSTRACT An integral membrane protein associated with sites of microfilament-membrane attachment has been identified by a newly developed IgG_1 monoclonal antibody. This antibody, MAb 30B6, was derived from hybridoma fusion experiments using intact mitotic cells of chick embryo fibroblasts as the immunization vehicle as well as the screening probe for cell surface antigens. In immunofluorescent experiments with fixed cells, MAb 30B6 surface labeling is uniquely correlated with microfilament distributions in the cleavage furrow region of dividing chick embryo fibroblasts and cardiac myocytes in culture. The MAb 30B6 antigen in addition is associated with microfilament-membrane attachment sites in interphase fibroblasts at the dorsal surface, the adhesion plaque region at the ventral surface, and at junction-like regions of cell-cell contact. It is also found co-localized with the membrane-dense plaques of smooth muscle. The MAb 30B6 antigen is expressed in a wide number of chicken cell types (particularly smooth muscle cells, platelets, and endothelial cells), but not in erythrocytes. Some of the molecular characteristics of the MAb 30B6 antigen have been determined from immunoblotting, immunoaffinity chromatography, immunoprecipitation, cell extraction, and charge shift electrophoresis experiments. It is an integral sialoglycoprotein with an apparent molecular mass of 130 kD (reduced form)/107 kD (nonreduced form) in SDS PAGE. Another prominent glycoprotein species with an apparent molecular mass of 175 kD (reduced form)/165 kD (nonreduced form) in SDS PAGE is co-isolated on MAb 30B6 affinity columns, but appears to be antigenically distinct since it is not recognized by MAb 30B6 in immunoblotting or immunoprecipitation experiments. By virtue of its surface distributions relative to actin microfilaments and its integral protein character, we propose that the MAb 30B6 antigen is an excellent candidate for the function of directly or indirectly anchoring microfilaments to the membrane.

Actin microfilaments form associations with the plasma membranes of a wide variety of cells, and these associations are important in the determination of cell shape, cell motility, and cell-cell adhesions. Microfilament-membrane associations are morphologically of at least two distinct classes (1, 2, 3, 13, 26, 31, 50), lateral (in which the microfilaments lie parallel to the cytoplasmic surface of the membrane) and end-on (in which they appear to terminate at the surface). For our present purposes, examples of lateral associations include (*a*) the cleavage furrow of dividing cells (21, 45, 47, 48), where a belt of microfilaments (the contractile ring) lies under the plasma membrane; and (*b*) the dorsal surfaces of well-spread interphase fibroblasts, where stress fibers (bundles of microfilaments) lying under the cell surface appear to be attached to the membrane (2, 3, 25, 29, 49). Examples of regions of end-on associations include (*a*) the plaque-like focal adhesion sites formed by fibroblasts with their substrata and with one another (23, 28); and (*b*) the dense plaques at sites of smooth muscle cell contacts (4, 24). These and other microfilament-membrane associations at the molecular level probably involve specific integral proteins within the membrane, whose cytoplasmic domains serve as anchoring sites for one or more peripheral proteins that form molecular bridges to the microfilament. As yet, however, only a few proteins have been identified as candidates for such linkage functions, and much remains to be done to obtain a complete understanding of the molecular composition and ultrastructure of these attachment sites.

One approach to the identification of integral membrane proteins that are involved in microfilament–membrane associations is to try to produce monoclonal antibodies (MAb's)[1] that are directed to such proteins; the selection of the appropriate MAb relying upon immunocytochemical methods to demonstrate a specific localization of the MAb to the cell surface regions where microfilaments are attached. In the present study, we report on the development of an IgG_1 MAb (30B6) directed to a cell surface antigen which by immunofluorescence measurements is localized at all of the four different sites of microfilament–membrane attachments mentioned above. The MAb 30B6 was one of a series of MAbs produced by immunization of mice with chick embryo fibroblasts (CEFs) arrested during cytokinesis (which largely avoided the production of antibodies to extracellular matrix antigens that are absent from the surfaces of the mitotic cells), and was ultimately selected by its specific and unique immunofluorescent labeling of the cleavage furrow of intact dividing cells.

The molecular identity of the cell surface antigen recognized by MAb 30B6 was determined by several approaches, including immunoblotting, immunoaffinity chromatography, and immunoprecipitation experiments. The relatively large concentration of the antigen in chicken gizzard smooth muscle, as compared for example to fibroblasts, expedited its characterization. The antigen appears to be a previously unknown sialoglycoprotein of 107 kD under nonreducing conditions, and 130 kD under reducing conditions, in SDS PAGE. In the reduced state, the epitope recognized by MAb 30B6 is inactivated. By its solubility properties, and by the criterion of charge-shift electrophoresis (32), the MAb 30B6 antigen has the properties of an integral membrane protein. The results of immunoaffinity/immunoblotting experiments suggest that this antigen may be associated with another, antigenically unrelated, higher molecular mass glycoprotein in the membrane. We have found the antigen, by its reaction with MAb 30B6, to be present in a wide range of chicken cells.

The unique cell surface localization and other properties of this antigen are consistent with the proposal that it is an integral membrane protein that is involved in specific attachments of microfilaments to the plasma membrane. This work has been presented in preliminary form (42. 44).

MATERIALS AND METHODS

Monoclonal Antibody Production

IMMUNIZATION: CEF mitotic cells, synchronized by thymidine shock, served as both the immunization vehicle and screening agent in enzyme-linked immunosorbent assays (ELISA) for the production of MAb's to CEF cell surface membrane antigens. A typical synchronization experiment yielded ~1×10^5 cells that were sufficient for one mouse injection. For each experiment 15–20 150 cm^2 T flasks containing secondary CEF cultures were incubated in Dulbecco's modified Eagle's (DME)/F_{12} medium with 10% fetal calf serum and 10 mM thymidine (54) for 17 h. The cultures were then rinsed and allowed to recover for 5 h in thymidine-free medium. Mitotic cells were accumulated in culture for 4–6 h in the presence of nocodazole at 60 ng/ml, and were then released by gentle tapping of the T-flask cultures. They were rinsed three times with phosphate-buffered saline (PBS) supplemented with 60 mM sucrose, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.5. Cells used for ELISAs were allowed to attach for 1 h at 4°C in 96-well microtiter plates previously coated overnight with poly-L-lysine (molecular weight = 32,600) (Miles-Yeda, Israel) at 1 mg/ml. Approximately 2×10^3 mitotic cells were bound to each well. They were subsequently fixed by the addition of 3% paraformaldehyde in PBS with 60 mM sucrose, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.5, for 20 min at room temperature. Mitotic cell microtiter plates were stored at 4°C for up to 1 wk.

Mice were injected by the following protocol: first injection, subcutaneous with complete adjuvant; second injection, subcutaneous with incomplete adjuvant 1 mo later; and the final injection, intraperitoneal, with incomplete adjuvant 10 wk after the first injection. 3 d after the final injection, the mice were sacrificed and the spleens were excised for fusion experiments.

ELISA: For primary screening and as well as for monitoring antibody production during cloning, ELISAs were performed with the mitotic cell microtiter plates. ELISA plates were first treated overnight in 20 mM Tris, 0.15 M NaCl, 0.1% azide, pH 7.4, with 3% BSA followed by another overnight incubation at 4°C with hybridoma culture supernatants diluted 1:1 with the same buffer. Bound antibodies were detected by using an affinity-purified rabbit anti-mouse IgG (10 μg/ml, 90 min at 37°C) followed by an affinity-purified goat anti-rabbit IgG conjugated to alkaline phosphatase (Miles-Yeda, Rehovot, Israel) (1:3,000 dilution, overnight at 4°C) and finally reacted with the fluorescent substrate 4-methylumbelliferil (Sigma Chemical Co., St. Louis, MO).

FUSION EXPERIMENT: Spleen cells from immunized mice were fused with NS-1 cells obtained from Dr. Gordon Sato, University of California, San Diego according to the methods of Fazekas de St. Groth and Scheidegger (19) and protocols outlined in the Cold Spring Harbor Laboratory manual for Monoclonal Hybridoma production. Supernatants from 614 cultures were analyzed by ELISA mitotic cell plates. Positive cultures were then further screened by immunofluorescence for surface staining criteria. Selected cultures were recloned 2–3 times by limiting dilution and were also monitored for continued antibody production by mitotic cell ELISAs.

ANTIBODY PURIFICATION AND CHARACTERIZATION: Subclass immunoglobulin classification of selected hybridomas was determined by an Ig typing kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Purification of IgG from mouse ascites fluid was performed by DEAE Affi-Gel Blue chromatography (10) (Bio-Rad Laboratories, Richmond, CA) of pooled 40% and 50% ammonium sulfate fractions. Purified IgG fractions were found to be protease free by assay with a protease casein substrate (Bio-Rad Laboratories).

Surface and Intracellular Expression of the MAb 30B6 Antigen as Determined by Immunofluorescence

For immunofluorescence studies, CEF cells were always prepared from 24–48-h-old primary cultures. Cardiac myocytes were derived from hearts of 8-day-old embryos. They were cultured for 24–36 h on glass coverslips in L-15 medium containing 10% fetal calf serum and 20 mM HEPES. Both interphase and mitotic cells were examined in the fibroblast and myocyte cultures. The cells were grown for 12–16 h on 18-mm^2 glass coverslips in DME/F_{12} medium with 10% fetal calf serum. Coverslip cultures were fixed for 30 min at room temperature with 3% paraformaldehyde in PBS, pH 7.4, rinsed three times with PBS-0.1 M glycine, and then incubated with either MAb 30B6 at 90 μg/ml, MAb 21D6 at 50 μg/ml, or MAb JG9 (27) at 200 μg/ml. They were subsequently rinsed three times for 10 min each with PBS-0.1 M glycine, pH 7.4, before incubation with an affinity-purified fluorescein-conjugate of F(ab')$_2$ fragments of goat anti-mouse IgG (CooperBiomedical, Inc., Malvern, PA) at 20 μg/ml in PBS-0.1 M glycine, pH 7.4. In some cases fixed cultures were permeabilized by brief treatment with 0.1% Triton X-100 before labeling with the MAb. In double-labeling experiments for the cell surface antigen and intracellular F-actin, coverslip cultures were fixed and labeled with MAb 30B6 as previously described for 30 min, then permeabilized with 0.1% Triton X-100 to permit intracellular access of a rhodamine-labeled phallacidin probe (Molecular Probes, Inc., Junction City, OR) at a 1:100 dilution in PBS-0.1 M glycine. Cells were then labeled with the fluorescein-conjugate of the F(ab')$_2$ fragment of goat anti-mouse IgG as described above. If secondary labeling of the bound MAb 30B6 was performed before the permeabilization step, labeling intensity in the focal adhesion plaque region was significantly lowered.

Semi-thin (0.5-μm) frozen sections of gizzard, cardiac, and skeletal muscle tissues were prepared according to the methods of Tokuyasu (52) from adult (6–8-wk-old) chickens. Tissues were cut into small blocks of ~1-mm dimension, and fixed for 30 min in 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The blocks were infused with 2.3 M sucrose, rapidly frozen in liquid N_2 and sectioned to ~0.5-μm thickness with a Sorvall MT-2 microtome equipped for cryosectioning (Sorvall Instruments, Norwalk, CT). Thawed sections mounted on glass slides were treated for 5 min with 0.1%

[1] *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]1-propane-sulfanate; CTAB, cetyltrimethylammonium bromide; DME, Dulbecco's modified Eagle's medium; DOC, deoxycholate; ELISAs, enzyme-linked immunosorbent assays; MAb's, monoclonal antibodies; PMSF, phenylmethylsulfonyl fluoride.

sodium borohydride in PBS-0.1 M glycine pH 7.4 for 5–7 min to reduce autofluorescence contributed by the glutaraldehyde treatment. After a brief rinse in PBS-0.1 M glycine, the sections were then pretreated with 2% gelatin in PBS-0.1 M glycine prior to reaction with MAb 30B6 at 90 μg/ml for 30 min. After rinsing in PBS-0.1 M glycine for 10 min, sections were secondarily labeled with affinity-purified fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG at 20 μg/ml for 30 min.

Immunofluorescent microscopy was performed with a Zeiss Photomicroscope III equipped with a vertical illuminator RS III for epifluorescence and a 63x 1.4 NA Planaphochromat objective for Nomarski differential interference contrast. Interference reflection microscopy was performed by modifying the Zeiss Photomicroscope III according to the methods of Bereiter-Hahn et al. (7).

Antigen Identification Studies

EXTRACTION OF THE ANTIGEN: In preliminary assays by nitrocellulose dot blots of CEF extracts obtained with a range of detergents including Triton X-100, Nonidet P-40, deoxycholate, 3-[(3-cholamidopropyl)-dimethylammonio]1-propane-sulfanate (CHAPS), it was found that the zwitterionic detergent CHAPS (Calbiochem-Behring Corp., La Jolla, CA) (33) most effectively extracted the antigen specific for MAb 30B6. It was therefore used in all subsequent experiments. Secondary CEF cultures were grown to ~70% confluence in 150-mm dishes in DME/F_{12} medium with 10% fetal calf serum. Prior to incubation with detergent the cultures were rinsed three times with cold PBS. Cultures were gently scraped for 5 min in the presence of 5 ml of buffered detergent containing 10 mM CHAPS in 40 mM Tris-HCl, 150 mM NaCl, 0.1% azide, pH 7.4, supplemented with 2 mM EDTA, 10^{-7} M pepstatin, 1 mM phenylmethysulfonyl fluoride (PMSF) and 0.1% aprotinin. Gizzard extracts were obtained by homogenization in a Waring blender of freshly obtained tissue in the same buffered detergent, using 15-s bursts at top speed. The gizzard homogenates were then passed once through a Dounce glass homogenizer with a B-type pestle and then stirred overnight at 4°C. CEF extracts and gizzard homogenates were finally centrifuged at 200,000 *g* for 45 min before the supernatants were used for immunoblotting or immunoaffinity chromatography.

IMMUNOBLOTTING: CEF extracts, gizzard homogenates, or immunoaffinity column eluates were prepared for SDS PAGE using a protocol that avoided the usual use of either reducing agents (β-mercaptoethanol or dithiothreitol) or of heating (50–100°C). A typical sample was made 0.1% in SDS at room temperature 10 min before electrophoresis. Blotting could be detected using final SDS concentrations from 0.1% to 2% SDS and with incubation at 50°C. After completion of electrophoresis, transfer from gels to nitrocellulose sheets (Millipore/Continental Water Systems, Bedford, MA) was effected in running buffer used for SDS PAGE (35) at 0.5 mA for 90 min. After transfer, the replicas were rinsed three times for 10 min with 20 mM Tris, 0.5 M NaCl, pH 7.4, and were blocked overnight at 4°C with 3% BSA in the same buffer. Before and after treatment with primary and secondary antibodies, transfers were rinsed four times with buffer. Reaction with the MAb 30B6 was for 2 h at room temperature at 10 μg/ml in 20 mM Tris, 0.5 M NaCl, 3% BSA. This was followed by either of two treatments: (*a*) an affinity-purified rabbit anti-mouse IgG for 90 min at 37°C followed by an affinity-purified horseradish peroxidase conjugate of goat anti-rabbit IgG (CooperBiomedical, Inc., Malvern, PA) at 10 μg/ml for 2 h at room temperature; or (*b*) an affinity-purified peroxidase-conjugate of goat anti-mouse IgG (CooperBiomedical, Inc.) at 10 μg/ml for 2 h at room temperature. In some experiments, blots of immunoaffinity eluates were reacted with the peroxidase-labeled lectins, ricin or concanavalin A (Sigma Chemical Co., St. Louis, MO). All blots were finally reacted with diaminobenzidine at 500 μg/ml in 50 mM Tris-HCl, pH 7.5, with 0.03% hydrogen peroxide.

IMMUNOAFFINITY CHROMATOGRAPHY: MAb 30B6 affinity columns were made by coupling 1 mg of the MAb that had been purified on DEAE Affi-Gel Blue to 1 ml packed Affi-Gel 10 (Bio-Rad Laboratories). Control columns were similarly prepared using either normal mouse IgG (Zymed Laboratories, San Francisco, CA) or MOPC-21 myeloma IgG (Bethesda Research Laboratories, Gaithersburg, MD). CEF extracts or gizzard homogenates prepared as described above were passed over the MAb 30B6 or control columns at room temperature at a flow rate of ~1 ml/min. The columns were then extensively washed with 50–100 ml of 10 mM CHAPS in 40 mM Tris-HCl/0.15 M NaCl containing 0.1% azide until the levels of the absorbance at 280 nm reached 0.002. The columns were then completely drained of buffer followed by gentle mixing of the column contents with 2 mM Tris-HCl, pH 8.0, 10 mM CHAPS (15). The columns were allowed to equilibrate for 5 min before fractions were collected. A peak fraction of eluted material was obtained with the first 2-ml eluate after which the absorbance at 280 nm rapidly decreased to background levels. The columns were then re-equilibrated with 50 ml of 40 mM Tris-HCl, 0.15 M NaCl, 0.1% azide before a new passage of either CEF or gizzard extracts. Elution with high salt or diethylamine gave identical results but did subsequently inactivate the binding capacity of the MAb 30B6 columns.

IMMUNOPRECIPITATION: In long-term labeling experiments, secondary CEF cultures at ~70% confluence were labeled with [^{35}S]methionine at 0.05 mCi/ml (New England Nuclear, Boston, MA) in Dulbecco's modified Eagle's medium (DME) containing 2% fetal calf serum and 1/10 the normal level of methionine (3 mg/liter medium) for 18 h. In short-term labeling experiments, CEF cultures were rinsed three times with DME followed by a half-hour incubation in the same medium to exhaust intracellular pools of methionine. The cultures were then labeled with 0.25 mCi/ml [^{35}S]methionine in DME containing 2% fetal calf serum for 15 min. Chase conditions were established by adding a 10-fold increase over normal levels of methionine in DME. Both kinds of metabolically labeled cultures were rinsed three times with cold PBS, and then extracted with 10 mM CHAPS in 40 mM Tris-HCl/0.15 M NaCl, 0.1% azide supplemented with 2 mM EDTA, 0.1% aprotinin, 1 mM PMSF, and 10^{-7} M pepstatin. Extracts were then centrifuged at 200,000 *g* for 45 min. The supernatants were concentrated by precipitation in 6 vol of ice cold acetone overnight at −20°C. The acetone precipitate was collected by centrifugation, air dried, and then dissolved in 1 ml of 10 mM CHAPS in 40 mM Tris-HCl, 0.15 M NaCl, 0.1% azide supplemented with 0.1% aprotinin and 1 mM PMSF. This extract was then preincubated in Eppendorf tubes with Sepharose 4B beads conjugated with goat anti-mouse IgG (CooperBiomedical, Inc.) with continuous end-over-end agitation for 30 min at room temperature. Either MAb 30B6 or normal mouse IgG was added to the absorbed extracts at a final concentration of 30–50 μg/ml. This mixture was then incubated for 2 h at room temperature with agitation. Afterwards, 50–100 μl of buffer-rinsed goat anti-mouse–Sepharose 4B beads were added followed by another 90 min of incubation at room temperature with agitation. The beads were then pelleted at 1,000 *g* for 3 min, rinsed three times with 40 mM Tris-HCl, 0.15 M NaCl containing 10 mM CHAPS, one time with 40 mM Tris-HCl, 0.5 M NaCl, and one time with 0.06 M Tris-HCl, pH 8.8. Each bead pellet was taken up in sample buffer containing dithiothreitol and 2% SDS, and then heated at 100°C for 5 min. This was followed by alkylation with iodoacetamide (36). Neuraminidase digestion of immunoprecipitates was performed as follows. The reacted beads were washed as above, except that in the final wash, the Tris buffer was replaced with 0.05 M Na acetate, pH 5.5, 154 mM NaCl, 4 mM $CaCl_2$. The beads were then incubated with 100 μl of neuraminidase, *Vibrio cholerae* (Calbiochem-Behring Corp.) for 30 min at 37°C. The beads were rinsed one time with 0.06 M Tris-HCl, pH 8.8, prior to the addition of electrophoresis sample buffer.

SDS PAGE

Solubilized samples were electrophoresed in 7.5% polyacrylamide slab gels by the method of Laemmli (35). Gels were stained with Coomassie Brilliant Blue or silver nitrate (38). Gels of radiolabeled proteins were processed for fluorography with EN^{3}HANCE (New England Nuclear, Boston, MA). Gels were dried down and exposed to Kodak BB-5 X-ray film (Eastman Kodak Co., Rochester, NY).

Charge Shift Electrophoresis

Gizzard homogenates in 10 mM CHAPS, 40 mM Tris-HCl, 0.15 M NaCl, 0.1% azide, pH 7.4, were dialyzed against 10 mM CHAPS in 0.01 M glycine, 0.05 NaOH, 0.1 M NaCl, 0.1% azide, pH 9.0 (32). Portions of the dialyzed extracts were subsequently made 0.4% in cetyltrimethylammonium bromide (CTAB) or 1.0% in deoxycholate (DOC). Three 1% agarose gels containing the appropriate detergent (either 10 mM CHAPS alone, or 10 mM CHAPS containing either 0.05% CTAB or 0.25% DOC) were run simultaneously for each detergent extract preparation at 30 mA for 5 h. The gels were then blotted onto nitrocellulose according to the methods previously described for SDS gels except that electrophoretic transfer was made for 1 h. Replicas were reacted with MAb 30B6 as described above to detect the position of antigen migration. From the same replicas, parallel blots were carried out to determine the migration behavior of the peripheral protein, vinculin, using affinity-purified rabbit anti-vinculin antibodies (23), and α-actinin using affinity-purified rabbit anti-α-actinin antibodies (13).

Two-Dimensional Iodinated Tryptic Peptide Mapping

Peptide mapping of selected bands from Coomassie Blue–stained gels followed the procedures outlined by Elder et al. (18) as modified by Zweig and Singer (55). Briefly, excised gel slices were washed in 10% methanol, lyophilized, iodinated with 0.5 mCi I^{125} using chloramine T, and extensively washed before

trypsinization. Peptide mapping was carried out using 1 cpm per unit molecular weight of the protein on 10 × 10-cm cellulose-coated glass thin layer chromatography plates (EM Laboratories, Gibbstown, NJ). Plates were exposed to Kodak BB-5 X-ray film using a DuPont Lightning Plus intensifying screen at −70°C.

RESULTS

Production and Selection of the MAb 30B6 Hybridoma

Intact mitotic CEF cells arrested in metaphase by thymidine shock and nocodazole treatment were accumulated in culture and used as the immunogen in BALB/c mice, as well as in whole cell ELISAs for the first screenings of hybridoma supernatants. The use of mitotic cells for ELISAs, under the conditions described in Materials and Methods, was found to give low background labeling, in contrast to the case where interphase cells were used. Of 614 hybridoma culture supernatants screened by the ELISAs, 110 were positive, with 30 strongly positive. Positive supernatants were further tested by indirect immunofluorescence labeling of CEF coverslip cultures synchronized as above but released from the nocodazole block 15 min before fixation to allow the arrested cells to undergo cytokinesis. Of the 110 positive hybridomas, only one (30B6) gave CEF cell surface labeling concentrated at three different areas: (*a*) the cleavage furrow of cells undergoing cytokinesis; (*b*) the regions of the dorsal surface lying directly over actin stress fibers; and (*c*) the focal adhesion sites on the ventral surface. These labeling patterns are documented as follows.

Immunofluorescent Labeling of the Surfaces of Dividing CEF and Dividing Cardiac Myocytes by MAb 30B6

Although the distribution of immunofluorescent labeling with MAb 30B6 on the surfaces of dividing CEF cells attached to a substratum was variable (see below), a concentration in the cleavage furrow was seen many times, particularly with cells in mid-to-late cleavage stages that exhibited a smooth bilobar shape on the substratum (Fig. 1, *A–F*). In addition, some polarization of MAb 30B6 labeling at sites of attachment to the substratum was also observed in these cells (Fig. 1 *B*, double arrows). This concentration in the cleavage furrow was a unique property of MAb 30B6; with all the other positive hybridoma supernatants on similar cells, the labeling distribution was essentially uniform over the entire surface of the dividing cell (as in the case of MAb 21D6, shown in Fig. 1, *G* and *H*).

The cell shape of mitotic CEF was frequently observed to be distorted by attachments to the substratum. In these cells, surface labeling by MAb 30B6 and the labeling of actin microfilaments as detected by rhodamine-phallacidin were often co-distributed, concentrated at sites on the cell periphery (Fig. 2, *A*, *E*, and *I*). Similar peripheral labeling was observed with cells at different stages throughout cytokinesis (Fig. 2, *B–L*). When double-labeling for MAb 30B6 on the cell surface and for actin in the cell interior was carried out, it was evident that despite the varied surface distributions of MAb 30B6 from cell to cell (Fig. 2, *F–H*), in most cases, a close correspondence with that of the actin underlying the cell surface in the cleavage furrow region was observed (Fig. 2, *J–L*).

In another type of cultured chick embryo cell that was examined, the cardiac myocyte, mitotic cells typically had smooth, unperturbed shapes (Fig. 3, *A–C*). In these cells, in contrast to the mitotic CEFs, a strikingly sharp concentration of MAb 30B6 labeling (Fig. 3, *D–F*) in the developing cleavage furrow was consistently observed without substantial labeling elsewhere on the cell surface. Actin labeling was also more concentrated in the cleavage furrow (Fig. 3, *G* and *H*). Surface labeling of mitotic cardiac myocytes with another monoclonal antibody JG9 (27), which is directed to a surface antigen complex involved in fibronectin-mediated cell adhesion (14),

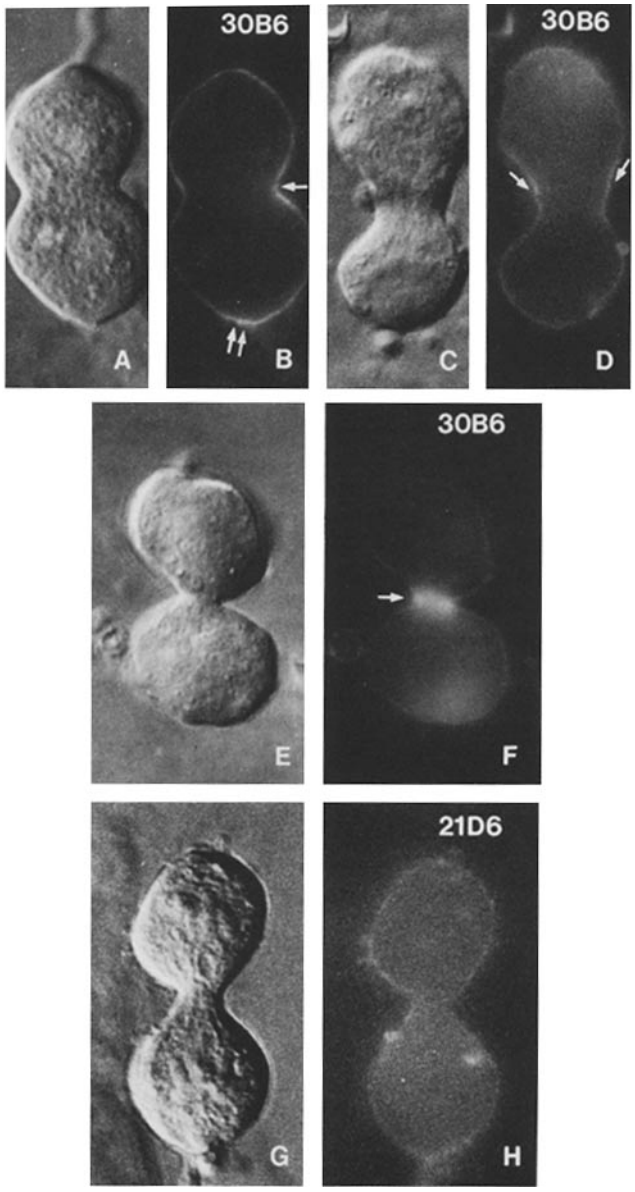


FIGURE 1 Cell surface staining of fixed CEFs during mid-to-late stages of cytokinesis using MAb 30B6(*B*, *D*, *F*) and another antibody directed to mitotic cells obtained from the same fusion experiment, MAb 21D6(*H*), as a control. Both MAb's were detected by indirect immunofluorescence, using affinity-purified fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG. Enriched staining in the cleavage furrow region was uniquely observed with MAb 30B6(*B*, *D*, *F*), whereas MAb 21D6(*H*) consistently led to uniform staining in this region. Note also MAb 30B6 labeling in other surface regions at sites of cell substratum contact (*B*, double arrow). The respective Nomarski images are shown in *A*, *C*, *E*, and *G*. × 1,254.

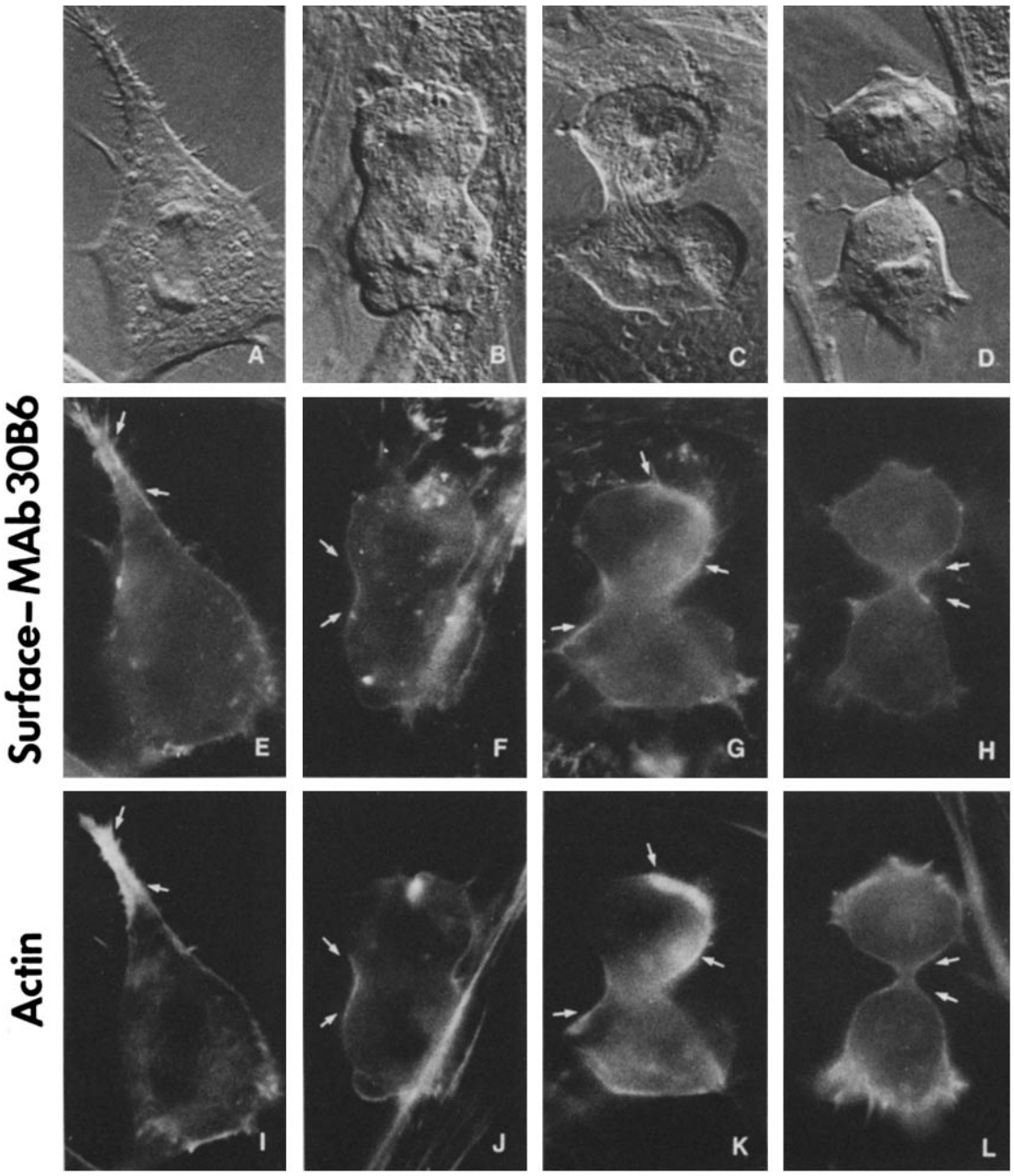


FIGURE 2 Co-distributions of surface-bound MAb 30B6 with intracellular actin microfilaments in CEFs prior to and during cytokinesis, demonstrated by double-label fluorescence. Fixed CEF cultures were initially labeled with MAb 30B6 followed by affinity-purified fluorescein-conjugated $F(Ab')_2$ fragments of goat anti-mouse IgG, before permeabilization with 0.1% Triton X-100 for 5 min to allow intracellular access of a rhodamine-phallacidin probe. The top row (*A–D*) represents the Nomarski image of the cells; the second row (*E–H*) distributions of the MAb 30B6 antigen; and the third row (*I–L*) distributions of actin. A polarized co-distribution of the MAb 30B6 antigen and actin (arrows) is shown in an early anaphase mitotic cell (*A*, *E*, and *I*). During early-to-late stages of cytokinesis, similar co-distributions (corresponding arrows) could be detected in the cleavage region (*B–L*), as well as in remote cell surface regions (*C*, *G*, and *K*). × 978.

showed only slight apparent concentration in the cleavage furrow region (Fig. 4, *A–D*), but not as sharply concentrated there as the labeling with MAb 30B6. Similar results were observed with MAb 21D6 (data not shown).

It should be emphasized that all surface immunolabeling and detection of actin distributions were performed after fixation of the cultured fibroblasts and cardiac myocytes. Hence, associations of MAb 30B6 in the cleavage furrow region are not the result of an antibody-induced redistribution of its cell surface antigen (8, 9).

The primary significance of these observations with mitotic cells is considered in the Discussion, where we conclude that there is a close correspondence of the distributions of MAb 30B6 and the contractile ring of actin microfilaments in the cleavage furrow.

Immunofluorescent Labeling of the Surfaces of Interphase CEF by MAb 30B6

Immunofluorescent labeling with MAb 30B6 showed specific and characteristic distributions on both the dorsal and

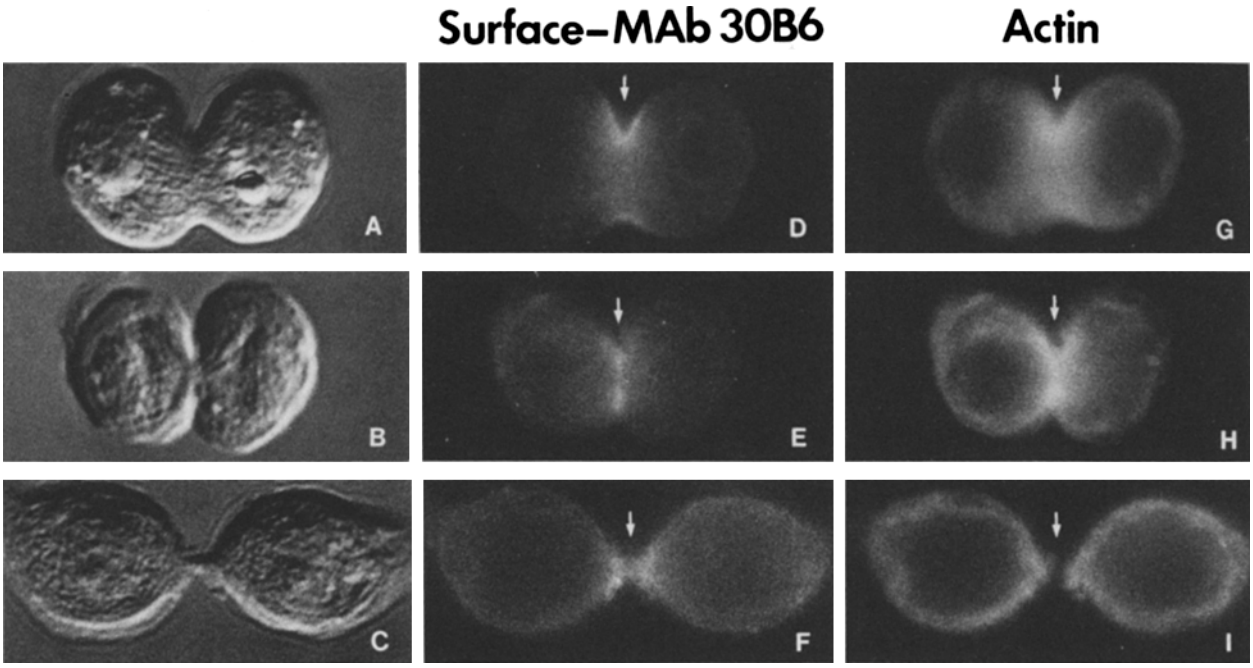


FIGURE 3 During cytokinesis of cardiac myocytes, surface-bound MAb 30B6, indirectly detected with fluorescein-conjugated F(ab')$_2$ fragment of goat anti-mouse IgG, is demonstrated to be sharply localized to the cleavage furrow region (arrows in *D–F*) where actin labeled with rhodamine–phallacidin is also concentrated (*G–I*). The corresponding Nomarski images are presented in *A–C*. × 1,610.

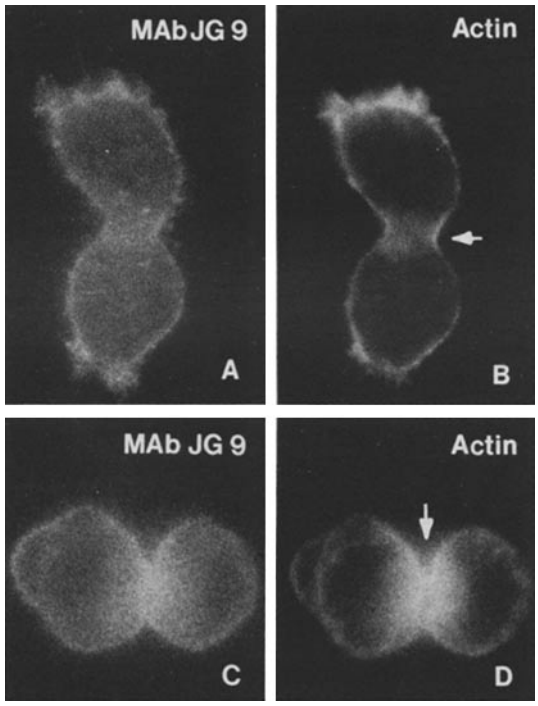


FIGURE 4 Dividing cardiac myocytes similarly doubly labeled as in Fig. 3 on the cell surface with MAb JG9 (*A* and *C*), and intracellularly for actin with rhodamine–phallacidin (*B* and *D*). Although actin is clearly concentrated in the cleavage region (arrow), MAb JG9 labeling is broadly distributed over the entire cell surface, with perhaps a slight concentration in the furrow as in *C*. (*A* and *B*) × 1,574. (*C* and *D*) × 1,610.

ventral surfaces of isolated intact fibroblasts that had been fixed prior to labeling. Using double-labeling for the 30B6 antigen and for actin, we observed a striking degree of co-distribution of the 30B6 antigen on the dorsal surface of the interphase cell (Fig. 5*A*) and of actin stress fibers (Fig. 5*C*) underlying the dorsal surface. On the ventral surface, the 30B6 antigen was organized in a more patchy distribution (Fig. 5*B*) than on the dorsal surface, and near the cell periphery appeared to be concentrated near the termini of the actin stress fibers (Fig. 5*D*). These termini are known to correspond to the sites of focal adhesion of the fibroblasts to the substratum (23, 28). In Fig. 6, the immunofluorescent labeling of MAb 30B6 (Fig. 6*B*) at the ventral surface is compared with interference reflection images (images *1*, *2*, *3* corresponding with those in Fig. 6*B*) of the same fields. There is a substantial degree of overlap of the labeling for the 30B6 antigen and of the dark patterns in interference reflection in the peripheral regions of the cell, further indicating an association at the light microscopic level of resolution of the 30B6 antigen with the focal adhesion sites (28). Such peripheral, focal adhesion-type labeling of the ventral surface with MAb 30B6 was much more intense if the cells were treated with detergent after fixation but prior to antibody binding. Since the 30B6 antigen is exposed at the cell surface, this increased labeling of the permeabilized (rather than the intact) cell is probably a reflection of increased accessibility of the labeling reagents to the narrow gaps between the cell and the substratum in the vicinity of the focal adhesion sites. If fixed but nonpermeabilized cells were mechanically disrupted with a stream of buffer, patches of ventral surface membrane that were still attached to the substratum showed a striking co-distribution of the MAb 30B6 antigen (Fig. 6*C*) and the residual actin labeling (Fig. 6*D*) at the residual sites containing the focal adhesion plaques. In addition to its presence in areas of cell–substratum contact, the MAb 30B6 antigen was also found in regions of fibroblast cell–cell contact that are associated with corresponding patches of actin labeling (data not shown),

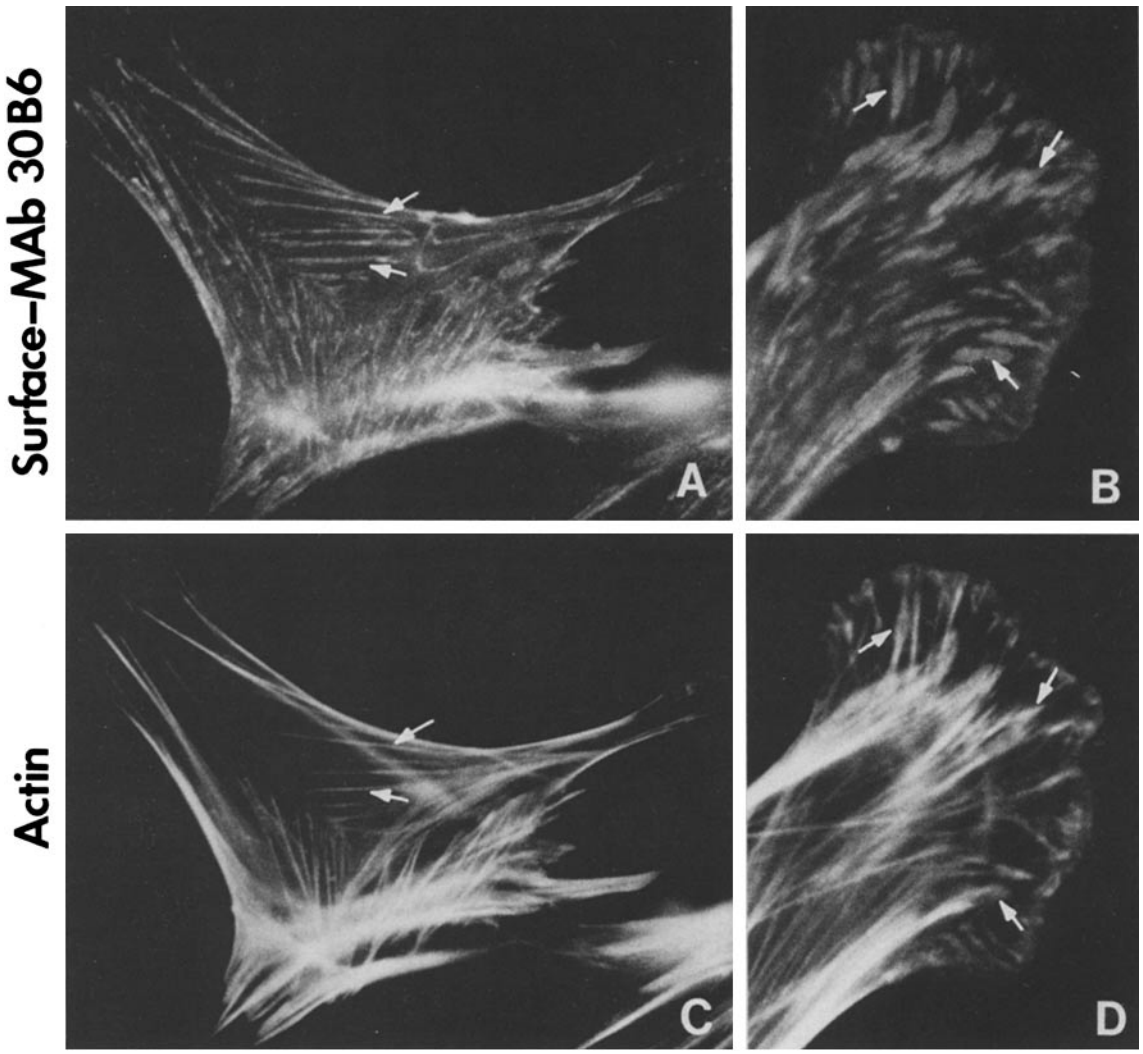


FIGURE 5 Surface MAb 30B6 and intracellular F-actin distributions associated with the dorsal and ventral surfaces of interphase CEFs examined by double fluorescence as described in Fig. 2. At the dorsal surface, the MAb 30B6 antigen (*A*) is found to be aligned (arrows) over the underlying actin microfilaments (*C*). At the ventral surface, the MAb 30B6 antigen (*B*) is present in a patchy distribution, with many of the patches co-localized with the termini of actin microfilaments at the ventral membrane (arrows) (*D*). × 1,281.

probably reflecting the termination sites of actin microfilament bundles at the cell membranes (30).

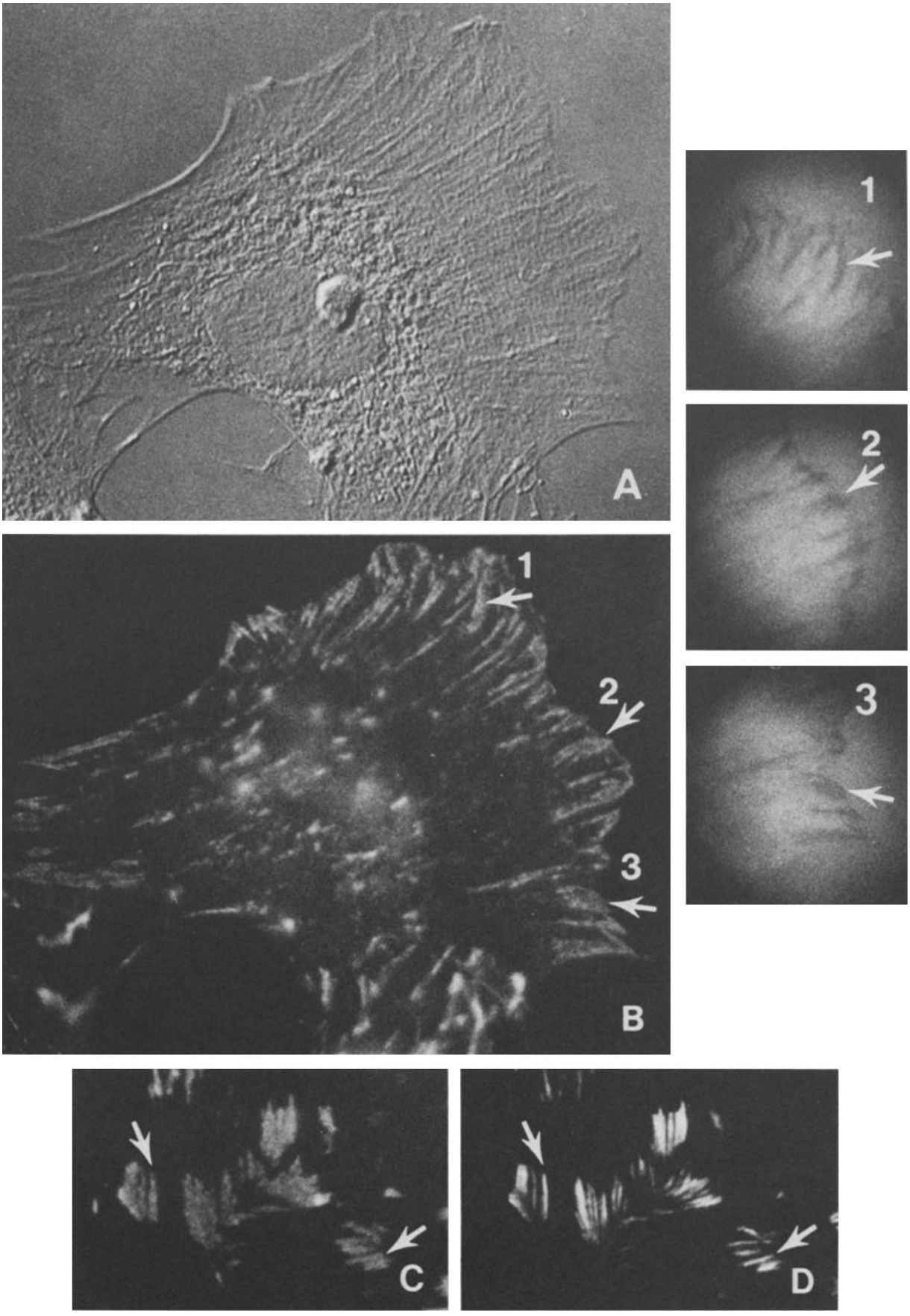
MAb 30B6 Antigen Distribution in Other Cell Types

In a preliminary survey by immunocytochemical methods, a number of cell types and tissues (examined in culture or by semi-thin frozen sections) were found to express the MAb 30B6 antigen. Epithelial, endothelial, lymphoid, platelet, and granulocyte cell types, smooth, cardiac and skeletal muscle, and the soma and processes of ciliary ganglion neurons (Jacobs, M., and A. A. Rogalski, unpublished observations) were all found to be immunolabeled with MAb 30B6; erythrocytes, however, were unlabeled. MAb 30B6 labeling was most significantly associated with platelets, endothelial cells, and smooth muscle cells. In semi-thin frozen sections of gizzard smooth muscle immunolabeled with MAb 30B6 (Fig. 7), regions of direct cell–cell contact associated with dense plaque regions were strongly fluorescently labeled.

Identification of the MAb 30B6 Antigen

To identify the cell surface antigen recognized by MAb 30B6, we have used several techniques, including immunoblotting, immunoaffinity chromatography, and immunoprecipitation, of detergent-solubilized CEF or gizzard smooth muscle. Detergent was required to solubilize the antigen and the choice of detergent was made by dot blot assays on nitrocellulose of CEF extracts prepared with Triton X-100, DOC, Nonidet P-40, or CHAPS. In these preliminary experiments, CHAPS was found to be most effective in extracting the MAb 30B6 antigen, and was used throughout the remaining studies. CEF and gizzard smooth muscle were extracted with 10 mM CHAPS in buffer containing protease inhibitors. There was no indication of any proteolytic breakdown of the MAb 30B6 antigen under these conditions.

If the CEF or gizzard smooth muscle extracts were then prepared for SDS PAGE in the usual manner (i.e., heating at 50–100°C in buffered SDS containing β-mercaptoethanol or dithiothreitol), no specific MAb 30B6 immunostaining of


A
1
2
3
B
C
D

transfers to nitrocellulose was observed. It was then found that the antigenic epitope specific for MAb 30B6 was inactivated by the treatment with reducing agent, and showed a significant loss of activity upon heating in SDS without reducing agents present. The extracts were therefore prepared for SDS PAGE in 0.1% buffered SDS without reducing agents and at room temperature. With such preparations after electrophoresis and blotting, MAb 30B6 bound to a major single band with an apparent molecular mass of 107 kD in both CEF and gizzard extracts (Fig. 8 (*left set*), lanes *c* and *d*). This band was much more prominent in the gizzard than the CEF extracts. In addition, a band with an apparent molecular mass of 200–210 kD showed weak binding of MAb 30B6, but the intensity of this band was quite variable from one extract to the next. In immunoprecipitation experiments to be described below, with samples that, after immunoprecipitation, were treated with reducing agents and heat prior to SDS PAGE, no such band at 200–210 kD was observed. It seems likely, therefore, that this band is due to some dimerization of the 107-kD component that resulted from the mild treatments (cf., 46) used to preserve the activity of the MAb 30B6 antigen in the immunoblotting experiments.

For affinity purification of the antigen, mild conditions were also used so as to preserve the antibody-binding capacity. CEF or gizzard extracts in 10 mM CHAPS passed slowly over a column of an Affi-Gel column coupled to MAb 30B6, followed by washing and elution, yielded the SDS PAGE results in Fig. 8 (*right set*). Two molecular species were specifically bound to the MAb 30B6, but not the control normal mouse IgG column, one of ~107 kD and the other 165 kD (lanes *A* and *C*), of which only the former reacted with MAb 30B6 in blotting experiments (lanes *E* and *G*).

The two bands were excised from SDS gels, electrophoretically eluted, reduced and alkylated, and re-run on another SDS gel (Fig. 9 [*left*]). Under these conditions, the component that had migrated with an apparent molecular mass of 107 kD (lanes *A* and *D*) under nonreducing conditions shifted to 130 kD (lanes *E* and *F*) under reducing conditions, and the component at 165 kD (lanes *A* and *B*) to 175 kD (lanes *C* and *F*). ^{125}I-tryptic peptide fingerprinting of the 107-kD/130-kD and 165-kD/175-kD bands showed that the two components were not related, consistent with their immunochemical discrimination by MAb 30B6 (Fig. 9 [*right*], *a* and *b*).

These results indicate that the antigen recognized by MAb 30B6 is a protein of 107 kD under nonreducing, and 130 kD under reducing, conditions; these results also suggest that it is associated in the intact cell surface with an immunochemically unrelated protein that has a molecular weight of 165 kD/175 kD in the nonreduced/reduced state.

The Glycoprotein Nature of the MAb 30B6 Antigen

Several types of experiments showed that the MAb 30B6 antigen is a glycoprotein. In one type, gizzard extracts were specifically absorbed to the antibody–affinity columns, eluted,

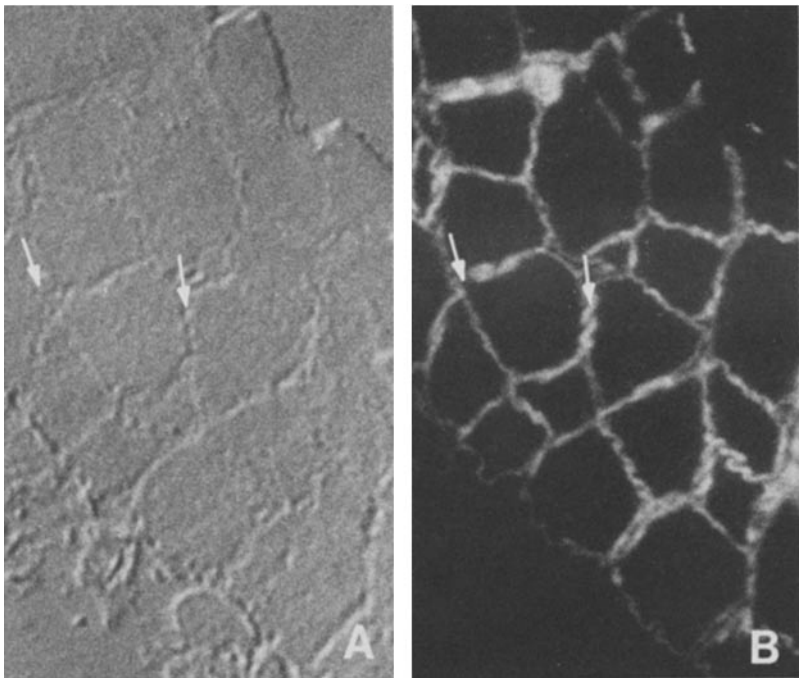


FIGURE 7 Indirect immunofluorescence labeling of the MAb 30B6 antigen (*B*) on semithin (0.5-μm) sections of fixed gizzard smooth muscle, seen in Nomarski optics in *A*. The prominent patch-like pattern of labeling only along regions of cell–cell contact (arrows) probably represents the membrane-associated dense plaque regions. × 1,989.

FIGURE 6 Peripheral regions of the ventral surface of CEFs revealed by the dark patterns in interference reflection microscopy to be sites of intimate contact with the substratum (arrows in *1–3*) are also immunolabeled with MAb 30B6 (*B*). The corresponding Nomarski image is presented in *A*. In this preparation, fixed CEFs were treated with 0.1% Triton X-100 for 5 min prior to primary and secondary antibody labeling. Ventrally attached membranes still bound to the substratum after mechanical dislodgement of CEFs show a direct correspondence (arrows) of MAb 30B6 antigen (*C*) and F-actin (*D*) distributions by double-label fluorescence as used in Figs. 2–5. In *C* and *D*, fixed CEFs were directly labeled with MAb 30B6 (without treatment with Triton X-100), followed by fluorescein-conjugated F(Ab')$_2$ fragments of goat anti-mouse IgG and rhodamine–phallicidin. × 1,421.

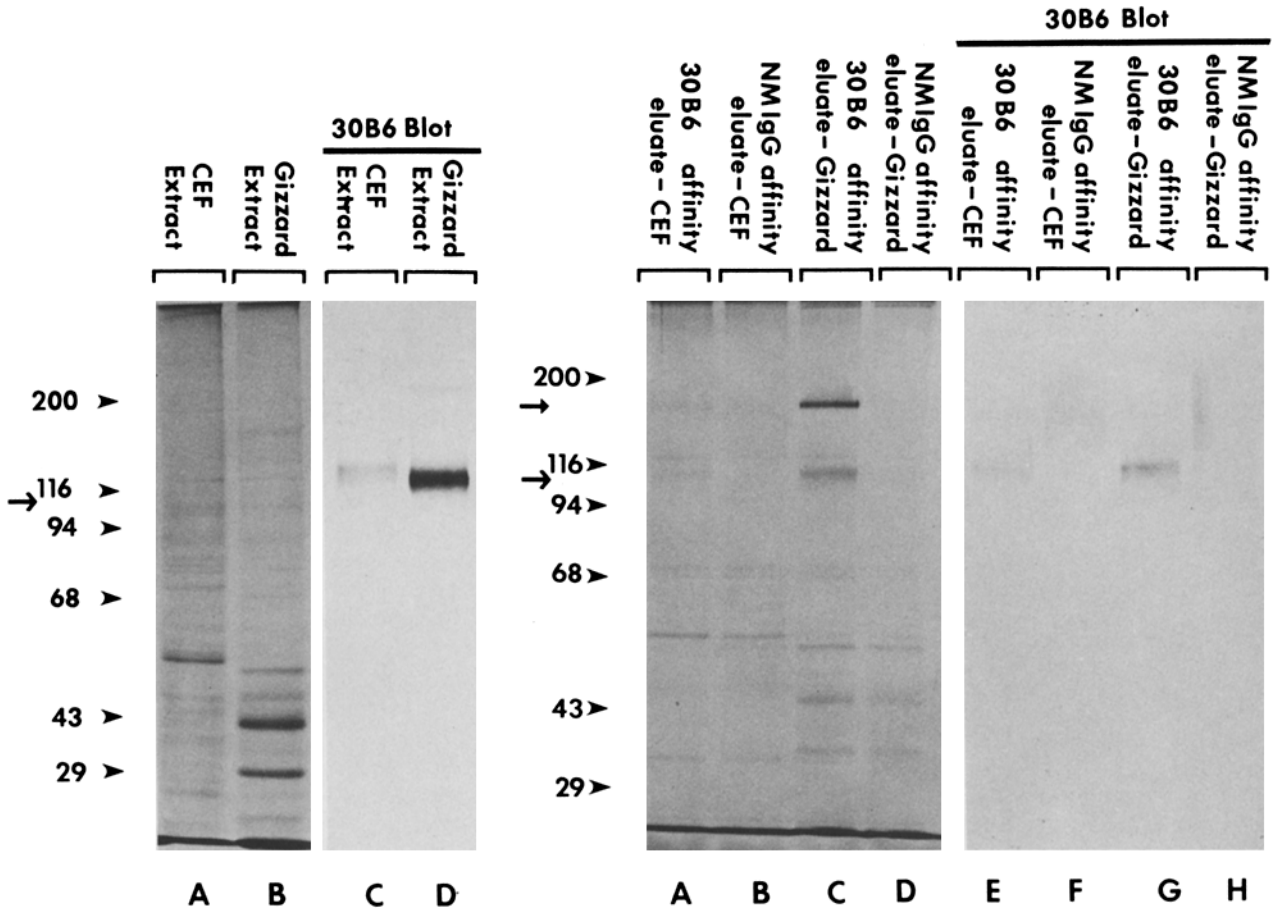


FIGURE 8 (*Left set*) Crude 10-mM CHAPS extracts of CEFs (*A*) and gizzard smooth muscle (*B*) separated in SDS PAGE under nonreducing conditions as detected by Coomassie Blue staining. After electrophoretically transferring to nitrocellulose, immunoblotting with MAb 30B6 was carried out. One band of ~107 kD is strongly stained (arrow) in both CEF (*C*) and gizzard (*D*) extracts. A variable number of species in the 200–210-kD range is also weakly detected. Immunoblotting is completely abolished under reducing conditions of SDS PAGE. (*Right set*) Combined immunoaffinity and immunoblotting analyses of the MAb 30B6 antigen isolated from CEFs and gizzard smooth muscle in SDS PAGE under nonreducing conditions. Two species of ~107 kD and 165 kD were specifically enriched from CEFs (*A*) and gizzard smooth muscle (*C*) and not detected with eluates from columns prepared with normal mouse IgG (*B* and *D*) in this silver nitrate–stained SDS gel. Immunoblotting of gel replicas with MAb 30B6 resulted in detection of only the 107-kD species purified from either CEF (*E*) or gizzard (*G*) extracts, but not in eluates from normal mouse IgG columns (*F* and *H*).

and the eluates electrophoresed in SDS PAGE under nonreducing conditions, and then blotted onto nitrocellulose (Fig. 10). The blots were treated either with MAb 30B6 (lane *B*), or with the peroxidase-labeled lectins ricin (lane *D*) or concanavalin A (lane *C*). The results show that the MAb 30B6 antigens of both the 107 kD and the co-eluted 165 kD species react with both lectins.

In another type of experiment (Fig. 11 [*left set*]), CEF cultures were metabolically labeled with [^{35}S]methionine for 18 h, extracted with CHAPS; the extracts (lane *A*) were immunoprecipitated with MAb 30B6 (lanes *B* and *C*) or with normal mouse IgG (lanes *D* and *E*), and the proteins separated on SDS PAGE after reduction. A radiolabeled doublet species was specifically immunoprecipitated by MAb 30B6, corresponding to molecular masses of ~130 kD and 125 kD (lane *B*). Suspecting that this heterogeneity was due to varying amounts of sialic acid residues on the protein (5), we treated the immunoprecipitates with neuraminidase. This resulted in conversion of the doublet to a single band of ~125 kD (lane *C*).

In a further experiment (Fig. 11 [*right set*]), instead of long-term metabolic labeling, the cultures were pulsed for 15 min with [^{35}S]methionine, and then chased for varying times up to 60 min. Under reducing conditions in SDS PAGE, only a single band of ~130 kD was found in the specific immunoprecipitates (lanes *A–E*). A slight but progressive shift to a lower molecular weight species close to the molecular weight of the de-sialylated form is also evident. The basis of this shift (processing of the peptide and/or in vivo removal of sialic acid residues) is under investigation.

These three sets of results are consistent with the proposal that the MAb 30B6 antigen is a glycoprotein, and that it undergoes progressive glycosylation, terminating in sialylation, as it is transported through the cell to the cell surface, in a manner that is typical of sialoglycoproteins.

This proposal led to the prediction that the MAb 30B6 antigen should be detected in organelles (e.g., the rough endoplasmic reticulum and the Golgi apparatus) that participate in the intracellular transport and processing of glycoproteins. Cultured CEF were fixed and permeabilized, then immunolabeled with MAb 30B6, and examined for the intracellular distribution of label rather than the surface distribution

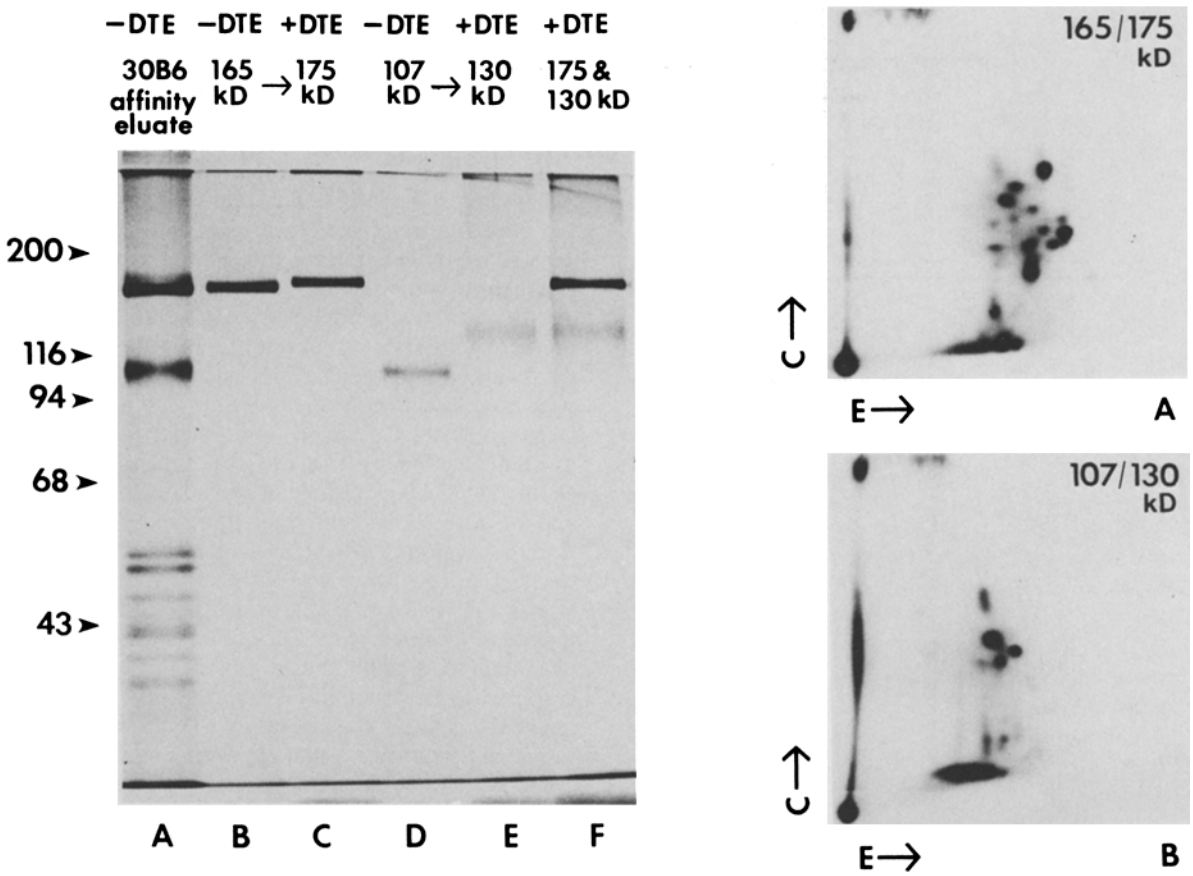


FIGURE 9 (*Left*) The protein purified on MAb 30B6 affinity columns from extracts of gizzard smooth muscle, examined by SDS PAGE under nonreducing (*−DTE*) or reducing (*+DTE*) conditions. Two species of ~107 kD and 165 kD under nonreducing conditions (lane *A*) are specifically enriched as previously shown. Each band was separately cut out of gels, eluted and re-electrophoresed under either nonreducing (lanes *B* and *D*) or reducing conditions (lanes *C*, *E*, and *F*). Both the 107-kD and 165-kD species assume a higher molecular mass under reducing conditions of SDS PAGE (i.e., 107 (*D*) to 130 kD (*E*), and 165 (*B*) to 175 kD (*C*). (*Right*) Two-dimensional maps of ^{125}I-tryptic peptides of the 165-kD/175-kD (*A*) and the 107-kD/130 kD (*B*) species reveal distinctive and unrelated patterns. In these experiments, electrophoresis (*E*) was performed in the horizontal direction and chromatography (C) in the vertical direction as indicated by the arrows.

shown in Fig. 5. The results in Fig. 12, *A* and *B* (plane of focus is above cell substratum level), show that the nuclear envelope (whose outer membrane is part of the rough endoplasmic reticulum) is labeled; results in Fig. 12, *C* and *D* show that a perinuclear structure that is very likely the Golgi apparatus (43) is also labeled, as expected for a molecular species that is either a secreted protein or a plasma membrane integral protein.

The MAb 30B6 Antigen Is a Membrane Integral Protein

The results presented so far, demonstrating that the MAb 30B6 antigen is a cell surface glycoprotein, do not discriminate between the possibilities that the antigen (*a*) is a protein that is secreted and as a peripheral membrane protein forms part of the extracellular matrix; or (*b*) is an integral protein of the plasma membrane. A peripheral protein might be released from a membrane by low or high salt concentrations or extreme pH; however, treatment of CEF cultures with low or high ionic strength buffers, or 0.1 N NaOH, pH 11.0 (51) or 0.1 M Na carbonate, pH 11.5 (20) did not solubilize a component that reacted with MAb 30B6 (data not shown). A more definitive discrimination was based on the method of charge-shift electrophoresis (32). Electrophoresis in agarose gels of gizzard extracts in 10 mM CHAPS was carried out in the zwitterionic CHAPS buffer alone, or in CHAPS containing either the cationic detergent CTAB or the anionic detergent DOC. The extent of migration of the MAb 30B6 antigen into these agarose gels was then detected by immunoblotting. The results (Fig. 13 [*top*]) showed that the cathodic mobility of the antigen in CHAPS (lane *B*) was increased in the presence of DOC (lane *A*), as well as decreased in the presence of CTAB (lane *C*). Such significant bidirectional electrophoretic shifts are usually diagnostic of integral proteins (32), demonstrating their ability to be incorporated into the micelles of neutral detergents such as CHAPS. Vinculin, a peripheral protein of the membrane, was found in parallel experiments (Fig. 13 [*bottom*]), to exhibit a mobility shift only in the presence of DOC (lane *A*) but not in the presence of CTAB (lane *C*). Similar results were obtained with α-actinin (data not shown).

DISCUSSION

We are interested in the identification and ultrastructural arrangements of the proteins that are involved in the attachments of actin microfilaments to membranes. These attach-

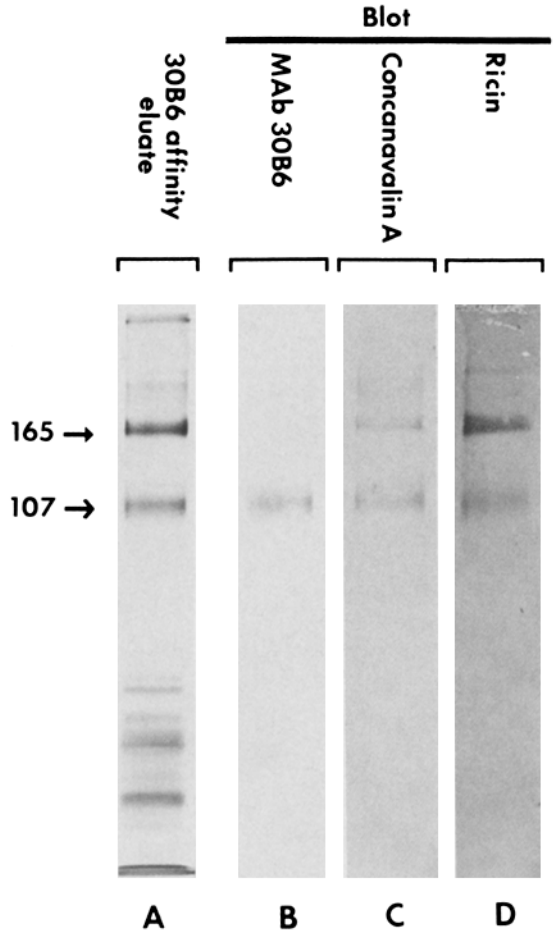


FIGURE 10 The 107-kD and 165-kD species affinity purified on MAb 30B6 columns from gizzard smooth muscle extracts (lane *A*), of which only the 107-kD species is specifically stained with MAb 30B6 in replica immunoblots (lane *B*), are both demonstrated to be ricin-binding (lane *D*) and concanavalin A-binding (lane C) glycoproteins. The specificity of binding was demonstrated (not shown) by their elimination in the presence of 0.1 M lactose (for ricin) or by 0.1 M α-methyl mannoside (for concanavalin A).

ments are likely to be specific; that is, likely to involve specific sets of peripheral and integral membrane proteins mediating particular types of attachments. A number of peripheral membrane proteins, such as vinculin (23), talin (11), and the 200-kD protein (37) have been isolated in recent years and shown by immunocytochemistry to be localized in the vicinity of membrane–microfilament sites; and a few cell surface proteins have also been implicated (12, 14, 41). Nevertheless, it is clear that we are still far from a complete molecular and ultrastructural definition of these sites.

In this study, the approach used to detect integral membrane proteins that might be involved in membrane–microfilament attachments was to use intact dividing fibroblasts for the induction and screening of appropriate monoclonal antibodies (MAbs) that were directed to such integral proteins. Dividing cells are characterized by a large band of actin microfilaments (the contractile ring) attached to the cell membrane under the cleavage furrow (47, 48). An integral protein that is involved in membrane–microfilament attachments might then be expected to be concentrated in that membrane domain.

The Distribution of MAb 30B6 Labeling on the Surfaces of Dividing Cells

In our preliminary observations (Fig. 1), we found MAb 30B6 to be unique among all other MAbs directed to mitotic CEFs by its labeling associated with the cleavage furrow region. Since the immunolabeling was carried out on fixed but impermeable cells, the antigen recognized by MAb 30B6 (*a*) is concentrated in the furrow to begin with, and not as the result of an antibody-induced capping phenomenon (8, 9); and (*b*) is exposed at the exterior cell surface. Further study of MAb 30B6 labeling of mitotic CEFs showed, however, that the situation was more complicated than indicated for the selected cells shown in Fig. 1. Quite frequently, MAb 30B6 labeling was not as clearly concentrated in the cleavage furrow as in other instances (Fig. 2). This puzzling observation was clarified by double-labeling for MAb 30B6 on the cell surface and for intracellular actin (Fig. 2). In many cells, in addition to a concentration of actin under the membrane of the cleavage furrow, there was substantial, often more intense, labeling for actin that was irregularly distributed at other regions of the cell surface. Such cells generally showed shapes that were not simply bilobar, as with the cells in Fig. 1, but appeared to be distorted. We suggest that these distortions reflect the variable attachments of the different dividing cells to the substratum, and that actin microfilaments were associated with these attachments sites and not only with the cleavage furrow. It is possible that the actin at the attachment sites was recruited from, and thereby depleted, the microfilaments underlying the cleavage furrow. These displacements may also account for the variability reported by other investigators for the presence of α-actinin and myosin in the cleavage furrow of CEFs (22, 40). In any event, the important point for our present purposes is that the labeling with MAb 30B6 on the cell surface closely co-distributed with that of membrane-localized actin in the dividing CEF cells, whether in the cleavage furrow or at the sites of apparent contact with the substratum.

With cardiac myocytes in culture, dividing cells often showed a more nearly bilobar shape than dividing CEFs (cf. Fig. 3 with Fig. 2). Correspondingly, a clear co-concentration of both MAb 30B6 labeling (Fig. 3, *C*, *F*, and *I*) and of actin labeling (Fig. 3, *B*, *E*, and *H*) in the cleavage furrow of the dividing myocytes was observed. Presumably, the attachments of the dividing myocytes to the substratum were less extensive, and involved less redistribution of the actin from the cleavage furrow, than with CEFs.

The Distribution of MAb 30B6 Labeling on the Surfaces of Interphase CEF Cells

We consider separately observations made at the dorsal and ventral surfaces of isolated interphase CEF cells on a substratum. With well-spread interphase fibroblasts, stress fibers containing bundles of actin microfilaments lie immediately under the dorsal cell surface and extend for some distance parallel to the surface (1, 29, 49). It seems likely that these stress fibers have multiple attachments to the membrane along much of their length via one or more specific integral proteins (50). Such protein(s) should therefore be found concentrated in the dorsal surface directly over the stress fibers. This is indeed the unique distribution found for the cell surface antigen recognized by MAb 30B6 on the dorsal surface of

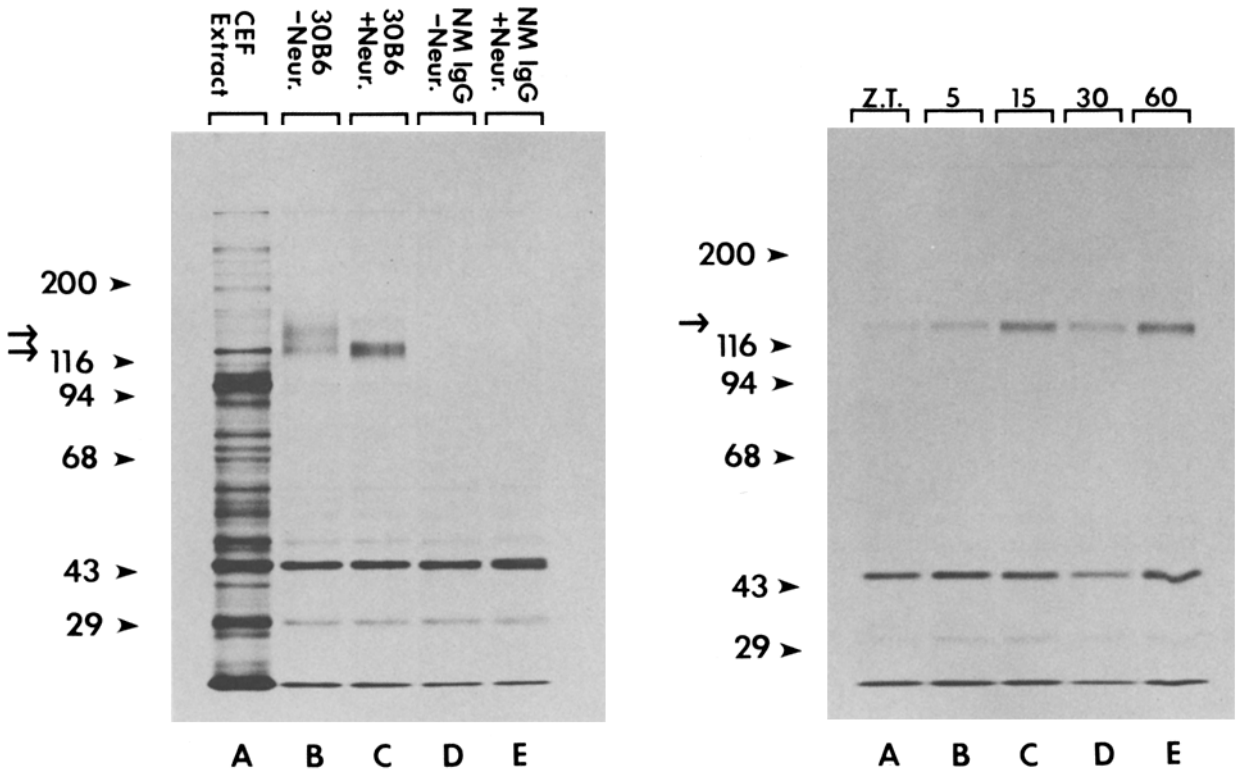


FIGURE 11 (*Left set*) CHAPS extracts of CEFs, after 18-h metabolic labeling with [^{35}S]methionine (*A*) immunoprecipitated by MAb 30B6 (*B*) and normal mouse IgG (*D*). Two closely migrating species (arrows) of ~130 and 125 kD under reduced SDS PAGE conditions (*B*) are specifically recognized and are not observed with normal mouse IgG immunoprecipitates (*D*). Results of neuraminidase digestion of the immunoprecipitates in *B* and *D* are presented respectively in *C* and *E*. The 130 and 125 kD species (*B*) appear to be converted into one prominent band of ~125 kD (*C*), indicating that the two bands in *B* result from a sialic acid-induced charge heterogeneity. Controls of neuraminidase digestion of normal mouse IgG immunoprecipitates are shown in *E*. (*Right set*) Immunoprecipitation of 10 mM CHAPS extracts of CEFs metabolically labeled with [^{35}S]methionine for a short term (15-min pulse) followed by chase periods of 5 (*B*), 15 (*C*), 30 (*D*), and 60 min (*E*). SDS PAGE was carried out under reducing conditions. Only one band (arrow) is observed of apparent molecular mass of 130 kD in *A*, which appears to undergo a slight decrease in apparent molecular weight throughout the course of the chase period.

fixed interphase CEF (Fig. 5, *A* and *C*).

On the ventral surfaces of well-spread fibroblasts, it is well known that patch-like contacts (focal adhesions) form between the cell and the substratum around the periphery of the cell. Inside the cell at these focal adhesion sites, bundles of actin filaments have been shown to approach obliquely to the membrane surface and to terminate there (1, 25). The cytoskeletal proteins vinculin (23) and talin (11), and the 200-kD protein (37) have been shown by immunofluorescence observations to be concentrated inside the cell at these focal adhesion sites. Specific integral proteins that were involved in the attachment of actin microfilaments to membranes might therefore be expected to be concentrated at the focal adhesions. Our observations show that immunolabeling with MAb 30B6 is indeed concentrated at the ventral cell surface in the region of the focal adhesions, as demonstrated by the co-distribution of MAb 30B6 labeling and the termini of actin bundles at the ventral surfaces of the CEFs (Fig. 5, B and *D*; Fig. 6, *C* and *D*), as well as by co-distribution of the MAb 30B6 labeling with the dark plaques seen in interference reflection patterns (Fig. 6) that are produced by the focal adhesions (7, 28).

The Distribution of MAb 30B6 Labeling in Chicken Gizzard Smooth Muscle

In smooth muscle tissue, neighboring cells are linked to one another at multiple discrete sites all along their surfaces, known as dense plaques. The dense plaques are sites where microfilament bundles terminate obliquely at the cell membrane (4). With semi-thin frozen sections of gizzard tissue, the immunofluorescent labeling with MAb 30B6 was intense and concentrated along the cell boundaries (Fig. 7). In further experiments with ultra-thin frozen sections of gizzard tissue, immunolabeling at the electron microscopic level of resolution has shown the MAb 30B6 antigen to be concentrated at the membrane of the dense plaque (Rogalski, A. A., and S. J. Singer, manuscript in preparation).

Before we further discuss the significance of our observations that the cell surface antigen recognized by MAb 30B6 co-distributes with actin microfilaments in a considerable number of different cellular systems, we will examine the molecular properties of this antigen.

The Molecular Characteristics of the MAb 30B6 Antigen

Immunoblotting experiments with MAb 30B6 identified a single protein antigen in detergent extracts of both CEF cells and chicken gizzard smooth muscle cells. More of this protein is present in the gizzard extracts (Fig. 8) which correspond to the intense immunolabeling with MAb 30B6 of the muscle tissue as compared to CEF (Fig. 7). That the epitope recog-

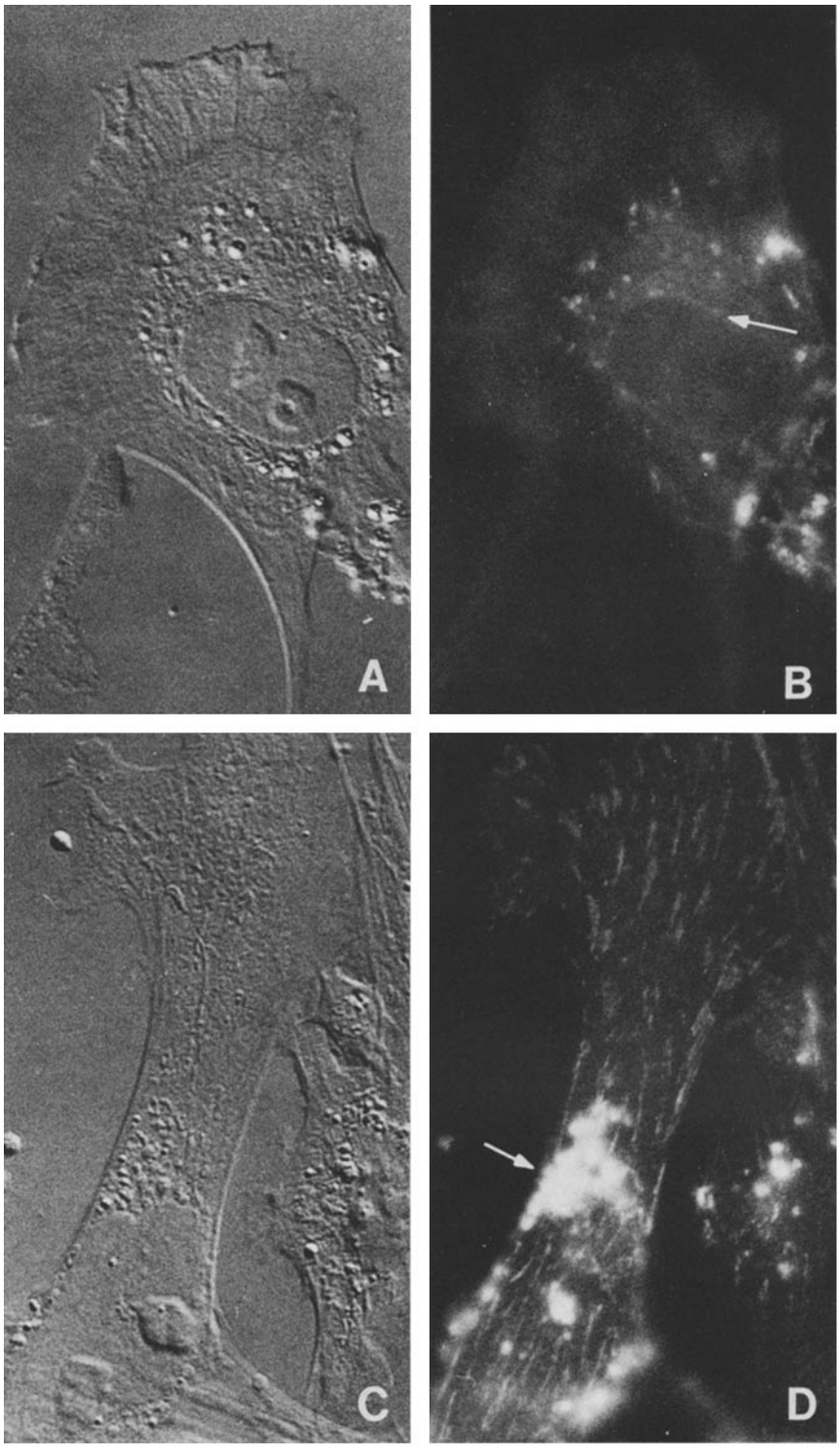


FIGURE 12 Intracellular expression of the MAb 30B6 antigen as detected by indirect immunofluorescence of 0.1% Triton X-100–permeabilized fixed CEFs. Antigen localization in the nuclear envelope (*B*, arrow) and in the perinuclear Golgi region (*D*, arrow) is observed. Plane of focus is above the cell substratum level in *B*. × 1,218.

nized by MAb 30B6 is a conformationally dependent site on a protein molecule is indicated by its sensitivity to heat and to reducing agents. The antigen is a sialoglycoprotein, by virtue of its interaction with the lectins ricin and concanavalin A (Fig. 10, lanes *C* and *D*) and its sensitivity to neuraminidase treatment (Fig. 11 [*left set*], lane *C*). The apparent molecular mass of the protein on SDS PAGE gels depends on a number of parameters. The fully glycosylated and nonreduced protein has a molecular mass of 107 kD, which after reduction shifts to 130 kD (Fig. 9 [*left*]). The protein is characterized by a distinctive ^{125}I-tryptic peptide fingerprint (Fig. 9 [*right*]).

On immunoaffinity columns prepared with MAb 30B6, the same unreduced 107-kD protein is specifically absorbed from CEFs as well as gizzard detergent extracts, accompanied however by another glycoprotein which is antigenically and structurally (Fig. 8 [*right set*] and Fig. 9 [*right*]) unrelated to it. This second protein has a molecular mass of 165 kD when in the nonreduced form, and 175 kD when reduced (Fig. 9 [*left*]). Since the 165/175-kD glycoprotein is neither immunoprecipitated nor immunoblotted by MAb 30B6, the conditions of immunoaffinity chromatography apparently permit high affinity interactions with the bound MAb 30B6 antigen (even

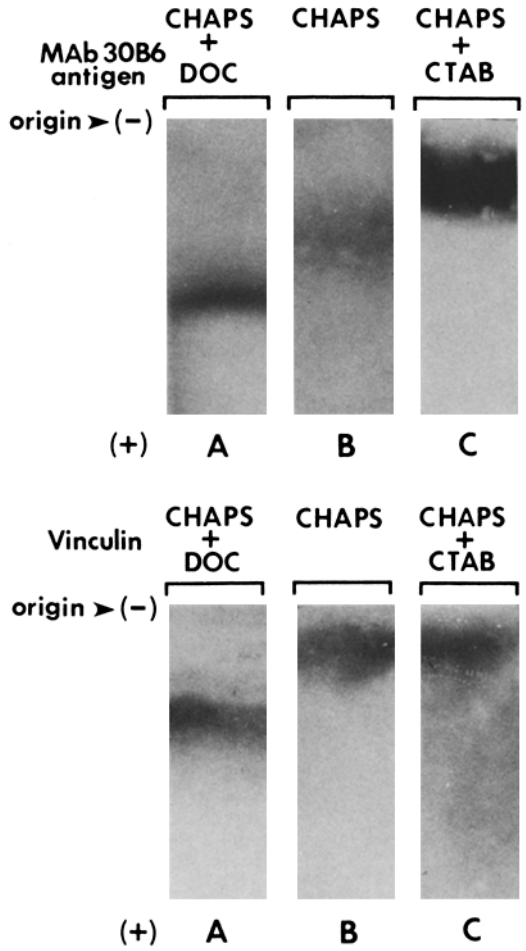


FIGURE 13 Charge-shift electrophoresis evidence for the integral nature of the MAb 30B6 antigen. (*Top*) Agarose gel electrophoresis of a 10-mM CHAPS extract of gizzard smooth muscle (lane *B*) to which was added either an anionic (1.0% DOC, lane *A*) or a cationic (0.2% CTAB, lane *C*) detergent. Each sample was then immunoblotted with MAb 30B6. The MAb 30B6 antigen shows an increased cathodic mobility in *A* and a decreased cathodic mobility in *C*, as compared to *B*. (*Bottom*) An experiment run parallel to the one described for top gels, using affinity-purified rabbit anti-vinculin antibodies to demonstrate the charge-shift electrophoretic behavior of the peripheral protein vinculin. Unlike the case with the MAb 30B6 antigen, a bidirectional shift of mobility is not found. Vinculin migrates into the agarose gel to the same extent in the presence of 10 mM CHAPS (*B*) or 0.2% CTAB (*C*), but shows a more cathodic mobility in the presence of DOC (*A*) (see text for discussion).

in the presence of 10 mM CHAPS). The MAb 30B6 antigen and the 165/175-kD glycoprotein species may interact similarly in the intact membrane.

The MAb 30B6 antigen is exposed at the cell surface, since it can be immunolabeled on intact CEF cells. Our experiments indicate that it is an integral protein of the plasma membrane rather than a component of the extracellular matrix, because it requires detergents to solubilize it from the membrane, and more directly, because it exhibits significant differential mobilities in charge-shift electrophoresis experiments (Fig. 13). In such experiments (32), Triton X-100 is usually used as the nonionic detergent that is supplemented with either an anionic or a cationic detergent. Because we found that the zwitterionic detergent CHAPS was more effective than Triton X-100 in the extraction of the MAb 30B6 antigen, however, we used CHAPS as the electrically neutral detergent. The facts that the active antigen of MAb 30B6 showed a greater cathodic mobility in CHAPS supplemented with the anionic DOC, and a smaller mobility in CHAPS supplemented with the cationic detergent CTAB, than in CHAPS alone demonstrates that the antigen bound CHAPS. It is a property of integral membrane proteins that they bind neutral and nonionic detergents (32), usually by intercalating their hydrophobic domains into the detergent micelles. In contrast, extracellular matrix proteins, and other peripheral membrane proteins, do not bind nonionic detergents and therefore, do not generally exhibit differential mobilities in charge-shift electrophoresis. As pointed out by Helenius and Simons (32), charge-shift electrophoresis experiments should be performed with both anionic and cationic detergents since some proteins bind one or the other detergents through ionic interactions. The magnitude of bidirectional shifts can also serve as an indicator of the hydrophobic character of a protein. For example, a peripheral protein with some hydrophobic domains, ankyrin (6), exhibits minor bidirectional shifts when compared with those of the integral protein, Band 3. In the present study, large bidirectional shifts demonstrated for the MAb 30B6 antigen were not found with either of the peripheral proteins, vinculin or α-actinin. Instead, both of these proteins bound only DOC, presumably by ionic interactions.

Most integral membrane glycoproteins that have so far been studied in sufficient detail have been shown to be transmembrane proteins, having hydrophilic domains exposed on both sides of the membrane. The MAb 30B6 antigen molecule has a domain on the extracellular surface of the plasma membrane that is recognized by the MAb; we have as yet no direct evidence, however, that it has another domain exposed at the cytoplasmic surface.

A Possible Function of the MAb 30B6 Antigen

The localization, at least at the light microscopic level of resolution, of the MAb 30B6 integral membrane protein to all of several different types of membrane domains where actin microfilaments are attached, is a most unusual result. To the best of our knowledge, no similar set of findings has been reported for any other protein.

Recently, two independent groups have developed monoclonal antibodies designated JG9 and JG22 (27), and CSAT (34, 39), which were found to recognize an unusual complex of three glycoproteins from detergent extracts of chick embryos. The relationship of these species to the MAb 30B6 antigen remains to be established. However, at both immunofluorescent and immunoelectron microscopic (14) levels of resolution, the JG9/JG22 MAb shows labeling characteristics that are distinct from those of MAb 30B6: (*a*) no intense concentration of the former within the cleavage furrow region (Fig. 4); and (*b*) immunoelectron microscopic labeling of membrane regions peripheral to the dense plaque of smooth muscle by the former (14), and within the dense plaque by the latter (Rogalski, A. A., and S. J. Singer, manuscript in preparation). Another MAb, ID-7.2.3. (53), which is specific for a 135-kD protein of adherens junctions of chicken cardiac muscle intercalated discs, fails to immunolabel the dense plaques of smooth muscle and the adhesion plaques of CEF and therefore, is most likely directed to a different antigen than that of MAb 30B6.

In future studies, it will be important to investigate the

molecular relationship between the MAb 30B6 antigen with that of a rat thymocyte glycophorin-like protein recognized by MAb W3/13 (17). This sialylated protein spontaneously caps on the uropod of rat and mouse thymocytes, a redistribution phenomenon whose basis is yet unclear, but that may be related to an association with actin microfilaments.

One possible explanation of the MAb 30B6 immunolabeling results would be that in many types of cells the antigen (either by itself or as part of a molecular complex with other integral proteins such as the 165-kD/175-kD glycoprotein with which it is likely associated) is a transmembrane protein that provides a unique and specific anchoring site on the cytoplasmic surface of the membrane for the attachment of actin microfilaments, probably via molecular bridges involving still other peripheral proteins. If this explanation is correct, it must account for the presence of the MAb 30B6 integral protein in the two types of membrane domains, one involving lateral associations of the microfilaments with the membrane (the cleavage furrow of dividing cells, and the dorsal surface of interphase CEF cells), and the other involving end-on associations (the dense plaques of smooth muscle cells, and the focal adhesions on the ventral surface of CEF). One could suggest that the MAb 30B6 protein participates either as a membrane anchoring site for both types of association, or as a membrane anchoring site only for the lateral type. In the latter case, the additional proposal would be made that lateral and end-on associations are closely interdigitated within the smooth muscle dense plaques and the fibroblast focal adhesions (28), and cannot be discriminated at the light microscopic level of resolution.

On the other hand, other explanations of our findings are not ruled out. It is apparently possible for a membrane integral protein to be co-localized with actin microfilaments under the membrane, without being anchored to them directly. The membrane immunoglobulins that are unique to lymphoid cells are probably a case in point. These integral proteins have been shown to have the unusual property of concentrating spontaneously into the cleavage furrows of dividing lymphocytes (16). The mechanism for such a concentration is not clear, but it is not likely to be due to a direct linkage of membrane immunoglobulins to the microfilaments. In other words, the co-localization of an integral protein with membrane sites where actin microfilaments are attached is a necessary but not sufficient condition to establish that the integral protein provides a membrane attachment site for microfilaments. One additional necessary condition is that the integral protein in question be ubiquitous, found on the surfaces of all cells capable of undergoing division. (Clearly the membrane immunoglobulins discussed above do not satisfy this condition.) In this connection, while we have not yet made a systematic study of its cellular distribution, we have found the MAb 30B6 antigen to be present on every type of chicken cell we have so far examined, except for the nondividing erythrocyte.

We conclude that the MAb 30B6 integral protein is an excellent candidate for the function of anchoring microfilaments to membranes, but that it requires additional study, including in vitro investigations of its interactions with other cytoskeletal proteins and actin microfilaments, to establish that function.

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