# Redistribution of a Major Cell Surface Glycoprotein during Cell Movement

KENNETH JACOBSON,*‡ DONNA O'DELL,* BRUCE HOLIFIELD,*
THERESA L. MURPHY,§ and J. THOMAS AUGUST§
*Laboratories for Cell Biology, Department for Anatomy and ‡Cancer Research Center, School of
Medicine, University of North Carolina at Chapel Hill 27514; and §Department of Pharmacology and
Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT   The distribution in living cells of an 80,000-dalton major cell surface glycoprotein of murine fibroblasts has been studied by use of monoclonal antibodies. The presence of the molecule throughout the plasma membrane and on the substrate attached surface of the cell was demonstrated by immunofluorescence. Cell growth kinetics were not altered and the cells remained motile in the presence of the antibody. The uniform distribution of the direct immunofluorescence stain persisted for long periods (>100 h), which indicates that the fluorescent monoclonal antibodies may be used to trace antigen surface distribution during cell functions. In motile cells, but not $G_0$ or confluent cells, the degree of fluorescent staining decreased toward the leading edge; this gradient increased markedly during the time that the antibody was bound to the cells. However, the gradation was not seen with the lipid probe, dihexadecylindocarbocyanine. The antigen was "patched" only by the application of a second antibody directed to the rat monoclonal antibody and the relationships of these patches to the underlying cytoskeleton were characterized.

Redistributions of plasma membrane components and movement of the membrane itself are intimately involved in cellular motility (for recent reviews, see references 1 and 2). Glycoprotein redistributions during motility have been studied using multivalent ligands. For example, surface immunoglobulins have been observed to redistribute in motile B lymphocytes (3) and fluoresceinated concanavalin A bound to locomoting polymorphonuclear leukocytes preferentially accumulated in the uropod as opposed to the lamellapodium (4). Gradients in the distribution of unspecified surface antigens toward the trailing edge of motile fibroblasts were also observed in the photographs of Heath (5; Fig. 1 i). Gradations in the concentrations of polymorphonuclear leukocyte surface proteins were also seen when fluorescein was used as a monovalent ligand (6).

A major cell surface protein of mouse embryo 3T3 cells as well as macrophages and epithelial cells of the mouse is a polymorphic glycoprotein of ~80,000 daltons (7, 8), designated Ly-24 (Pgp-1)[1]. This glycoprotein is the predominant iodinated component of 3T3 cells labeled by lactoperoxidase

and constitutes ~0.1% of total cell protein, with over $10^6$ antigenic sites distributed throughout the surface of the NIH/3T3 cell (9). Trypsin treatment of intact cells releases a 65,000-dalton fragment (10). The molecule is an integral membrane component and is likely to have the topology of a transmembrane glycoprotein, with extracellular, hydrophobic, and cytoplasmic domains. The gene-controlling expression of the antigen is on chromosome 2, close to the locus encoding the cell surface markers H-3, Ir-2, $\beta2$ microglobulin, and Ly-23 (11). Trowbridge et al. (12, 13) have independently identified the same glycoprotein of mouse lymphoid tissues. Expression of the antigen is differentiation-related. It is present in much higher concentrations in NIH/3T3 cells, G8-1 myoblasts, and IC-21 macrophages than in many other tissue culture cells. In vivo, the antigen is prominent on macrophages and lymphocytes and strong surface staining is observed on most epithelial cells.[2]

The present studies have utilized a monoclonal antibody to describe the cell surface distribution and lateral mobility of this 80,000-dalton glycoprotein. The results provide evidence of its redistribution during cell motility. Such experiments

---

[1] Morse, H. C., I. F. C. McKenzie, U. Hammenburg, and F.-W. Shen, personal communication.

[2] Murphy, T. L., T. W. Bauer, and J. T. August, unpublished data.

were possible because this monoclonal antibody remained localized on the cell surface for extremely long periods without either becoming rapidly internalized or significantly perturbing cell division or motility.

## MATERIALS AND METHODS

*Antibodies:* AMF-15 monoclonal IgG$_{2a}$ antibody which binds the 80,000-dalton plasma membrane antigen (anti-GP80)[3] was produced as previously described (7). Antibody in mouse ascites fluid was purified by ammonium sulfate precipitation and DEAE cellulose column chromatography.

Rabbit anti-rat antibodies and goat anti-rabbit antibodies were obtained from Cappel Laboratories, (Cochranville, PA) as antisera and were purified as above. Heat-inactivation of possible complement contaminants was at 56°C for 30 min.

The fluorescence labels, fluorescein isothiocyanate (FITC), dichloro-triazinylaminofluorescein (DTAF), and tetramethylrhodamine isothiocyanate (TRITC), were obtained from Research Organics (Cleveland, OH). Antibody conjugated to FITC was first dialyzed overnight at 4°C against 0.1 M borate, pH 9.3. FITC was added to the antibody at a ratio of 10 mol FITC:1 mol IgG and the antibody and FITC were mixed with slow stirring for 4–6 h at room temperature in a container protected from light. The fluorescein conjugated antibody was separated from the free FITC by chromatography on a Sephadex G-25/G-50 column. Dye to protein ratios ranged from 0.8 to 1.4. DTAF conjugates were prepared in a similar manner except that the ratio of DTAF to IgG was 5:1, the buffer was 0.05 M borate, and the reaction time was 1 h. The purified antibody was conjugated to TRITC at a ratio of 10 μg TRITC per mg of protein for 6 h at 4°C. Free dye was removed by dialysis against PBS and adsorption onto Norite-A activated charcoal (0.43 mg charcoal per mg protein for 1 h at room temperature). The Norite-A activated charcoal was removed from the antibody solution by centrifugation. Dye to protein ratios ranged from 3.4 to 3.8.

*Other Reagents:* Dihexadecylindocarbocyanine [diI-C$_{16}$(3)] was a generous gift from Dr. A. Waggoner (Carnegie Mellon University, Pittsburgh, PA). FITC-succinyl concanavalin A was obtained from E-Y Labs, Inc. (San Mateo, CA). NBD(7-nitrobenz-2-oxa-1,3-diazole)-phallacidin was purchased from Molecular Probes, Inc. (Junction City, OR). Mounting medium was prepared by dissolving 20 g Gelvatol (Monsanto, St. Louis, MO), a semipermanent, nonfluorescent polyvinyl alcohol solution, in 80 ml PBS, pH 7.2, with mixing for 16 h; 40 ml glycerol was added to this solution and mixed for at least 16 h. The undissolved particles of Gelvatol were removed by centrifugation at 12,000 rpm for 15 min; the supernatant was collected and sodium azide (0.01% final concentration) was added. The final pH of the Gelvatol was 6.5.

*Cultured Cells:* Stock cultures of C3H/10T1/2 cells (received from Dr. John Bertram, Roswell Park Memorial Institute, Buffalo, NY) were maintained in basal medium Eagle (BME) with Earle's Salts (GIBCO Laboratories, Grand Island, NY) supplemented with 5–10% fetal bovine serum (FBS, Reheis Chemical Company, Phoenix, AZ), 0.292 mg/ml L-glutamine (GIBCO Laboratories), and either 0.25 μg/ml gentamicin (Schering Corporation, Kenilworth, NJ), or 100 U per ml penicillin, 0.25 μg per ml fungizone, and 100 μg per ml streptomycin (GIBCO Laboratories). Stock cultures were subcultured every 7 d at a density of 1.5 × 10$^5$ cells per 75-cm$^2$ flask (Corning, Corning, NY, or Falcon Labware, Oxnard, CA) or 5 × 10$^4$ cells per 25-cm$^2$ flask (Falcon Labware) and fed on the third or fourth day following subculture. Cells used in experiments were seeded onto coverslips at a density of 2.5 × 10$^4$ cells per 35-mm petri dish (Falcon or Corning) and allowed to grow for at least 48 h.

Stock cultures of NIH/3T3 cells were grown in Dulbecco's minimal essential medium, supplemented with 10% FBS, 0.292 mg/ml L-glutamine, and the above antibiotic-antimycotic solution. The cells were subcultured every 3 d; an equivalent seeding density of 8 × 10$^5$ cells per 75-cm$^2$ flask was substituted for the conventional seeding density of 3 × 10$^5$ cells per 60-mm petri dish. Experiments were conducted with 5 × 10$^4$ cells seeded onto coverslips. All cell cultures were maintained at 37°C in humidified atmosphere of 5% CO$_2$-95% air.

*Arresting Cells In G$_0$:* C3H/10T1/2 cells were arrested in G$_0$ through serum deprivation. Cells were seeded onto coverslips at a density of 5 × 10$^4$ cells per 35-mm petri dish in enriched BME (containing L-glutamine and antibiotic-antimycotic solution) plus 5% FBS. After 12 to 24 h, the cell monolayers were rinsed three times with PBS and fed with enriched BME plus

1% FBS. The cells were monitored daily by cell counts for entry into G$_0$. The total number of cells per 35-mm petri dish was counted with a hemacytometer following removal of the cells by trypsinization. At least two separate counts from each of two duplicate dishes were averaged to give the mean cell number. When the total cell number did not increase significantly in a 2–3-d period, the cells were assumed to be in G$_0$. The plateau in total cell number usually occurred after 72–96 h in low serum, at which time there was no further increase in cell number. After the cells were determined to be in G$_0$, they were stimulated to re-enter the cell cycle by replacing the low-serum media with enriched BME supplemented with 15% FBS.

*Cell Growth in the Presence of Surface Bound Monoclonal Anti-GP80:* NIH/3T3 cells and C3H/10T1/2 cells were seeded at densities of 2 × 10$^4$ and 10$^4$ cells per 35-mm petri dish, respectively, and allowed to grow for 48 h. One half of the cells were labeled with anti-GP80; the other half were mocklabeled using only PBS. All cells were then rinsed three times with sterile complete BME, fed, and returned to the incubator. The number of cells per dish was then determined at various times as follows. While the cultures were sparse, the cells were counted in three random fields of view in each of three control and three experimental dishes; confluent cultures was assayed by hemacytometer counts after trypsinization. Four counts were made from each of two control and two experimental dishes.

*Preparation of Ventral Surfaces of C3H/10T1/2 Cells:* C3H/10T1/2 cells were seeded onto coverslips in 35-mm petri dishes at a density of 5 × 10$^3$ cells per dish; cultures were allowed to grow for 6 d with one change of media on the fourth day. The ventral surface preparation was initiated by rinsing the cell monolayer twice with PBS, pH 7.4, and then briefly with 10 mM Tris, pH 7.2. The cells were then incubated in 10 mM Tris, pH 7.2, for 2 min at room temperature. The buffer solution was removed and a piece of Whatman filter paper (no. 2; Whatman Chemical Separation, Inc., Clifton, NJ) was gently placed over the cells. After 10 s, the filter paper was removed very carefully with a peeling motion; the top surface of most cells came off with the filter paper. The remaining ventral surfaces were washed gently with PBS, pH 7.4, two or three times, and labeled for immunofluorescence microscopy.

*Immunofluorescence Microscopy:* Direct immunofluorescence microscopy was performed with cells grown on coverslips, washed three times with PBS, and then incubated with the appropriate fluorescence-conjugated antibody for 5–10 min at room temperature with constant mixing on a rocker plate. Cells were then rinsed three times with PBS and mounted on the stainless steel slides described in the following paper. Experiments were limited to 30 min so that data would be collected from cells that were still in good condition.

The relatively faint direct immunofluorescence stain required the use of indirect immunofluorescence technology for photography. Cell monolayers growing on coverslips were washed three times with PBS and placed in 3.7% formaldehyde in PBS for 20–30 min at room temperature. The fixed cells were rinsed briefly with PBS; the coverslips were then placed in a staining rack and the cells were washed extensively in PBS. The coverslips were returned to the petri dishes and the cells were incubated with 100 μg of the first antibody in 1 ml PBS for 5 min at room temperature with constant mixing. The coverslips were washed well with PBS three to four times in the petri dish. The cells were then incubated with 100 μg of fluorescence labeled second antibody for 5 min at room temperature with constant mixing and in the dark. The coverslips were washed extensively in PBS, rinsed in double distilled H$_2$O (ddH$_2$O), and mounted on acetone-cleaned slides with Gelvatol. The slides were allowed to harden for at least 1 h at room temperature before observation.

Cells to be stained for F-actin were permeabilized in 0.1% Triton X-100 for 5 min at room temperature after fixation and 6.6 ng NBD-phallacidin in 20 μl PBS was applied to each coverslip for 20 min at room temperature. Coverslips were then washed three times with PBS and mounted.

Fluorescence microscopy was performed with a Leitz Orthoplan microscope and a Zeiss X63/1.4 objective. Photographs of the fluorescent images were made with Kodak Tri X Pan black and white film, ASA 400, and exposure times of 30–60 s.

## RESULTS

### Immunoprecipitation of the 80,000-dalton Glycoprotein in C3H/10T1/2 and NIH/3T3 Cells

The presence in C3H/10T1/2 cells of an 80,000-dalton glycoprotein homologous to that previously characterized with NIH/3T3 cells was demonstrated by immunoprecipitation and NaDodSO$_4$-polyacrylamide gel electrophoresis of antigen labeled at the cell surface by lactoperoxidase-catalyzed iodination. The $^{125}$I-labeled polypeptides precipitated from each cell (data not shown).

---

[3] *Abbreviations used in this paper:* BME, basal medium Eagle; DTAF, dichlorotriazinylaminofluorescein; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GP80, 80,000-dalton cell surface glycoprotein; TRITC, tetramethylrhodamine isothiocyanate.

## Persistence of Antibody Binding

The duration of antibody binding to the cell surface was analyzed by photometry on single cells using the focused laser spot of the fluorescence recovery after photobleaching microscope as the source of exciting radiation (Fig. 1). Fluorescence (above background) was measurable for over 100 h. The kinetics of fluorescence loss presumably reflect both dilution of label due to cell division and gradual degradation of the antigen-antibody complex. The persistence of antibody binding was also demonstrated by visible fluorescence of the labeled monoclonal antibody on cells as shown below. To demonstrate that the antibody was, in fact, still on the surface of the cells after several days, cells that had been labeled with DTAF-anti-GP80 for various times were then labeled with a rhodamine second antibody that cross-linked the accessible first antibody and formed patches. There was little or no fluorescein fluorescence observed that was not patched and coincident with rhodamine fluorescence up to 72 h after the antibody was bound to the cell surface. After longer periods of incubation with anti-GP80, there was a significant decrease in the amount of first antibody accessible to the cross-linking second antibody as judged from the density of patches. This decrease paralleled an increase in perinuclear fluorescence observed with fluorescein optics. However, even after 120 h, there was antibody that was cross-linked by the second antibody and was therefore still on the cell surface.

## Cell Growth in the Presence of Bound Antibody

Cell growth curves for either C3H 10T1/2 or NIH 3T3 cells were not significantly altered by the presence of bound antibody (Fig. 2) in either the kinetics of growth or the density of cells at confluence. Immunofluorescence revealed, as described below, that antibody remained bound to the cells for the duration of the experiment.

## Cell Motility in the Presence of Bound Antibody

The effect of anti-GP80 on the motility of C3H/10T1/2 cells was studied with the phagokinetic assay of Albrecht-Buehler (14). Cells were plated on scored coverslips coated with gold particles at a density of $5 \times 10^3$ cells per 35-mm petri dish and allowed to grow for 48 h; the tracks of the cells were recorded photographically. After labeling with anti-GP80 the cells were allowed to grow for an additional 24–48 h, and the tracks of the cells were again recorded photographically. Cells continued to move and to divide after antibody was bound to their surfaces.

## Lateral Distribution of the 80,000-dalton Glycoprotein in Motile and Stationary Cells

Cells directly labeled with fluorescein-tagged anti-GP80 displayed a visible membrane fluorescence stain that persisted for over 96 h (Fig. 3). The initial stain was smooth yet graduated across the cell from the leading to trailing edge with the strongest fluorescence appearing near the trailing edge. This gradient grew larger when the antibody remained on the cells for 12–24 h (Fig. 3B). The marked gradation was maintained until the cells became more confluent and less motile (Fig. 3C). When wounded monolayers were labeled with fluoresceinated anti-GP80, the cells migrating into the wound displayed a pronounced gradient while the cells in the monolayer had a more even surface stain (data not shown). Finally, it should be pointed out that any antibody exchange between initially labeled and newly inserted antigen during the long incubations required would only serve to diminish the observed gradation.

Control studies showed that staining was blocked by incubating first with unlabeled monoclonal antibody. Moreover, the fluoresceinated antibody did not bind to either Nil hamster or BG-9 human fibroblasts. Neither did it bind to BALB/c 3T3 fibroblasts, which provides an excellent staining control since an allelic 80,000-dalton protein is present on these cells but lacks the antigenic determinant recognized by the anti-mouse fibroblast (AMF)-15 antibody (8, 15).

Ventral fragments were also prepared. These fragments were visualized by scanning electron microscopy as membraneous sheets in close apposition to the substrate (data not shown) as reported earlier (16). The substrate attached surface fragments could be stained with the antibody, indicating the presence of the antigen on the bottom surface as well as its accessibility to antibody (Fig. 3D).

We attempted to use TRITC-labeled anti-GP80 because it is more photo-stable than fluorescein-conjugated antibody and therefore preferable for photography and photobleaching.
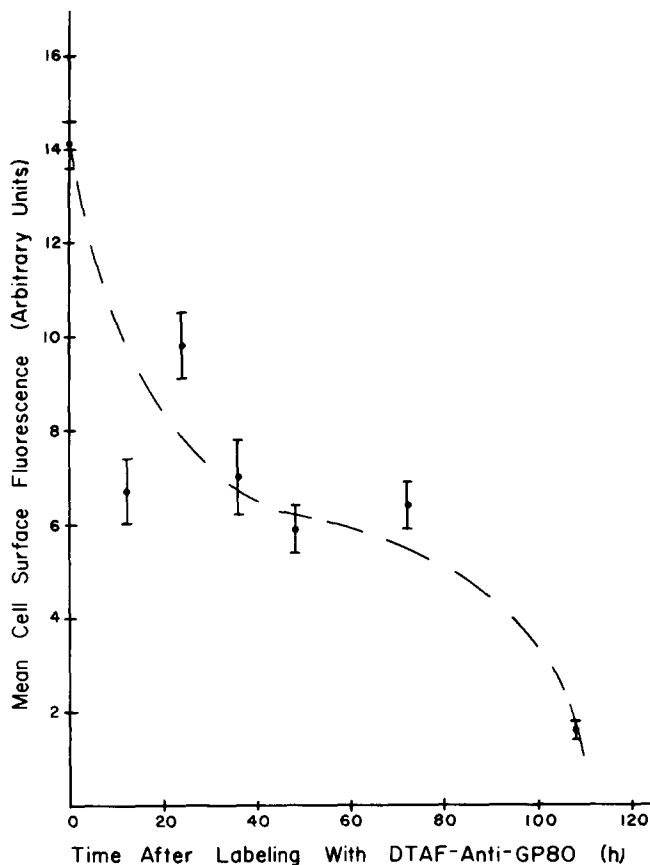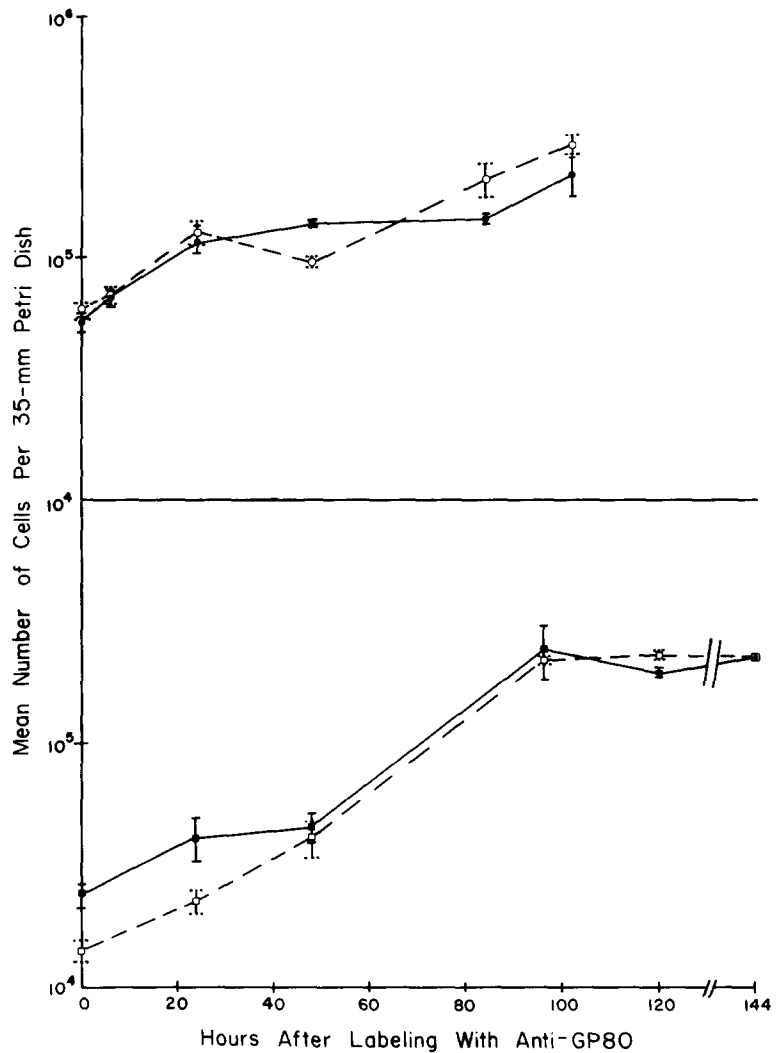


FIGURE 1 The persistence of DTAF-anti-GP80 antibody on the surface of C3H/10T1/2 cells. The mean cell surface fluorescence is the average of the trailing edge and the leading edge fluorescence as measured by spot photometry using the focused Gaussian profile laser beam of the FRAP microscope ($1/e^2$ spot diameter of ~2 $\mu$m). Laser power and amplification of the signal were constant throughout the experiment. Measurements were made in regions of the cell depicted in the diagram in Fig. 4. The error bar represents the SEM. Cells were incubated with 100 $\mu$g/ml anti-GP80 for 5 to 10 min at room temperature.

FIGURE 2 The effect of anti-GP80 on the growth of C3H/10T1/2 (bottom) and NIH/3T3 (top) cells. The number of cells per 35-mm petri dish was determined for cells labeled with 100 μg/ml anti-GP80 (●, C3H/10T1/2; ■, NIH/3T3) and control cells (○, C3H/10T1/2; □, NIH/3T3) at various times. The error bar represents the SEM.

In spite of careful preparation (Materials and Methods), the TRITC-labeled antibodies stained human BG9 fibroblasts, cells that do not express the antigen. The staining of the BG9 fibroblast was on the surface since the label was aggregated by a second anti-rat antibody. Thus, it appeared that the TRITC label caused nonspecific adsorption of the anti-GP80 antibody to the surface of cells, possibly enhanced by TRITC's positive charge being attracted to the negative cell surface.

## Photometric Measurement of the Antigen Gradient in Motile Cells

Visual observation suggested an antigen concentration non-uniformity in motile cells (Fig. 3). This gradated distribution of fluorescence in motile cells was substantiated by spot photometric measurements of fluorescence intensity in leading and trailing regions of single cells. Assuming that fluorescence intensity is linearly proportional to antigen concentration, these studies showed that the density of antigen was over twofold greater in the trailing edge as compared with the leading edge of motile cells (Fig. 4). This gradation was largely abolished in confluent or $G_0$ cells which were not actively motile; however, it was re-established in stimulated $G_0$ cells (Fig. 4). Log-phase cells that had been left at room tempera-

ture for 2 h after labeling, showed a more evenly distributed staining pattern; if the cells were returned to the incubator for several hours, the gradation was re-established (data not shown). The gradient was less pronounced in motile cells stained with FITC-succinyl-Con A and no gradation was seen with the lipid probe, dihexadecylindocarbocyanine (Fig. 4).

The effect of extended antibody presence on the magnitude of the gradient is shown in Fig. 5. Initially the fluorescence in the trailing edge was twice as intense as in the leading edge. Within the first 48 h after labeling, the fluorescence in the trailing edge increased to as much as seven times the fluorescence of the leading edge. At longer times after labeling when the cells were becoming confluent and most no longer displayed the classic fan shape of motile cells, the gradient decreased to its initial level where the "trailing edge" was only approximately twice as bright as the "leading edge."

To insure that we were measuring predominantly surface fluorescence, measurements were repeated at 12, 24, and 48 h on fixed cells labeled by indirect immunofluorescence and similar results were obtained. In addition, photobleaching studies (see following paper) indicated no change in either lateral diffusion coefficient or mobile fraction after 12-, 24-, or 48-h incubation with DTAF anti-GP80 (data not shown). Significant internalization would be expected to decrease either or both of these values.
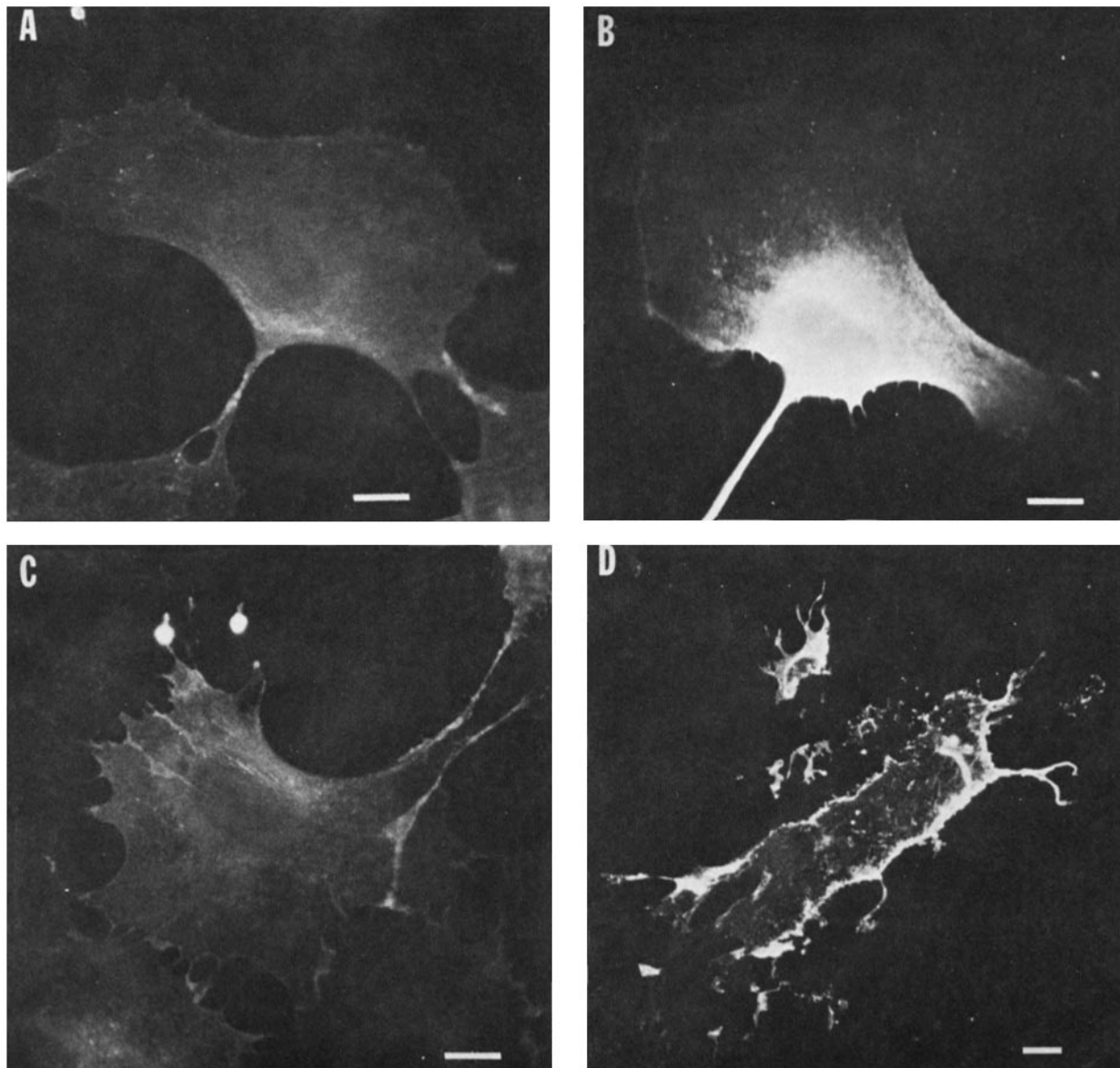
Figure 3  Time course of DTAF-anti-GP80 persistence on the surface of C3H/10T1/2 cells. Cells were labeled with DTAF-anti-GP80, placed in complete media, and incubated at 37°C for various times until fixation with 3.7% formaldehyde. Additional labeling with DTAF-goat anti-rat IgG was employed to facilitate photography of the faint direct labeling. (A) Initial stain; (B) immunofluorescence after DTAF-anti-GP80 is on cells for 12 to 24 h; (C) immunofluorescence after 96 h incubation with antibody; (D) the distribution of GP80 on the ventral surfaces of log phase cells. Bar, 10 μm.

## Redistribution of the 80,000-dalton Glycoprotein Caused by Addition of Cross-Linking Antibodies

Cross-linking of the monoclonal antibody with a purified second antibody resulted in patching of the immune complexes within minutes at 37°C or room temperature (Fig. 6A). The patches could be localized to both dorsal and ventral surfaces by optical sectioning, consistent with labeling of ventral surface fragments (Fig. 3D) and immunoelectron microscopy. The patches were frequently observed to form linear patterns on the dorsal surface (17) during the first 30 min after cross-linking (Figs. 6B, 7, A and C). These patterns were never detected on the ventral surface and rarely remained distinct beyond 1 h after cross-linking. Double labeling studies using NBD-phallacidin to visualize F-actin showed that linear pattern formation was related to the presence of fine actin

bundles in the same focal plane as the patches (Fig. 7, A–D). This pattern formation was never observed in cells apparently lacking such membrane-associated actin bundles. In addition, we have noted another relationship between GP80 patches and actin distribution: patches found on cells containing abundant stress fibers appeared significantly smaller than patches on cells lacking stress fibers. This size difference was observed among different cells (Fig. 7, A–B) and on single cells (Fig. 6, C and D). The distribution of stress fibers generally corresponded to the region of smaller patches.

Continued incubation at room temperature or 37°C resulted in coalescence of the patches into larger aggregates. In initial studies this progressive aggregation of patches was accompanied by rounding of most cells. Aggregates were usually found near the cell periphery at 2 h after cross-linking and by 4 h most cells had undergone extensive retraction and

FIGURE 4  Gradations in the surface density of various probes on the surface of C3H/10T1/2 cells. In subconfluent cultures, the fluorescence intensity was measured in random areas of the leading and trailing edges of cells with a typically motile shape as shown in the diagram. In confluent cultures, the fluorescence intensity was measured in random areas of opposite edges of cells. The spot photometry was described in Fig. 1. Cells were labeled with the stated probe for 5–10 min at room temperature. Staining concentrations were as follows: 100 μg/ml DTAF-anti-GP80; 1 μg/ml FITC-S-Con A; 5 μg/ml dil-$C_{16}$ (3) introduced in ~0.5% ethanol. Motile cells were defined as those cells in subconfluent, log phase cultures with the classic fan shape. Confluent cultures were defined as those in which close to 100% of the growing surface was covered by the cell monolayer. The protocol for arresting cells in $G_0$ is given in the text. Measurements were made on stimulated $G_0$ cells at least 24 h after stimulation.
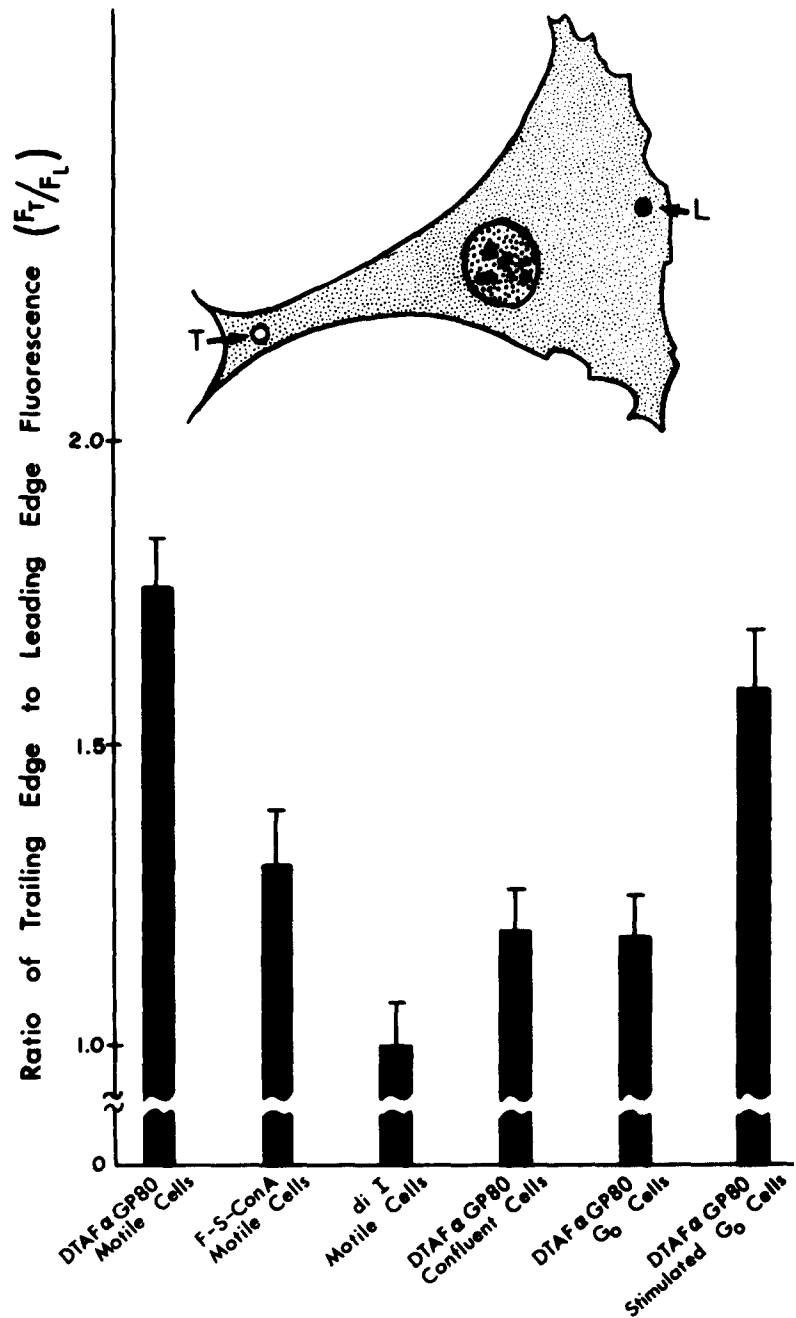
were easily detached from the substrate by PBS washes. However, in later studies the cross-linking induced rounding of cells did not occur. The former and latter results were obtained using either complement-inactivated or noninactivated antibody preparations. Redistribution of aggregates in the later studies was apparently random on cells of nonmotile morphology; aggregates were redistributed to areas opposite leading lamellae on motile cells such as those migrating into a wound in a confluent monolayer. These indirect immunofluorescence studies also demonstrated the absence of cross-linked GP80 from subsequently extended lamellipodia (Fig. 7, E and F).

Some internalization of cross-linked immune complexes was observed. Anti-GP80 bound to living cells was reacted with TRITC-rabbit anti-rat IgG and the incubation was then continued at 37°C for 1–3 h. Cells were then stained with DTAF-goat anti-rabbit IgG. Some rhodamine fluorescence

was detected without coincident fluorescein fluorescence, indicating that some cross-linked antigen was not accessible to DTAF-goat anti-rabbit IgG during labeling due to internalization. However, the amount of internalized rhodamine label was slight compared to label remaining on the surface even hours after cross-linking and was located in spherical vesicles of varying sizes in the perinuclear region of the cell (data not shown).

DISCUSSION

Probing the Lateral Distribution of the 80,000-dalton Major Cell Surface Glycoprotein Using Monoclonal Antibodies

These studies describe the lateral distribution of a major cell surface glycoprotein of murine fibroblasts. This glycoprotein is present in ~$10^6$ (8, 10) copies per cell distributed
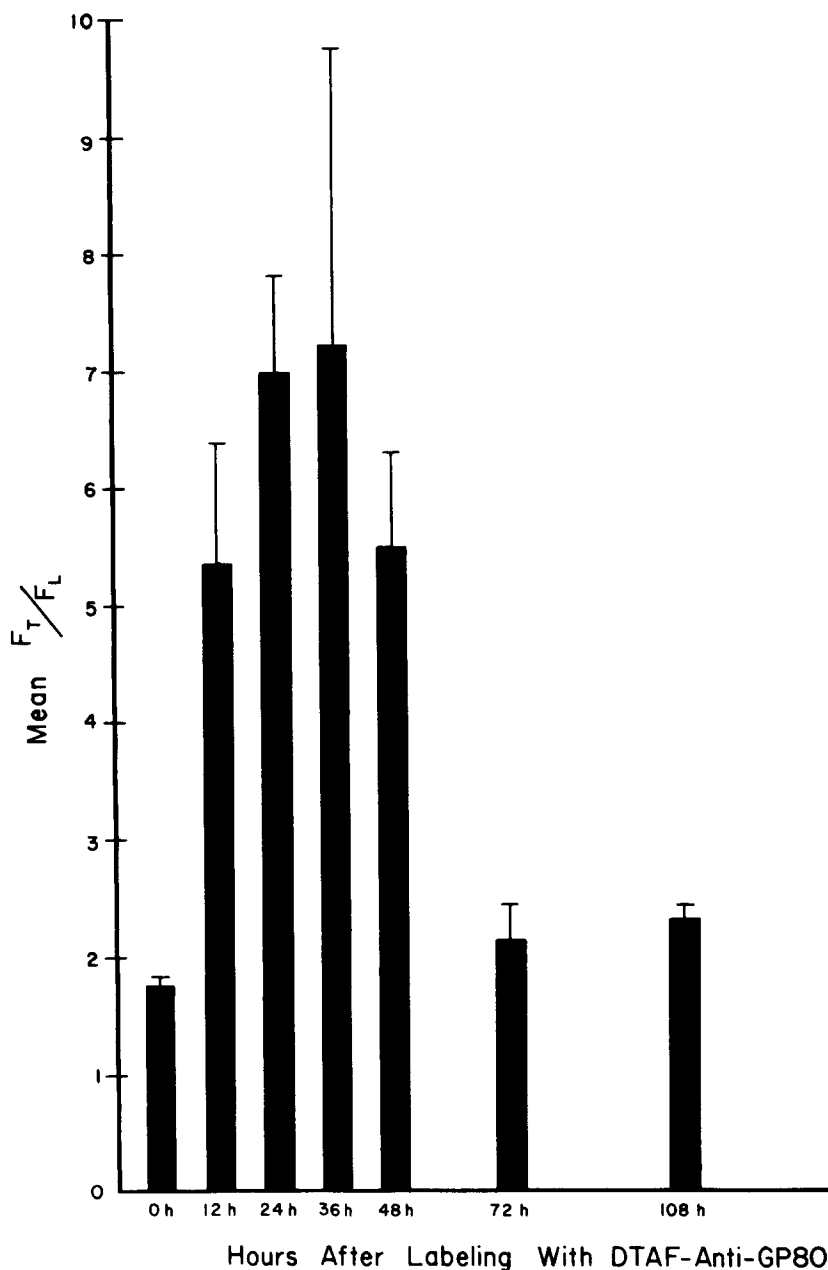
FIGURE 5 Variation of the DTAF-anti-GP80 gradient with time in culture after labeling. The fluorescence in the leading and trailing edges of C3H/10T1/2 cells was measured by spot photometry as described in the caption of Fig. 4 at various times following initial labeling with DTAF-anti-GP80. Cells were treated as stated in the text. The error bar represents the SEM.

throughout the surface of the cell as seen by immunoelectron microscopy (9) and immunofluorescence. The antigen is located on both the dorsal and ventral surface and on ventral surface fragments of the cell. The binding to cultured cells of a fluorescent monoclonal antibody against the 80,000-dalton glycoprotein persisted over long periods ($\geq100$ h), consistent with long lifetimes of many cell surface components (18, 19). Since the cells continued to divide and move in the presence of bound antibody, this monoclonal antibody provides a valuable tool to study the distribution of a well characterized surface antigen during physiological functions in living cells.

## The Relationship of Glycoprotein Lateral Distribution to Cell Motility

These observations on the lateral distribution of GP80 in motile cells may bear on the various mechanisms proposed for membrane movement in cellular motility (for recent re-

views, see references 1, 2, and 4). At least two possibilities exist to explain the gradient in labeled antigen which develops with time. The membrane flow hypothesis (20, 21) argues that slowly diffusing membrane proteins will be swept toward the tail of motile fibroblasts by a rearward lipid flow associated with membrane insertion at the leading edge of the cell and subsequent endocytosis occurring over the cell's surface. Evidence of G protein insertion into the leading edge of motile, vesicular stomatitis virus (VSV) infected normal rat kidney (NRK) cells (22), and transferrin and low density lipoprotein receptor insertion into the leading edge of giant Hela cells (23), has recently been reported. However, earlier experiments tracking the Thy-1.1 antigen on the surfaces of motile rat fibroblasts failed to reveal evidence for membrane insertion at the leading edge (24). Equations can be applied to approximate the flow situation in cells (20) and predict an essentially infinite antigen gradient in the steady state for a 50-$\mu$m long cell moving with a velocity of 1 $\mu$m/min when the mobile
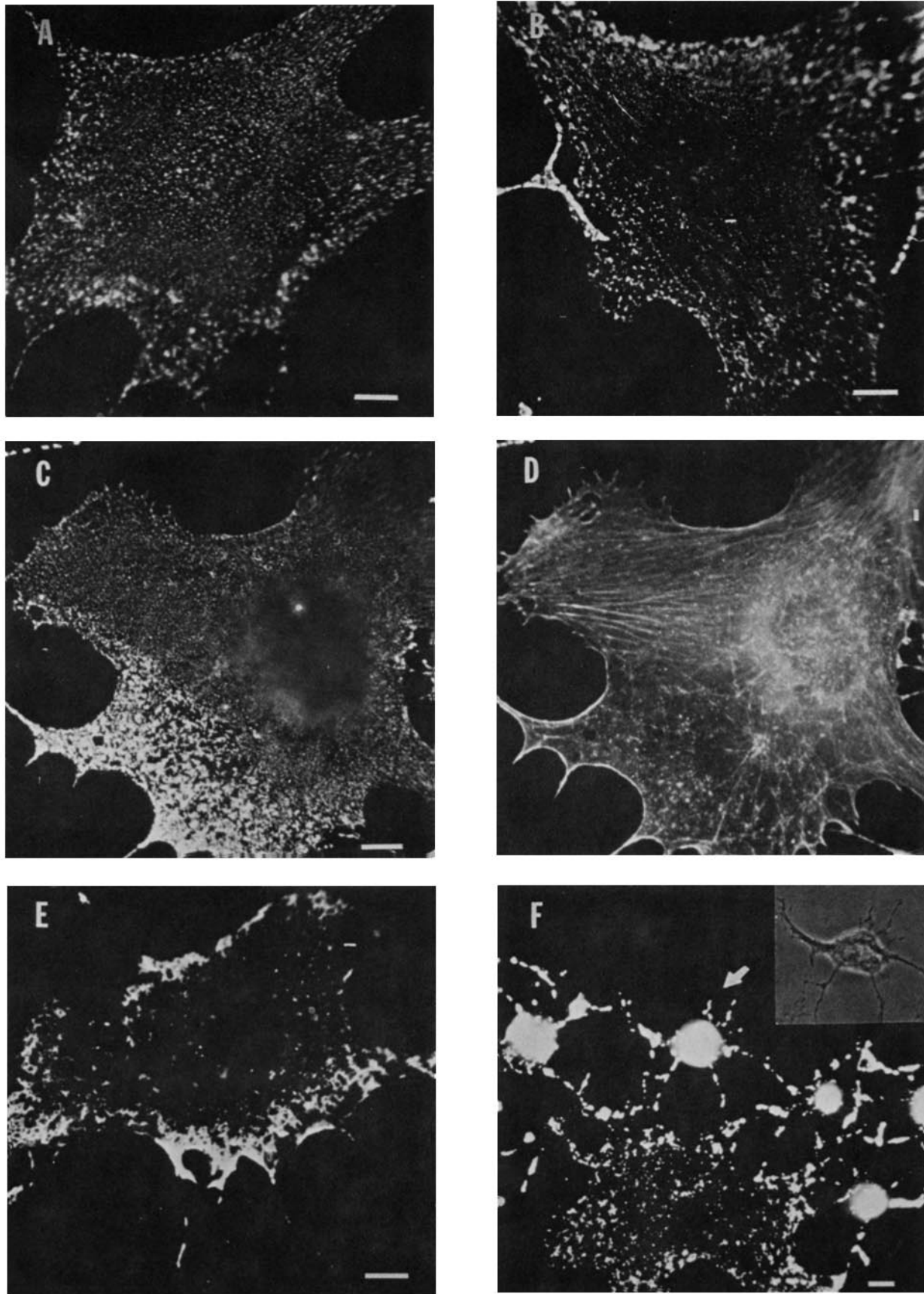
FIGURE 6  Time course of second antibody-induced redistribution of GP80 on C3H/10T1/2 fibroblasts. Cell were treated with monoclonal anti-GP80 followed by rabbit anti-rat IgG-TRITC, and were returned to 37°C for various times before fixation: (A–D) 15 min; (E) 2 h; (F) 4 h. D shows the corresponding F-actin distribution of the cell shown in C. Extensive retraction and rounding of cells resulted after longer periods of incubation (F); inset shows phase contrast of cell indicated by arrow. The time course described was that observed for the vast majority of cells. Bar, 10 μm.

membrane protein has a diffusion coefficient of $1 \times 10^{-10}$ cm$^2$/s (see following paper). This hypothesis views membrane flow as central to the propulsion of cells but its existence can only be inferred from the capping of membrane receptors and retrograde transport of particles placed on the dorsal surface of moving cells. In fact, cytoskeletal rearrangements accompany both of these membrane movements so that the cytoskeleton, and not membrane flow, could be providing the driving force for these surface movements. (5, 25).

An alternate view for locomotion would ascribe a more central role for the cytoskeleton and a more passive role to the plasma membrane. We propose that gradient formation may be related to the phenomenon termed "retraction induced spreading" in which protrusions of the leading ruffled membrane are coordinated with retraction of the trailing portion of the cell to the cell body (26). Presumably, retraction provides the reservoir of membrane, temporarily stored in folds and microvillae (26, 27), to allow new leading lamellae to form rapidly. The formation of such new lamellae may be driven by the rapid extension of cortical actin (28, 29). We hypothesize that the concomitant flow of the lipid bilayer into leading lamellae around slowly diffusion and anchored membrane proteins gives rise to leading lamellae initially deficient in these proteins. Although GP80 can diffuse laterally about 1 $\mu$m in a minute (see succeeding paper), leading lamellae extension can proceed at a rate on the order of 5 $\mu$m per minute (30) so that a concentration gradient in such slowly diffusing molecules will arise. Lipid probes flow with the bilayer that forms the leading lamellae; hence, no diffusion gradient can be observed for such probes. Thus, the cell advances in an "inchworm" fashion presumably garnering the cytoskeletal components required for rapid lamellar extension from disassembly at the rear portion of the cell. In this view, the relatively small initial gradient of labeled antigen will become progressively enhanced as lateral diffusion will not be rapid enough to counteract the sequestration of the antigen toward the rear of the cell produced by retraction induced lamellar spreading. Consider that it would take ~17 h for a membrne protein having a diffusion coefficient of $10^{-10}$ cm$^2$/s to move a net distance of 50 $\mu$m.

It is important to remember that we are examining only the fate of antigen labeled at a particular time and it is this subset of GP80 molecules that we observe being conveyed to the rear of motile cells. The smaller gradients measured after a second labeling indicate that fresh antigen has been inserted between the first and second labeling. Of course, it is presumed that this antigen, too, is moved rearward in the advancing cell.

A quantitative framework to consider such a redistribution stems from the theory of Dembo et al. (31) which predicts the progressive accumulation of cell substrate adhesion molecules toward the rear of motile cells. This gradation develops because molecules that attach ventral cell surfaces to the substrate are conveyed and concentrated at the rear of the cell by the cell's relative forward motion. The kinetics and extent of receptor redistribution depend on the size of the cell, its locomotory velocity, the lateral diffusion coefficient ($D$) of the cell surface adhesive molecules and the association and dissociation rate constants for binding to the substrate sites. Using "best guess" estimates of these parameters including a $D \sim 10^{-10}$ cm$^2$/s (see next paper), Dembo et al. (31) calculate that redistribution will be completed after the cell moves 1–10 cell lengths. We can make use of this description since it

only requires transient binding of membrane components to an immobile structure with respect to the cell (e.g., substrate); thus, binding to members of the cell's cortical cytoskeleton which remain essentially stationary during excursions of the cell limited to less than one cell length could also produce the observed redistribution. Indeed, preliminary video experiments suggest that those cytoskeletal components responsible for tethering cell surface patches do not move during limited cell movement (Holifield, B., G. McGregor, and K. Jacobson, unpublished results). If we consider a 50-$\mu$m-long fibroblast moving at 1 $\mu$m/min, complete receptor redistribution would occur roughly within 1 to 8 h. This time is consistent with our first observation of the large gradient 12 h after labeling (Fig. 5).

Thus, these two views of membrane involvement in cellular motility can both account, in a semi-quantitative fashion, for the development of the observed gradient. The two mechanisms are, however, fundamentally different with respect to the direction of membrane flows and the role of these flows and cytoskeletal dynamics in cellular locomotion. The predictions of both of these hypotheses should be testable using fluorescent-labeled membrane and cytoskeletal probes with modern ultra-low light level digitized fluorescence microscopy and photobleaching techniques.

## Cell Surface Glycoprotein Redistribution Induced by Cross-Linking Antibody: Relationships to the Cytoskeleton

The distribution of the anti-GP80 complexes changed very quickly from uniform to patched upon the addition of second antibody (anti-rat IgG). Patches of relatively uniform size, resulting in a uniform punctate pattern of fluorescence, formed over the entire cell surface within minutes after cross-linking. Secondary structures, composed of coalesced patches and referred to as aggregates, formed with increasing time. Patches became displaced from the region overlying the nucleus and the resulting aggregates were located at or near the periphery of the gradually retracting cell. Patching and capping in a variety of cells have been studied for over a decade (for recent review, see reference 2). In fibroblasts, the pattern of large aggregate or cap formation appears to depend on how motile the cells are (2). Caps, consisting of collections of cross-linked ligand-receptor complexes, have been hypothesized to form by continuous front to back lipid flow (20), membrane flow (21), "surfboarding" on cell surfaces waves (4), or by connection to a contractile cytoskeleton. The latter is exemplified by the recent proposal of a continuous flow of a dorsal cortical actin-microfilament sheath (5) which would drive cap formation.

Our studies have revealed some intriguing relationships between patch and actin distributions. Cellular processing of the patches presumably begins with the cytoskeleton which somehow perceives the array of cytoplasmic domains of the cross-linked glycoproteins as different than the norm. This is not surprising considering the size of these patches. For example, in this situation, we have cells of ~35-$\mu$m radius with $10^6$ glycoprotein molecules per cell; our micrographs indicate roughly $10^3$–$10^4$ patches on these cells, meaning that an average patch contains an array of 100 to 1,000 cross-linked antigens. Organized actin in the form of sharply defined stress fibers visible in immunofluorescence appears correlated with the formation of smaller patches which were often aligned
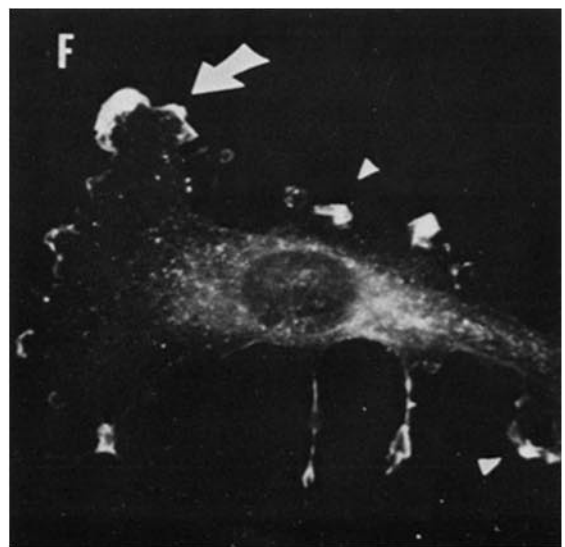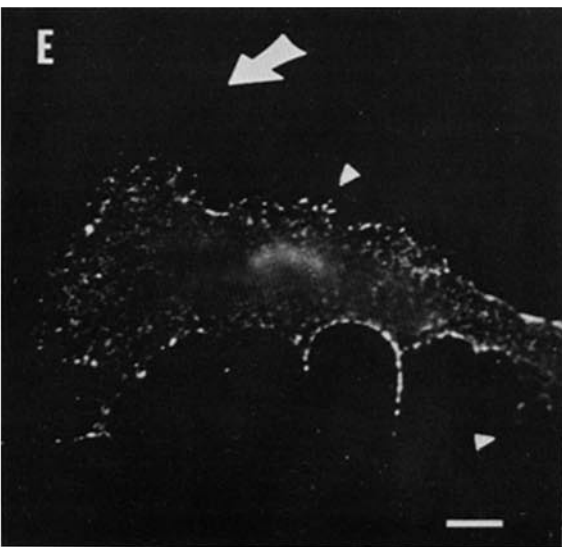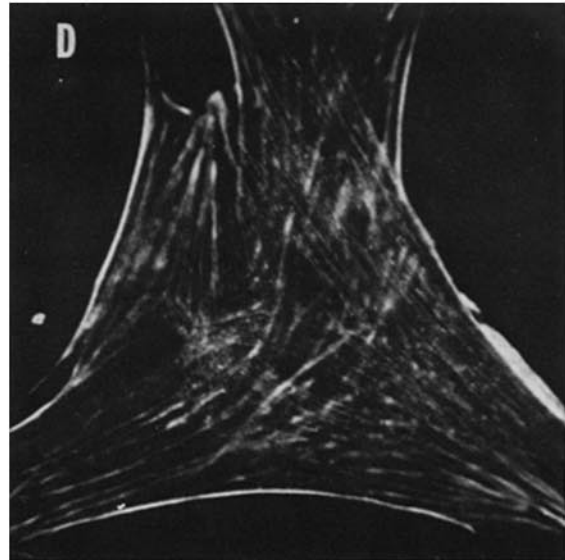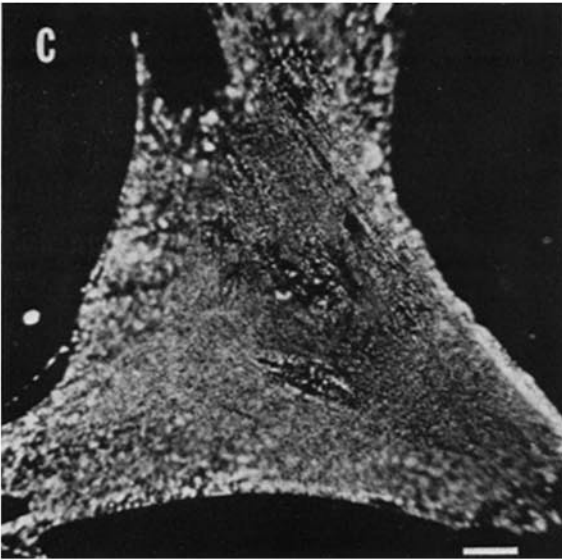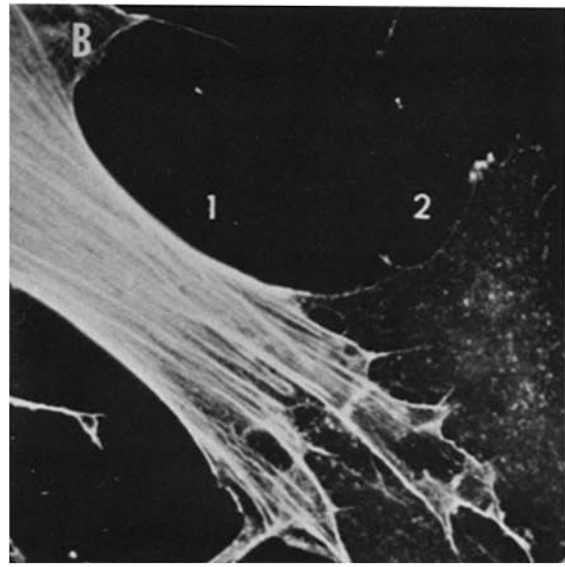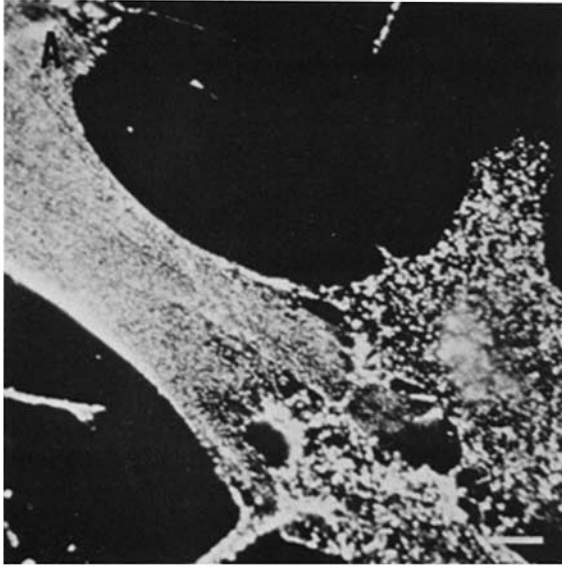
with the underlying stress fibers. Conversely, the formation of larger, randomly arrayed patches was correlated with a distinct lack of organized actin. Patches were never observed in membrane ruffles, which we attribute to their failure to diffuse into these rapidly extended regions owing to their anchorage to actin or to their sheer size. Similarly, some leading lamellae, were also devoid of patches. It seems unlikely that patches were actively cleared back from the leading lamellae prior to our observation since no orderly patch accumulation toward the cell body (5) was observed.

Larger patches developed where F-actin was not organized into stress fibers. Thus, stress fibers may serve not only to align the patches but also to restrict patch growth. Patch size reduction may be related to the fact that stress fibers render the lateral diffusion of cross-linked molecules anisotropic, with the fastest diffusion occurring in a direction parallel to the fibers (32). Thus, recruitment of receptors to the patch nucleus may be effectively restricted to the "channels" between the stress fibers. In addition, the large domains for interaction between the patch (see above) and the actin (in the form of stress fibers) may provide enough binding energy to allow a long term alignment of the patches with the fibers.

REFERENCES

1. Oliver, J. M., and R. D. Berlin. 1982. Mechanisms that regulate the structural and functional architecture of cell surfaces. Int. Rev. Cytol. 74:55–94.
2. Weatherbee, J. 1981. Membranes and cell movement: interactions of membranes with the proteins of the cytoskeleton. Int. Rev. Cytol. (Suppl.) 12:113–176.
3. Schreiner, G. F., J. Braun, and E. R. Unanue. 1976. Spontaneous redistribution of surface immunoglobulin in the motile B lymphocyte. J. Exp. Med. 144:1683–1688.
4. Oliver, J., and R. Berlin. 1982. Distribution of receptors and functions on cell surfaces: quantitation of ligand-receptor mobility and a new model for the control of plasma membrane topography. Philos. Trans. R. Soc. Lond. B Biol. Sci. 299:215–235.
5. Heath, J. 1983. Direct evidence for microfilament mediated capping of surface receptors on crawling fibroblasts. Nature (Lond.). 302:532–534.
6. Ryan, G. B., J. Z. Borysenko, and M. J. Karnovsky. 1974. Factors affecting the redistribution of surface-bound conconavalin A on human polymorphonuclear leukocytes. J. Cell Biol. 62:351–365.
7. Hughes, E. N., and J. T. August. 1981. Characterization of plasma membrane proteins identified by monoclonal antibodies. J. Biol. Chem. 256:664–671.
8. Hughes, E. N., G. Mengod, and J. T. August. 1981. Murine cell surface glycoproteins. Characterization of a major component of 80,000 daltons as a polymorphic differentiation antigen of mesenchymal cells. J. Biol. Chem. 256:7023–7027.
9. Murphy, T. L., G. Decker, and J. T. August. 1983. Glycoproteins of coated pits, cell junctions, and the entire cell surface revealed by monoclonal antibodies and immunoelectronmicroscopy. J. Cell. Biol. 97:533–541.
10. Hughes, E. N., A. Colombatti, and J. T. August. 1983. Murine cell surface glycoproteins. Purification of the polymorphic Pgp-1 antigen and analysis of its expression on macrophages and other myeloid cells. J. Biol. Chem. 258:1014–1021.
11. Colombatti, A., E. N. Hughes, B. A. Taylor, and J. T. August. 1982. Gene for a major cell surface glycoprotein of mouse macrophages and other phagocytic cells is on chromosome 2. Proc. Natl. Acad. Sci. USA. 79:1926–1929.
12. Trowbridge, I. S., R. T. Nilsen-Hamilton, and M. J. Bevan. 1977. Preliminary characterization of two thymus-dependent xenoantigens from mouse lymphocytes. Biochem. J. 163:211–217.
13. Omary, M. B., I. S. Trowbridge, and M. P. Scheid. 1980. T200 cell surface glycoprotein of the mouse. Polymorphism defined by the Ly-5 system of alloantigens. J. Exp. Med. 151:1311–1316.
14. Albrecht-Buehler, G. 1977. The phagokinetic tracks of 3T3 cells. Cell. 11:395–404.
15. Mengod, G., E. N. Hughes, and J. T. August. 1981. Murine cell surface glycoproteins: immunochemical analysis of a major differentiation alloantigen of phagocytic cells. Arch. Biochem. Biophys. 209:718–722.
16. Geiger, B., Z. Avnur, and J. Schlessinger. 1982. Restricted mobility of membrane constituents in cell-substrate focal contacts of chicken fibroblast. J. Cell Biol. 93:495–500.
17. Singer, S. J., J. F. Ash, L. Y. W. Bourguiguon, M. H. Heggeness, and D. Louvard. 1978. Transmembrane interactions and the mechanisms of transport of proteins across membranes. J. Supramol. Struct. 9:373–389.
18. Doyle, D., J. Cook, and H. Baumann. 1982. Regulation of membrane proteins in hepatocytes. In The Liver: Biology and Pathobiology. I. Arias, H. Popper, D. Schacter, and D. A. Shafrite. Raven Press, New York. pp. 185–204.
19. Baumann, H., and D. Doyle. 1980. Metabolic fate of cell surface glycoproteins during immunoglobulin-induced internalization. Cell. 21:897–907.
20. Bretscher, M. 1984. Endocytosis: relation to capping and cell locomotion. Science (Wash. DC). 224:681–686.
21. Harris, A. K. 1976. Recycling of dissolved plasma membrane components as an explanation of the capping phenomenon. Nature (Lond.). 263:781–783.
22. Bergmann, J. E., A. Kupfer, and S. J. Singer. 1983. Membrane insertion at the leading edge of motile fibroblasts. Proc. Natl. Acad. Sci. USA. 80:1367–1371.
23. Bretscher, M. 1983. Distribution of receptors for transferrin and low density lipoprotein on the surface of giant HeLa cells. Proc. Natl. Acad. Sci. USA. 80:454–458.
24. Middleton, C. A. 1979. Cell-surface labeling reveals no evidence for membrane assembly and disassembly during fibroblast locomotion. Nature (Lond.). 282:203–205.
25. Heath, J. P. 1983. Behavior and structure of the leading lamella in moving fibroblasts. J. Cell Sci. 60:331–354.
26. Chen, W.-T. 1981. Surface changes during retraction-induced spreading of fibroblasts. J. Cell Sci. 49:1–13.
27. Chen, W.-T. 1979. Induction of spreading during fibroblast movement. J. Cell Biol. 81:684–691.
28. Small, J. V., G. Rinnerthaler, and H. Hinsen. 1981. Organization of Actin Meshworks in Cultured Cells: The Leading Edge. In Cold Spring Harbor Symposia on Quantitative Biology. 46:599–611.
29. Stossel, T. 1982. The structure of the cortical cytoplasm. Philos. Trans. R. Soc. Lond. B. Biol. Soc. 299:275–289.
30. Abercrombie, M., J. E. M. Heaysman, and S. M. Pegrum. 1970. The locomotion of fibroblasts in culture. I. Movements of the leading edge. Exp. Cell Res. 59:393–398.
31. Dembo, M., L. Tuckerman, and W. Goad. 1981. Motion of polymorphonuclear leukocytes: theory of receptor redistribution and the frictional force on a moving cell. Cell Motility. 1:205–235.
32. Smith, B. A., W. R. Clark, and H. M. McConnell. 1979. Anisotropic molecular motion on cell surfaces. Proc. Natl. Acad. Sci. USA. 76:5641–5644.

FIGURE 7 Relationship of underlying F-actin to surface GP80 patches. Cells at 15-min time points were treated as described in Fig. 3. GP80 was visualized by TRITC indirect immunofluorescence (A, C, E); F-actin, in the same cells, with NBD-phallacidin (B, D, F). A and B contrast the effects of underlying actin on size and arrangement of GP80 patches. Smaller patches ordered into linear patterns were found on cells that displayed F-actin organized predominantly into bundles, shown by cell 1. Larger, apparently unordered, patches were found on cells lacking obvious actin bundles, such as cell 2; the punctate actin pattern shown here was not atypical since it was commonly found in control cells stained for actin alone. C and D are additional examples of this extensive ordering process. Typical unordered patches are shown in E. The corresponding actin distribution, (F) shows a large ruffling lamella (arrow) and several smaller ruffles (arrowheads) with characteristic actin densities. GP80 patches are absent from the ruffles and a large area of the leading lamella. Bar, 10 μm.