Comparative Behavior of Membrane Protein–Antibody Complexes on Motile Fibroblasts: Implications for a Mechanism of Capping

Bruce F. Holifield,* Akira Ishihara,* and Ken Jacobson*‡

*Department of Cell Biology and Anatomy, and ‡Lineberger Cancer Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Abstract. A characteristic feature of fibroblast locomotory activity is the rearward transport across the leading lamella of various materials used to mark the cell surface. The two processes most frequently invoked as explanations for this transport phenomenon, called capping, are (*a*) retrograde membrane flow arising from directed membrane insertion and (*b*) rearward cortical cytoskeletal flow arising from cytoskeletal assembly and contraction. The retrograde lipid flow hypothesis, the most current form of the membrane flow scheme, makes explicit predictions about the movement of membrane proteins subjected to the postulated rearward lipid flow. Several of these predictions were tested by comparing the behavior of four membrane proteins, Pgp-1, Thy-1, H-2, and influenza HA_0, identified by fluorescent antibodies. With the exception of Pgp-1, these proteins were uniformly distributed under nonaggregated conditions but were capped when aggregated into patches. In contrast, Pgp-1 was capped in similar time frames in both nonaggregated and aggregated states where the lateral diffusion coefficients were very different. Furthermore, the capping behavior of two tagged membrane proteins was markedly different yet both had similar diffusion coefficients. The results from these tests disprove the bulk membrane flow hypothesis and are at odds with explicit predictions of the retrograde lipid flow hypothesis for the mechanism of capping. This work, therefore, supports the alternative cytoskeletal-based mechanism for driving capping. Requirements for coupling cytoskeletal movement to membrane components are discussed.

It is known from a large body of literature that a variety of plasma membrane proteins, when appropriately aggregated to form patches, are transported rearward away from protrusive margins of motile fibroblasts. This transport process is commonly described as capping or membrane flow and has prompted a variety of explanations of how such transport is driven. Existing schemes for a mechanism of capping fit into two general categories. One scheme includes variations of a source/sink mechanism, in which a flow is generated due to physical separation of a sink for internalizing membrane and a source, at the leading cell margin, for returning recycled and newly synthesized components to the plasma membrane (Abercrombie et al., 1970; Bretscher, 1976; Harris, 1976). The other general scheme for driving the rearward transport of patches involves coupling them to a retrograde cortical cytoskeletal flow (Harris, 1976; Abercrombie, 1980; Dunn, 1980; Heath, 1983*a*, *b*). Insertion of membrane components at the leading cell margin and cortical flow of cytoskeletal elements are not mutually exclusive processes. While there is compelling evidence that both occur, the question of their roles in the capping of plasma membrane components remains a topic of debate (for example, see Bretscher, 1984, 1988; Ishihara et al., 1988).

This study examined the behavior of several tagged membrane proteins to test predictions of the source/sink mechanisms. The results from these tests disprove the bulk membrane flow hypothesis and are at odds with explicit predictions of the retrograde lipid flow hypothesis for the mechanism of capping. The proteins examined included Pgp-1, Thy-1, H-2, and influenza hemaglutinin (HA).[1] Pgp-1 is a major murine plasma membrane glycoprotein (Hughes et al., 1981; Trowbridge et al., 1982); it has been cloned and sequenced and has a 266-residue ectodomain, a single-pass transmembrane domain, and a 72-residue cytoplasmic domain (Wolffe et al., 1990). The human homologue is the CD44 cell surface antigen, a collagen binding receptor which shows cell–cell recognition properties (for review, see Stoolman, 1989). Thy-1 is a glycoprotein (molecular mass ~25 kD), which is integrated into the plasma membrane by a phosphatidylinositol-glycan moiety (Low and Kincade, 1985; Tse et al., 1985). H-2 is a molecule of the murine class

Dr. Holifield's present address is Department of Cell Biology, Baylor College of Medicine, Houston, TX 77031.

1. *Abbreviation used in this paper*: HA, hemaglutinin.

I major histocompatability complex and is a transmembrane protein of 45 kD (Kimball and Coligan, 1983). HA, the influenza virus hemagglutin, is a transmembrane protein and was artificially expressed in mouse cells (Sambrook et al., 1985).

Materials and Methods

Cells

C3H 10T1/2 and Balbc/3T3 mouse cell lines were obtained from American Type Culture Collection (Rockville, MD). A clonal cell line (GP_4F) derived from NIH 3T3 cells and transfected to express Japan strain influenza hemagglutinin (HA_0) was obtained from J. White (University of California San Francisco). C3H 10T1/2 cells were grown in Eagle's basal medium with Earle's salts (BME) containing 5% FBS and 10 mM Hepes. Balbc/3T3 and GP_4F cells were grown in DME containing 10% FBS. Cells were cultured at 37°C in 5% CO_2. For experiments, trypsinized cells were seeded on 22-mm glass coverslips and were used 24–48 h after plating.

Antibodies

The three fibroblastic cell lines used in this study were selected for their expression of specific cell surface antigens for which we had antibodies available. C3H 10T1/2 cells expressed Pgp-1 and Thy-1, Balbc/3T3 cells expressed H-2 and GP_4F cells expressed HA_0, the uncleaved, nonfusogenic, form of the fusogenic hemagglutinin HA (see White and Littman, 1989).

Pgp-1 (previously called GP80), is expressed as two allotypes among various mouse strains, namely pgp-1.1 and pgp-1.2 (Hughes et al., 1981; Trowbridge et al., 1982). We used the 5D2.27 monoclonal rat IgG_{2a} developed by Hughes et al. (1981) and referred to in the text as anti-Pgp-1. This antibody binds to Pgp-1.2, but not Pgp-1.1, indicating that it recognizes a polymorphic antigenic site. A 5D2.27 hybridoma cell line was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Ames, IA). The hybridoma was grown at 37°C in DMEM containing 20% FBS, 10% NCTC 135 (Gibco Laboratories, Grand Island, NY), 10 mM Hepes, 0.2 U/ml bovine insulin, 0.05 mg/ml sodium pyruvate, and 0.15 mg/ml oxaloacetic acid. The hybridoma supernatant was collected and concentrated ~10-fold by ultrafiltration. Immunoglobulin was precipitated by ammonium sulfate (40–50%) and collected by centrifugation. After dissolving in PBS at pH 7.4, the immunoglobulin was further purified by immunoaffinity column chromatography, using purified goat antibody against rat IgG Fc region (Jackson ImmunoResearch Laboratories, West Grove, PA) coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA). Immunoglobulin bound to the column was eluted with 0.1 M sodium citrate at pH 3.0 and dialyzed against PBS. Purity of eluted IgG was confirmed by SDS-PAGE. A portion of this anti-Pgp-1 preparation was used to prepare f(ab) fragments (see below). Purified anti-Pgp-1 was conjugated with lissamine-rhodamine B sulfonyl chloride as previously described (Scullion et al., 1987).

In addition to the 5D2.27 mAb, we used another monoclonal which recognizes a different epitope on Pgp-1 for one set of experiments. I42/5.1, a generous gift from I. S. Trowbridge (Salk Institute, San Diego, CA), is a rat IgG that binds to both Pgp-1.1 and Pgp-1.2, indicating that it recognizes a nonpolymorphic antigenic site.

Anti-Pgp-1 F(ab) fragments were prepared by digestion of purified anti-Pgp-1 using immobilized papain-4b (Pierce Chemical Co., Rockford, IL) at 37°C for 2 h in the presence of 10 mM L-cystine and 2 mM EDTA in PBS, pH 7.4. The digestion was stopped by addition of 20 mM iodoacetamide and the resulting solution passed over an anti-rat Fc immunoaffinity column described above. The column eluate contained F(ab) fragments and was free of intact IgG as determined by Coomassie blue and silver staining of SDS-PAGE gels.

Anti-Thy-1, a rat monoclonal IgG (T724/40), was also obtained from I. S. Trowbridge. Anti-H-2 (K^dD^d), a mouse monoclonal IgG, was kindly provided by J. A. Frelinger (University of North Carolina at Chapel Hill). Anti-HA was generously provided by J. White as a rabbit polyclonal antiserum raised against whole Japan strain influenza virus. An IgG ammonium sulfate fraction of anti-HA antiserum was prepared and a portion was conjugated with lissamine rhodamine B sulfonyl chloride as described. Rabbit anti-fluorescein antiserum was obtained from Lynn Cassemeris (University of North Carolina at Chapel Hill). Secondary antibodies were purchased from Organon Teknika-Cappel (West Chester, PA) and Jackson ImmunoResearch Laboratories.

Table I. Effect of Varying Exposure to Primary Antibody on Antigen Redistribution

Protein	Primary antibody	Cell type	[Ab]	Time of Ab incubation	Time of 37°C incubation	Patching	Capping
			μg/ml	*min*	*min*		
Pgp-1	Rat IgG mAb (5D2.27)	10T1/2	0.5	1	60	No	Yes
			100	60	60	No	Yes
			1,200	10	60	No	Yes
		3T3	200	1	45	No	Yes
			400	1	45	No	Yes
	(Airfuged)		100	1	60	No	Yes
	F(ab) (5D2.27)	10T1/2	50	60	60	No	No*
	Rat IgG mAb (I42/5.1)	10T1/2	100	5	60	No	Yes
HA	Rabbit IgG pAb (polyclonal)	3T3	20	1	45	No	No
			20	45	45	Yes	Yes
			50	1	45	Partial	Partial
			500	1	45	Yes	Yes
H-2	Mouse IgG mAb	Balb/c	80	1	60	No	No
			800	10	60	No	No
Thy-1	Rat IgG mAb	10T1/2	50	1	60	No	No
			500	10	60	No	No

* See text for the interpretation of this conclusion.

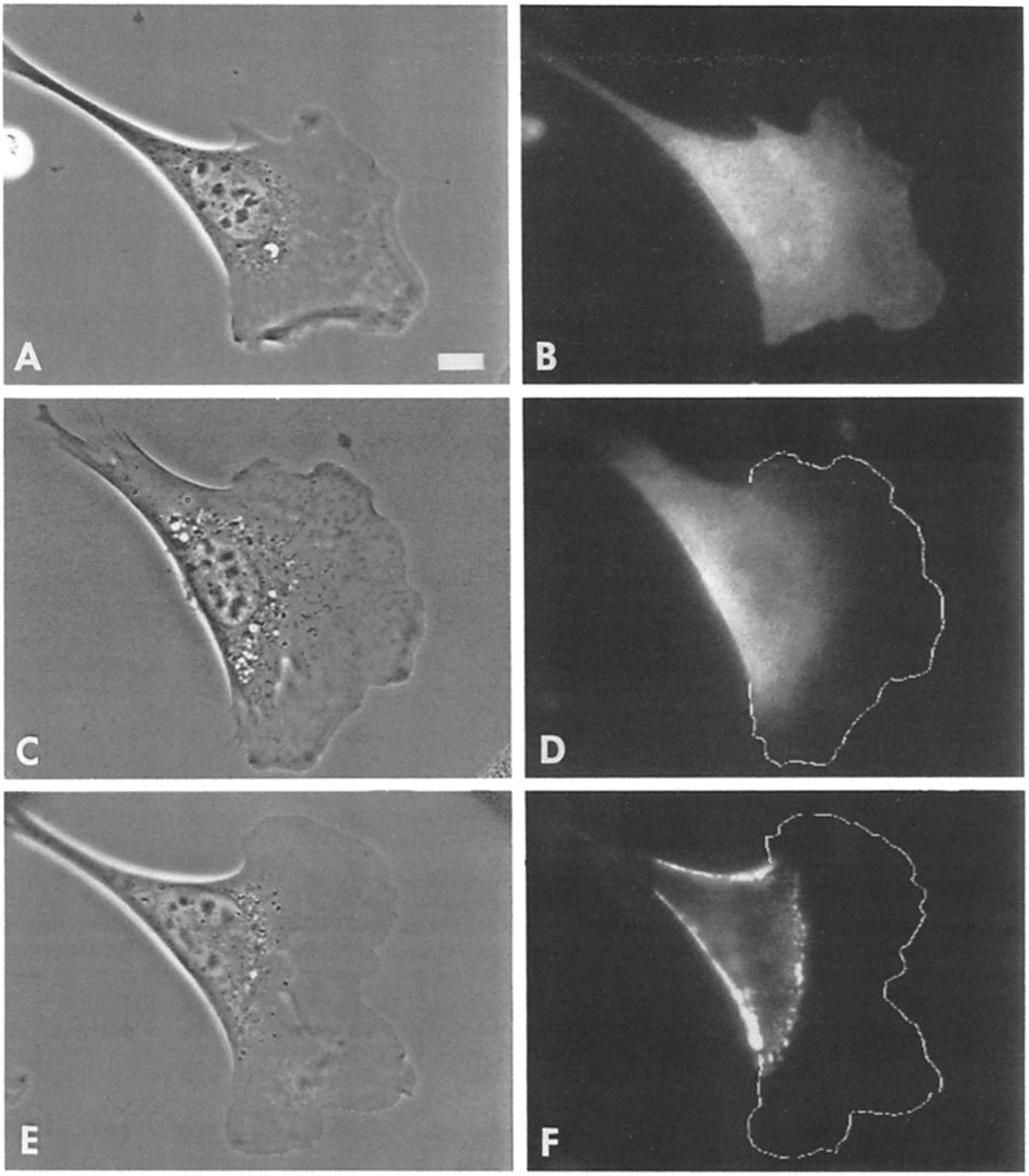

Figure 1. Induced redistribution of Pgp-1 antibody complexes. Shown are paired digitized phase-contrast and fluorescence images of C3H 10T1/2 fibroblasts on which Pgp-1 was tagged with antibodies under differing protocols. On a cell fixed before applying rhodamine-conjugated mAb, Pgp-1 is present throughout the entire surface of the leading lamella (*a* and *b*). On a living cell, Pgp-1–mAb complexes are cleared from the leading lamellar region during a 30-min incubation after applying the mAb, forming a cell surface gradient (*c* and *d*). This clearance of IgG mAb anti-Pgp-1 complexes occurs over a surface region and time period similar to the capping of Pgp-1–mAb aggregated with anti-IgG secondary antibodies (*e* and *f*), a treatment that induces formation of discrete aggregates of antigen–antibody immune complexes. White outlines in *d* and *f* indicate margins of the cell as observed in the companion phase contrast micrographs. Bar, 10 μm.

Immunofluorescence Labeling of Cell Surface Antigens

Stock solutions of antibodies at 0.5–1.2 mg/ml were diluted into growth medium to the desired concentrations (cited in text); in most cases this was >1:10 dilution. The resulting staining solutions were warmed to 37°C before being applied to cells. This step minimized perturbation of cell morphology. Coverslips were removed from 35-mm culture dishes and 20–30 μl of the primary antibody staining solution was applied to the cells. Coverslips were placed in a humidified chamber for incubation at room temperature for 1–5 min as listed in Table I and then were rinsed twice with 200 μl warmed growth medium. Labeled cells were then returned to warmed fresh growth medium and incubated for the appropriate time period (see Table I). Cells were then fixed with 3.6% formaldehyde in PBS without Ca^{2+} and Mg^{2+} for 30 minutes at room temperature. Appropriate fluorescent secondary antibodies were applied for 5 min at room temperature and cells were rinsed with PBS and mounted on slides using Gelvatol (Monsato Corp., St. Louis, MO). Where appropriate, secondary antibodies were applied before incubation at 37°C and fixation. For observation of living cells labeled with rhodamine-conjugated anti-Pgp-1, cells were either mounted into aluminum

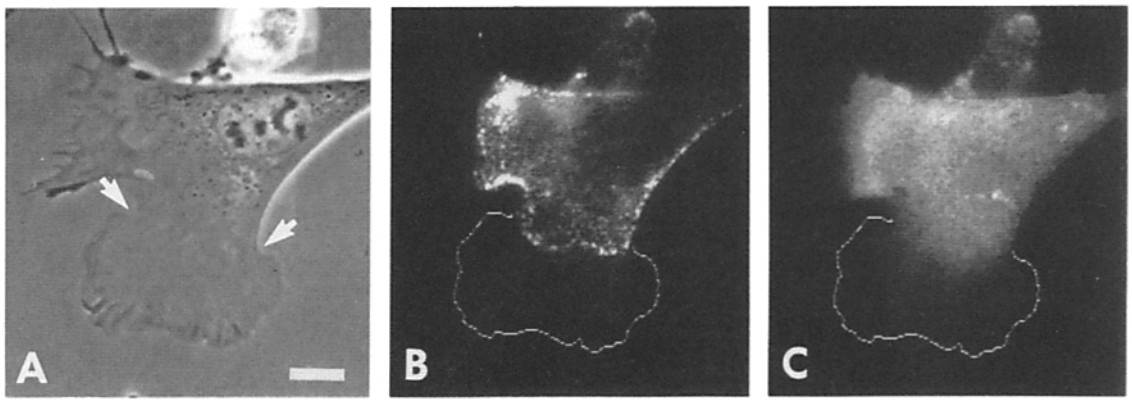

Figure 2. Gradient formation and capping of patched Pgp-1 on the same cell. Cells were labeled with a mixture of rhodamine-conjugated and fluorescein-conjugated anti-Pgp-1. Anti-fluorescein antibody was then applied to aggregate only the fluorescein-conjugated mAb, leaving the rhodamine conjugate diffuse. Shown is a living cell in phase contrast (*a*) 30 min after applying antibodies. Patches of fluorescein-tagged immune complexes (*b*) accumulated at the neck of the leading lamella (*arrows* in *a*). Nonpatched rhodamine-tagged Pgp-1–mAb complexes (*c*) were cleared back to the same site on the cell surface. White outlines in *b* and *c* delineate edge of leading lamella from phase contrast. Bar, 10 μm.

observation chambers after applying the primary Ab, or the entire labeling procedure was performed with the coverslip already mounted in the observation chamber situated on the stage of the inverted microscope.

Cells to be stained for F-actin were permeabilized in 0.1% Triton X-100 for 5 min at room temperature after fixation and a 1:50 dilution of the stock solution of fluorescein phalloidin (Molecular Probes, Junction City, OR) was applied to each coverslip for 30 min at room temperature. Coverslips were then washed three times with PBS and mounted.

Microscopy

The basic video microscopy system was described previously (DiGuiseppi et al., 1985; Ishihara et al., 1988). For the present study we added a beam splitter at the video port of the Nikon Diaphot inverted microscope so the specimen image could be sent to either a video camera (model 66 ISIT; Dage Corp., Michigan City, IN) (for fluorescence) or a chalnicon video camera (model 77; Dage) (for phase-contrast). Images of illuminated featureless scenes were stored for background subtraction from fluorescence images and mottle removal from phase-contrast images. Spot photobleaching (FRAP) measurements were made as previously described (Scullion et al., 1987).

Results

Monoclonal IgG Induces Capping of Pgp-1

Patches of Pgp-1 produced by antibody-induced cross-linking accumulate at a specific site on the dorsal cell surface as they are cleared from the leading lamella (Holifield and Jacobson, 1991). This site, usually just anterior to the nucleus, corresponds morphologically to the demarcation between endoplasmic and lamelloplasmic regions of a substratum-spread cell. When Pgp-1 is tagged with a fluorescent monoclonal IgG, the Pgp-1-mAb complex undergoes a similar redistribution (Jacobson et al., 1984*b*; Ishihara et al., 1988). Fig. 1 illustrates similarities between redistributions of Pgp-1-mAb and Pgp-1-mAb-2nd Ab complexes. When fluorescently labeled monoclonal IgG is used to label Pgp-1 on prefixed cells, fluorescence is found throughout the cell surface (Fig. 1 *b* represents the 5–10% gradient occasionally present on some cells fixed before being labeled with the mAb; however, its presence did not correlate in any obvious way with motility of the cells, unlike the mAb-induced gradient seen on living cells [see Fig. 3].) Fig. 1, *c* and *d* show a live cell 45 min after applying the fluorescent monoclonal anti-Pgp-1 alone. From the leading cell margin to points ~30 μm behind the leading edge, fluorescence intensity is not above background level, but then gradually increases at points closer to the nucleus, forming a gradient. This gradient does not represent internalized Ag–Ab complex since it can be traced by indirect immunofluorescence (Jacobson et al., 1984*b*). Note that the profile of the anterior cell margin is not detectable in the fluorescence image, as it is in prefixed cells. Fig. 1 *e* and *f* show the distribution of patches 45 min after applying mAb plus the second antibody to a cell of similar morphology. Note the accumulation of patches just anterior to the nucleus. Patches do not continue rearward movement after accumulation at such a site (Holifield and Jacobson, 1991). Upon comparing the distributions of Pgp-1 tagged with mAb alone (Fig. 1 *d*) and Pgp-1 tagged with mAb plus secondary Ab (Fig. 1 *f*), it is apparent that the induced redistributions of both types of immune complexes are spatially similar processes.

The rate of gradient formation was compared to the rate of patch clearance. By scanning different cell populations it was apparent that these rates were similar, with clearance of patches and Pgp-1-mAb complexes both occurring within 30 min after applying appropriate antibodies. However, variation in the extent of clearing of Pgp-1-mAb complex among different cells (see below) made a more objective comparison difficult. A direct comparison of clearance rates was made in the following way. Cells were first labeled with a solution containing both rhodamine- and fluorescein-conjugated monoclonal anti-Pgp-1. These cells then received rabbit anti-fluorescein IgG followed by fluorescein sheep anti-rabbit IgG. This second set of antibodies selectively patched the fluorescein-conjugated monoclonal anti-Pgp-1 on the cells, whereas the rat rhodamine mAb remained diffuse. Cells treated in this way were replaced in the incubator at 37°C and were fixed or observed live at various time intervals up to 60 min. Fig. 2 shows a live cell 30 min after applying antibodies, at which time the majority of cells have completed clearance of patches from their lamellar surfaces. Note that these different types of immune complexes redistributed at

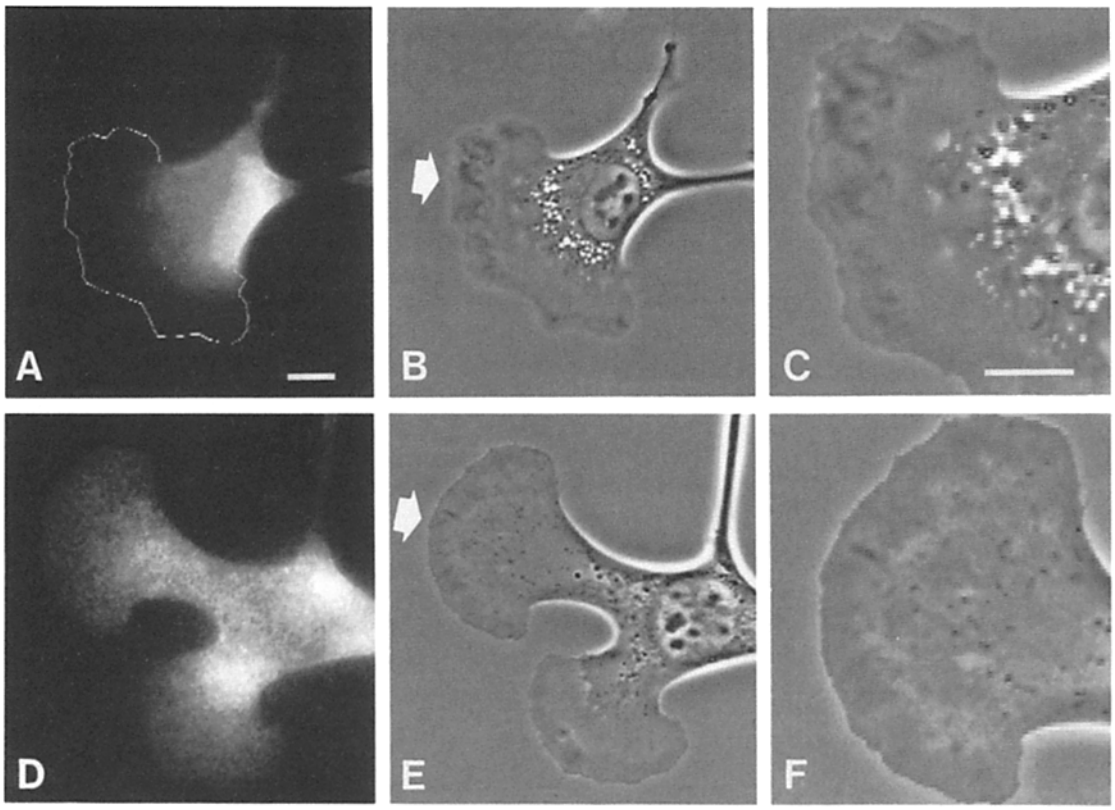

Figure 3. Clearance of Pgp-1–mAb complexes varies with occurrence of a phase-dense submarginal zone at the leading edge. Typical cells are shown that differ in completeness of clearance of Pgp-1–mAb complexes. These cells were located on the same coverslip and were imaged within minutes of one another. Clearance of Pgp-1–mAb complexes is more complete (compare *a* with *d*) on the cell showing a prominent phase-dense submarginal zone (Compare the phase dense submarginal zones immediately to the right of the large arrows in *b* and *e*.) The indicated submarginal regions are shown at higher magnification in *c* and *f*; such images acquired at 20-s intervals and displayed in time-lapse showed a prominent rearward flux of phase-dense structure associated with the cell in *a–c*, but not with the cell in *d–f* (data not shown). White outline in *a* delineates edge of leading lamella from phase contrast *b*. Bar, 10 μm.

similar rates. Note that the surface distribution of patched (Fig. 2 *b*) and nonpatched (Fig. 2 *c*) immune complexes are coincident. Both types of immune complexes were cleared from the lamellar region back to the same site (*arrows*, Fig. 2 *a*) during the 30-min incubation period. Cells fixed at shorter time intervals showed similar results, indicating that both complexes are cleared at approximately the same rate.

In the examples shown above, clearance of fluorescent Pgp-1-mAb complexes from the lamellar region was nearly quantitative, based on the absence of detectable fluorescence signal over much of the lamellar region. The cell surface fluorescence only rose above background level 20–30 μm behind the leading cell margin, near the nucleus. However, on a minority of cells in a population, clearance was not complete even after 60 min, although a more shallow gradient of fluorescence was formed. This indicated that the average rate of Pgp-1-mAb clearance varied among cells in a population. During clearance of Pgp-1-mAb complexes, there is no discrete fluorescent structure to track in order to measure transport rates at short time intervals. However, during clearance of Ag-Ab complexes, and in the absence of any surface marker at all, rearward movement of phase densities can be observed by time-lapse phase-contrast microscopy, and the movement of a specific type of phase-dense structure, the arc, has been correlated with the rearward transport of patches (Heath, 1983*b*). Therefore, we examined whether the variation in clearance of Pgp-1-mAb complexes was reflected in a variation of movement of phase densities.

From examination of several hundred cells, it was evident that cells having the steepest gradient, or more complete clearance of Pgp-1-mAb complexes, also had a characteristic phase-grey zone at the anterior lamellar margin. This finding is illustrated in Fig. 3. Two cells located on the same coverslip are shown to differ markedly in the extent of clearance of monoclonal anti-Pgp-1 from their lamella. The cell showing more complete clearance of Pgp-1-mAb (Fig. 3, *a–c*) has a phase-dense zone just behind its leading cell margin. Such a prominent submarginal zone is absent from the lamella of the cell which has cleared Pgp-1-mAb less completely (Fig. 3, *d–f*). Higher magnification time-lapse recording of the submarginal region of the first cell revealed a vigorous rearward flux of phase densities; critical focussing showed these densities to be small projections of the dorsal cell surface. Such a flux across the anterior region was much less apparent in the second cell (data not shown).

This highly active anterior region of a motile fibroblast has been called the submarginal thickening of the leading lamella and is associated with formation of arc-shaped microfilament

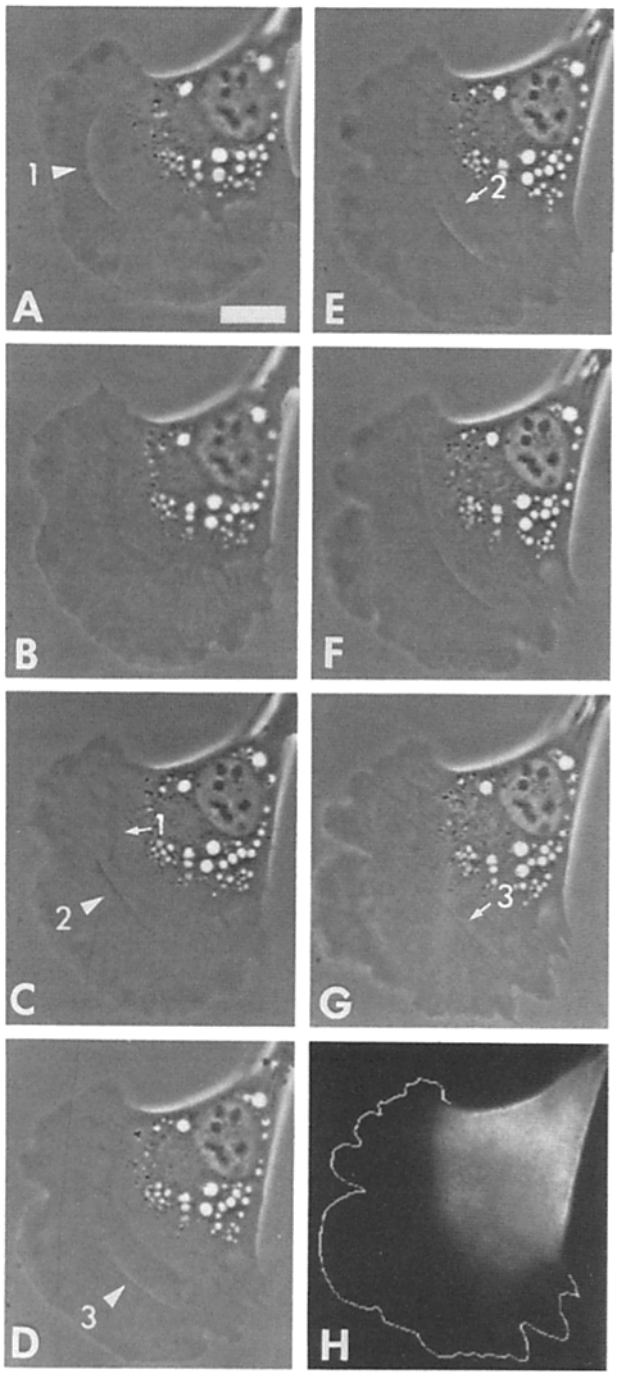

Figure 4. Correlation of Pgp-1-mAb distribution with rearward movement and disappearance of phase-dense arcs. A succession of three arcs (*numbered arrowheads*) formed near the leading edge during a 30-min time period, moved rearward, and sequentially faded from view (*numbered arrows*) upon reaching the perinuclear region (*a–g*). Fluorescently tagged Pgp-1–mAb complexes are concentrated on the cell surface posterior to the site where arcs disappeared (*h*). Outline of leading lamella from phase contrast (*g*). Bar, 10 μm.

bundles in the cortical cytoplasm (Heath, 1983*a*). Fig. 4 shows a cell in which a succession of arcs (*arrowheads*) formed, moved rearward across the lamella, and faded from view (*arrows*) as they approached the perinuclear area. The distribution of rh-anti-Pgp-1 mAb shows a very sharp increase in intensity near the site were the arcs disappeared (Fig. 4 *h*). Fig. 5 shows the organization of F-actin in a cell fixed after clearing Pgp-1-mAb. Note that the cytoplasm underlying the surface from which immune complex has been cleared contains several prominent arc-shaped F-actin bundles and an arrangement of very fine arc-shaped fibers that extends from the leading cell margin back approximately to the site where Pgp-1-mAb complexes are detectable.

We have shown that formation of the gradient of Pgp-1-mAb complex has several similarities with the capping of Pgp-1-mAb patches. Redistribution of both types of complexes is induced by the binding of antibodies. Both complexes are cleared from lamellar surfaces at similar rates and accumulate at the same cell surface location just in front of the nucleus. Like capping of patched antigen, clearance of Pgp-1-mAb complexes is associated with the generalized rearward movement of phase densities, which is commonly

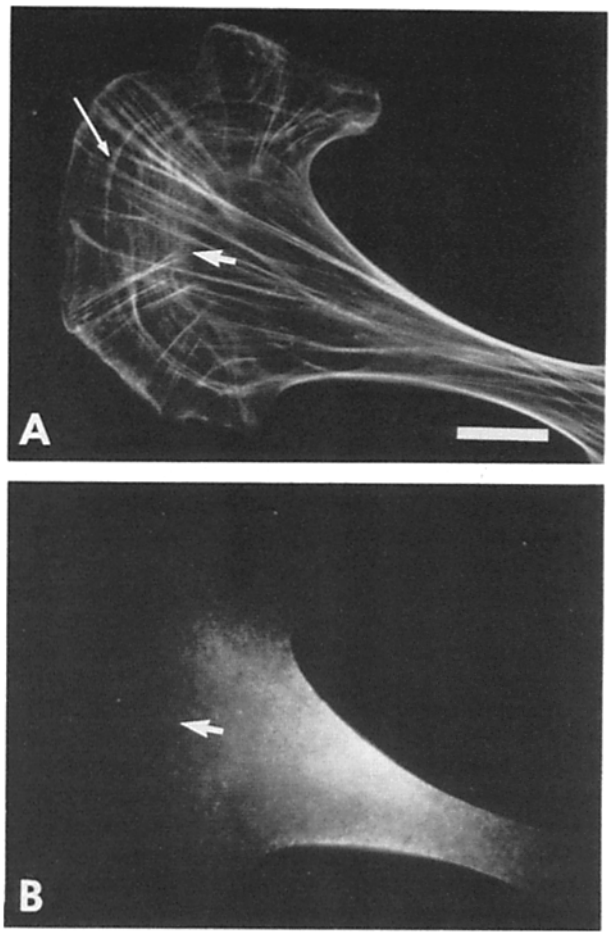

Figure 5. Organization of F-actin and clearance of Pgp-1–mAb. Pgp-1 on C3H 10T1/2 fibroblasts was tagged with rh-anti-Pgp-1-mAb. After a 45-min incubation at 37°C, cells were fixed and F-actin was labeled with fluorescein–phalloidin (*a*). Note the prominent arc-shaped F-actin bundles oriented approximately parallel to the nearby cell margin (for example, *smaller arrow*). Similarly oriented but less prominent fine bundles make up a more or less continuous sheath underlying the dorsal cell surface region from which Pgp-1–mAb complexes (*b*) have been cleared. The posterior edge of this sheath corresponds generally to the anterior extent of the Pgp-1–mAb complexes (compare larger arrows). Bar, 10 μm.

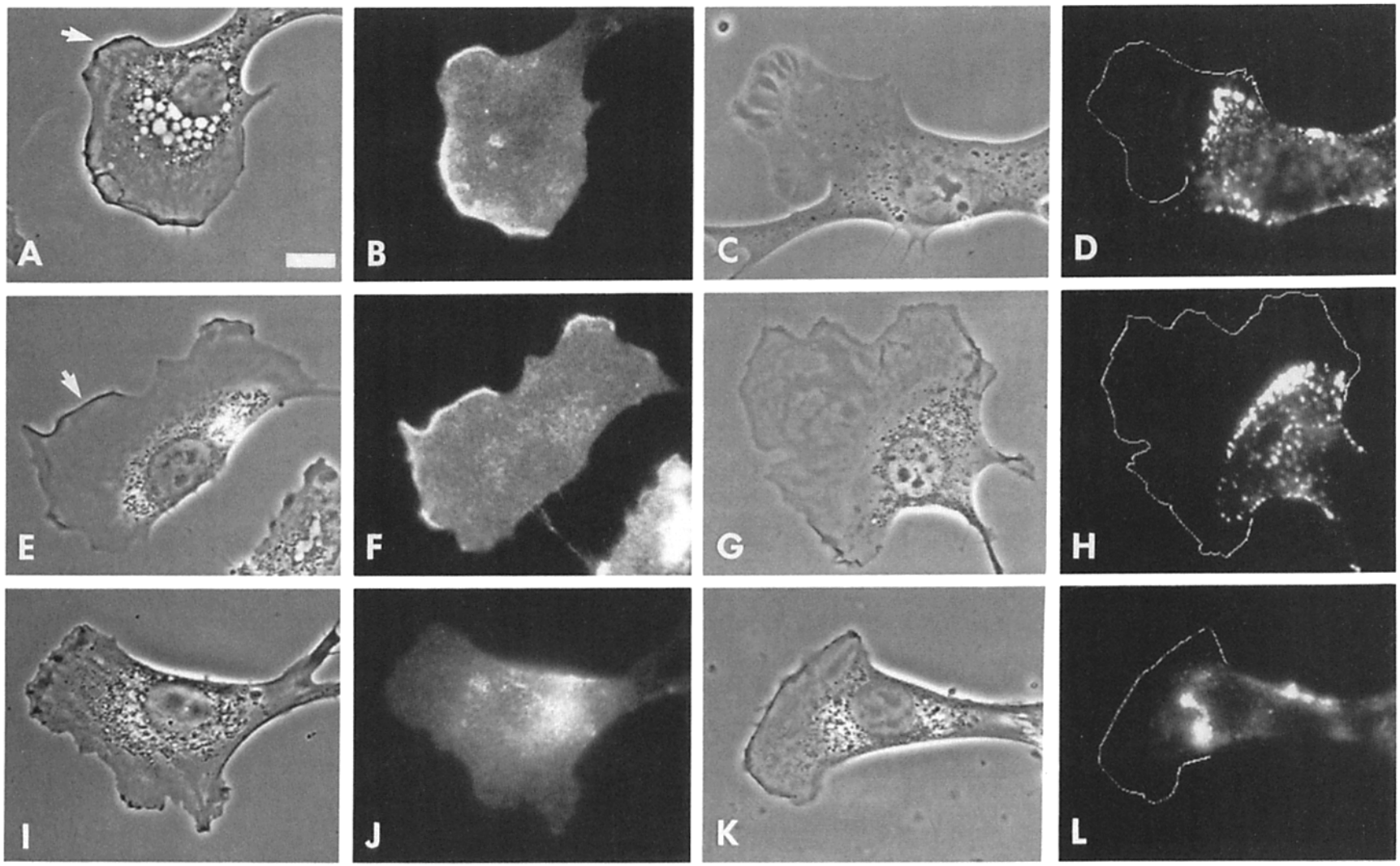

Figure 6. Primary antibodies against Thy-1, H-2, and HA do not induce capping of their antigens. Appropriate cell types (see text) were labeled with mAbs against Thy-1 (*a–d*) or H-2 (*e–h*) or polyclonal antibodies against influenza HA (*i–l*). Paired digitized fluorescence and phase-contrast images are shown. Cells were then either incubated at 37°C for 60 min, fixed, and labeled with rhodamine-conjugated secondary antibodies (*b*, *f*, and *j*), or labeled with the secondary antibodies and then incubated for 30 min before fixation (*d*, *h*, and *l*). Indirect immunofluorescence distributions show that primary antibodies alone did not induce clearance of these membrane proteins from surfaces of leading lamellae (*a* and *b*; *e* and *f*; *i* and *j*). Concentrations of primary antibodies applied were: anti-Thy, 500 μg/ml; anti-H-2, 800 μg/ml; anti-HA, 20 μg/ml (see Table I for effects of other concentrations of primary antibody). Note pronounced labeling of the upturned lamellipodia in *b* and *f* as shown by arrows in *a* and *e*. All three membrane proteins were capped if primary antibodies were aggregated with secondary antibodies before incubation (*c* and *d*; *g* and *h*; *k* and *l*). Note that patches of all three membrane proteins accumulated at similar locations anterior to the nuclei. Outlines of leading lamella in *d*, *h*, and *i* from phase contrast micrographs *c*, *g*, and *k*, respectively. Bar, 10 μm.

*Table II. FRAP Measurements of Membrane Protein–Antibody Complex Diffusion**

Protein	Cell type	Diffusion coefficient	Mobile fraction	Number of measurements
		cm^2/s	%	
HA‡	3T3	2.1 (± 0.8) × 10^{-10}	44 (± 17)	30
Pgp-1 (Before capping)§	3T3	4.1 (± 2.2) × 10^{-10}	79 (± 15)	29
Pgp-1 (Before capping)§	10T1/2	2.7 (± 1.2) × 10^{-10}	66 (± 20)	21
Pgp-1 (Capped)§	10T1/2	2.7 (± 0.7) × 10^{-10}	76 (± 19)	8
Pgp-1 (Patches)‖	10T1/2	−‖	11 (± 5)	7
Pgp-1 F(ab) labeling	10T1/2	3.4 (± 2.1) × 10^{-10}	68 (± 20)	32

* IgG labels used except where noted; measurements made at 25°C.
‡ HA labeled with pAb at a concentration that did not cause visible aggregation.
§ Measurements made either immediately after antibody labeling (before capping) or after a 1-h incubation with antibody that caused capping. See also Jacobson et al. (1984*a*). No significant difference was found between those IgG and F(ab) preparations that were airfuged before labeling and those which were not.
‖ Measurements made 1 h after incubation of primary and secondary antibody to cause overt patching. Very small recoveries preclude accurate diffusion coefficient calculations.

seen in motile fibroblasts. For these reasons, we conclude that gradient formation and capping of patches are reflections of a common rearward transport process, and we now refer to the redistribution of Pgp-1-mAb as capping.

However, two major differences exist between the two types of redistributed antigen-antibody complexes. First, Pgp-1-mAb complexes on living cells do not form aggregates resolvable by the light microscope, whereas discrete aggregates can be resolved within minutes after subsequently applying anti-IgG secondary Ab. A second difference, probably related to the first, is found in the diffusion coefficients of the two types of immune complexes and this is examined in detail below. With a few exceptions (for example, Ryan et al., 1974), aggregation of membrane proteins to form discrete patches has been necessary to induce capping. Therefore, we examined whether other membrane proteins were induced to cap when tagged with a nonaggregating primary Ab alone.

Primary Antibodies against Three Other Membrane Proteins Fail to Induce Capping

We examined whether primary antibodies induced capping of Thy-1 on C3H 10T1/2 cells, H-2 on Balbc/3T3 cells, and influenza HA on cells derived from the NIH 3T3 line. In all cases, cells were labeled with the appropriate primary antibody, incubated for 45–60 min in growth medium at 37°C, fixed, and then labeled with appropriate secondary anti-IgG to visualize the primary Ab. Unlike Pgp-1, none of these proteins were induced to redistribute by their primary Ab tag (Fig. 6), and their distributions after 45–60 min were indistinguishable from distributions on control cells fixed before applying primary antibodies (not shown). However, like Pgp-1, these three membrane proteins were capped within 30–45 min after applying secondary anti-IgG antibodies (Fig. 6). Internalization controls for patched antigens showed that fluorescence distributions, such as depicted in Fig. 1 *f* and Fig. 6, *d*, *h*, and *l*, represent primarily surface label rather than internalized label. The control staining used a second anti-Ig to trace the surface distribution of the first patching anti-Ig and showed that patched Pgp-1, Thy-1, H-2, and HA were not appreciably internalized during the time period in which capping occurred (data not shown; Holifield and Jacobson, 1991).

We examined whether differences in labeling conditions might be responsible for the unique behavior of tagged Pgp-1 as compared with the other three proteins. These results are summarized in Table I. Pgp-1 could be capped by a 100-fold dilution of the antibody concentration (50 μg/ml) used to produce results shown in Fig. 1 *D*. A 24-fold increase in concentration (to 1.2 mg/ml) did not induce visible patching of capped Pgp-1. High concentrations of primary antibodies against Thy-1 or H-2 did not induce patching or capping (Table I). These results indicate that the exceptional capping behavior of Pgp-1-mAb complex was not dependent on the antigen labeling protocols used. The possibility that Pgp-1-mAb capping was induced by multivalent aggregates in the mAb preparation was checked for by airfuging the preparation, at 100,000 *g*; capping still occurred after this treatment.

We also examined whether Pgp-1 gradient formation is dependent on the particular anti-Pgp-1 mAb preparation and its epitope (see Table I). First, a second mAb (I42/5.1; see Materials and Methods) which binds to a different antigenic determinant on the Pgp-1 molecule also induced capping of Pgp-1. Second, we used F(ab) fragments prepared from the original mAb and these did not induce complete clearance of F(ab) label the lamellar surface. However, the interpretation of this result is not unequivocal. Under the standard labeling protocol, F(ab) fragments produced a very weak indirect immunfluorescence signal, suggesting that the fragments dissociated to some extent from cells during the 37°C incubation step. To favor a level of ligand binding similar to that obtained with intact mAb, we incubated cells continuously with F(ab) during the 60-min incubation at 37°C. The control treatment of continuous incubation with intact mAb was done in parallel (not shown), and in this case cells displayed the typical gradient, whereas the F(ab)-labeled cells did not. It appears likely that failure of a steeper gradient to form was either due to a shorter dwell time of F(ab) fragments on the Pgp-1 molecules or to the inability of F(ab) fragments to induce Pgp-1 dimer formation. (Note that the lateral mobility of Pgp-1 tagged with the F(ab) fragment was similar to that measured for the Pgp-1 mAb complex [Table II].)

The failure of Thy-1 and H-2 mAb complexes to cap regardless of antibody concentrations contrasts with the capping behavior of HA tagged with a primary polyclonal Ab. In this case, capping of HA was markedly dependent on ap-

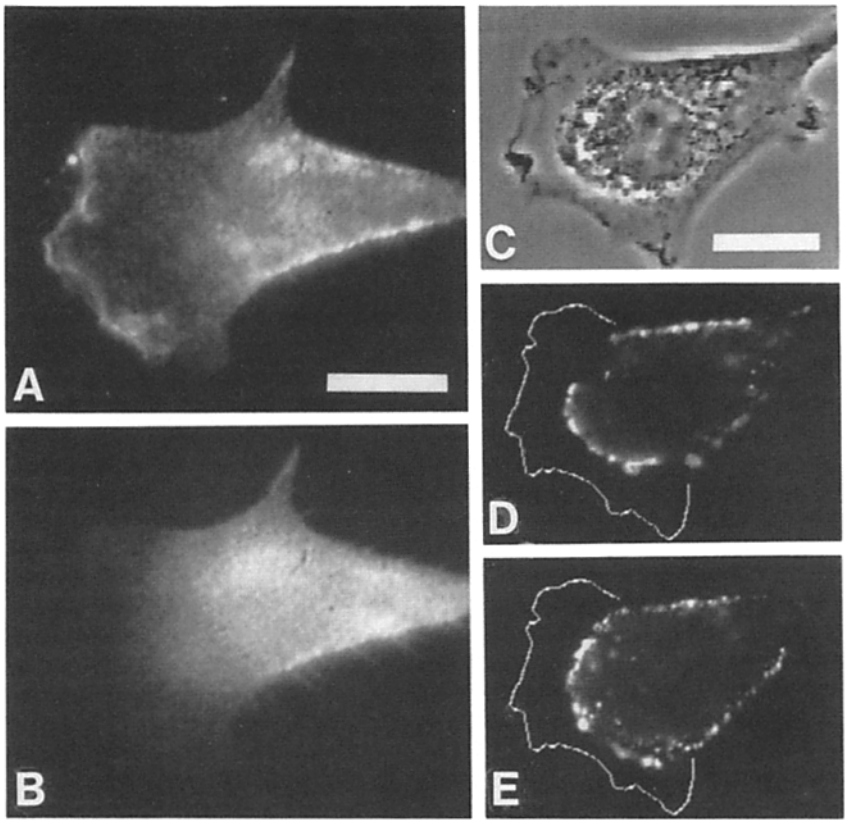

Figure 7. Selective and sequential capping on cells labeled both for Pgp-1 and HA. The cell in *a* and *b* was labeled with a mixture of rabbit rh-anti-HA (*a*) and fl-rat anti-Pgp-1 IgG antibodies (*b*). Rh-HA-pAb complexes remained on the lamellar surfaces (*a*) (note the staining of membrane ruffles at the leading edge), whereas nonpatched (*b*) fl-Pgp-1 was capped. In *c–e*, sequential capping of Pgp-1 (*d*) and HA (*e*) demonstrates that Pgp-1 is not required for capping of another membrane protein (see Discussion). White outlines of leading lamella in *d* and *e* from phase-contrast micrograph in *c*. Bars, 10 μm.

plied antibody concentration and incubation time (Table I), suggesting that a threshold in size exists for two-dimensional immunoprecipitates (patches) to be subject to the transport mechanism, at least for some proteins. Note, however, that Pgp-1 capping did not show this dependence on applied antibody concentration or incubation time. The lowest concentration of anti-Pgp-1 mAb that gave a significant level of staining (0.5 μg/ml) still induced capping.

Nonaggregated Pgp-1–Antibody and HA–Antibody Complexes Have Similar Diffusion Coefficients

The failure of rh-anti-HA to be cleared from the lamellar surface while patches of Pgp-1 and Pgp-1-mAb complexes were cleared can be explained by the most current form of the source/sink mechanism, the retrograde lipid flow hypothesis (Bretscher, 1976, 1984), if these different membrane markers have large differences in their diffusion coefficients. Table II lists the diffusion coefficients and mobile fractions obtained by the FRAP technique on cells labeled separately for Pgp-1 and HA. When tagged with their respective primary antibodies, both Pgp-1 and HA showed similar diffusion coefficients, the values of which are typical of many plasma membrane proteins tagged with antibodies (reviewed in Peters, 1981; Jacobson et al., 1987). According to the retrograde lipid flow hypothesis, both HA and Pgp-1 immune complexes should have been capped, since proteins with low diffusion coefficients of $\sim 3 \times 10^{-10}$ cm²/s would not be able to overcome the proposed rearward lipid flow by diffusion (Bretscher, 1981, 1984; Ishihara et al., 1988). Thus the failure of rh-anti-HA to redistribute into a gradient, whereas Pgp-1-mAb complexes do form a gradient, cannot be explained by this mechanism.

Double-Labeling Studies Reveal Selectivity of the Transport Mechanism

Results above suggest that the rearward transport mechanism may act selectively on some Ag-Ab complexes while others are not susceptible to it, and that this difference is not determined by the diffusion coefficient alone. To test this possibility directly, we performed double-label experiments in which two different membrane proteins, Pgp-1 and HA, were tagged on the same cell. Cells were labeled simultaneously for Pgp-1 and HA and incubated for 45 min at 37°C. In this situation Pgp-1-mAb complex was capped, forming a gradient, whereas HA-pAb complex was not capped, illustrating a selectivity in the capping mechanism (Fig. 7, *a* and *b*). This result also serves as a positive control for possible perturbation of a rearward transport mechanism under conditions where an antibody complex failed to cap (Fig. 6, *b*, *f*, and *j*).

The unique capping behavior of Pgp-1 under conditions used in this study could indicate a possible functional role in the mechanism of capping. A membrane protein having a similar apparent molecular weight and sharing immunological cross-reactivity with Pgp-1 was suggested as a candidate to link a variety of patched membrane proteins to nonerythrocyte spectrin in lymphoma cells (Kalomiris and Bourguignon, 1988; see Discussion). We asked whether removal of Pgp-1 from the lamellar surface by capping would perturb subsequent capping of another patched membrane protein. First, patching and capping of Pgp-1 was induced by applying primary and fluorescein-conjugated secondary an-

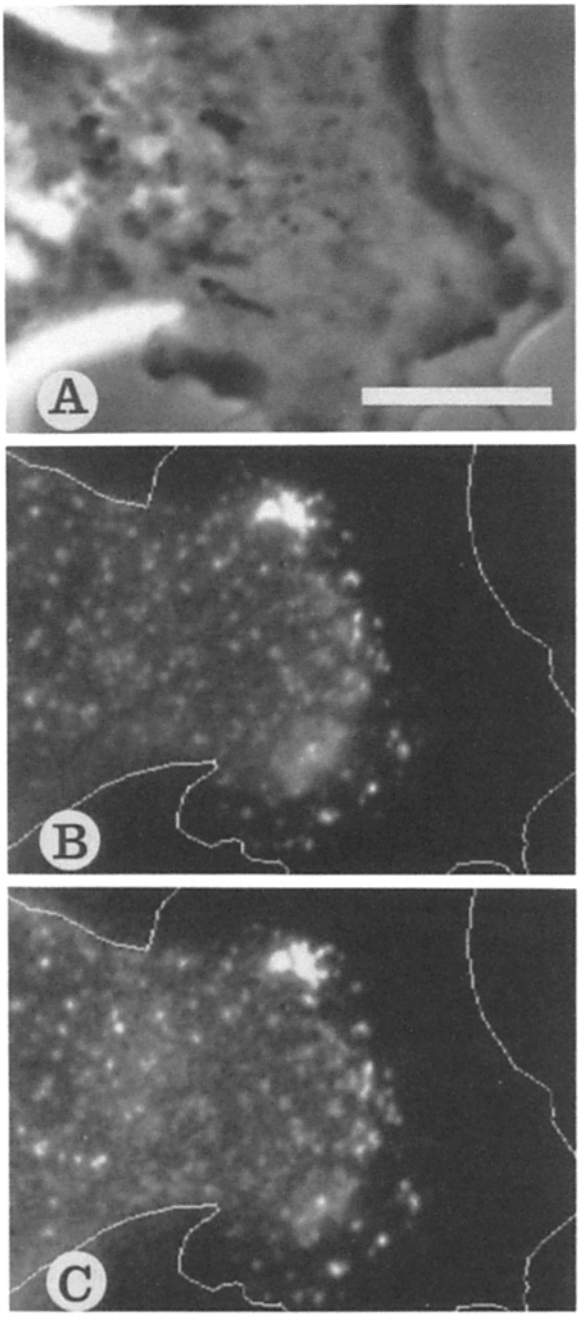

Figure 8. Failure of rh-anti-HA to be cleared from the leading lamella is not due to continuous recycling and insertion at the leading edge. Cells were labeled with rh-anti-HA rabbit polyclonal IgG and incubated at 37°C for 60 min. Capping did not occur under these circumstances (see Fig. 7). Subsequently, a brief pulse of fl-anti-rabbit IgG secondary antibodies induced patching clearance of fluorescein label from a portion of the leading lamella within 8 min. Shown are digitized images of the leading lamella (*a*), and distributions of fluorescein-labeled secondary antibody (*b*) and rhodamine-labeled primary anti-HA (*c*). If the failure of rh-HA-pAb to be capped during the 60-min incubation step was due to continual reinsertion of internalized immune complexes at the leading edge, then inserted rhodamine label should have appeared in the zone from which fluorescein patches were cleared. This did not occur and the distributions of fluorescein and rhodamine labels are nearly identical at the ventral focal plane shown, as well as at higher focal planes (not shown). White outlines of cell in *b* and *c* from phase-contrast micrograph (*a*). Bar, 10 μm.

tibodies. After a 30-min incubation at 37°C, primary and rhodamine-conjugated secondary antibodies were applied to patch and cap HA. Cells were fixed after another 30-min incubation step. As shown in Fig. 7, *c–e*, capping of Pgp-1 (Fig. 7 *d*) did not perturb the subsequent capping of HA (Fig. 7 *e*) in any obvious way, and both antigens accumulated at the same site on the cell.

The results shown in Fig. 7, *a* and *b* are consistent with the failure of nonaggregated HA-pAb to be engaged by the rearward transport mechanism. However, if HA-pAb were participating in a cycle of internalization and reinsertion at the leading edge, then failure to be cleared from the lamellar region could be explained by the continuous return of internalized fluorescent HA-pAb complexes to the leading edge. If such recycling rh-anti-HA complex indeed occurs, then insertion of fluorescent label at the leading edge should be detectable in the following way. Fig. 7, *a* and *b* demonstrate that rh-HA-pAb complexes are present on the most anterior region of the leading edge after 45 minutes after applying the antibodies. On such a labeled cell, after a brief pulse with fluorescein anti-rabbit IgG and a brief time for clearance of the resulting patches, we should be able to detect insertion of nonpatched rh-HA-pAb in the extreme anterior region by comparing the distributions of fluorescein and rhodamine antibodies. Fluorescein should report the location of patched surface label only. On the other hand, rhodamine should report the location of patched surface label (coincident with fluorescein patches) plus diffuse label present in the cytoplasm, and present, due to reinsertion at the leading edge, on the surface region cleared of patches. As Fig. 8 shows, no diffuse rh-HA-pAb was detected in the extreme anterior surface region. This result indicates that no appreciable reinsertion of internalized rh-HA-pAb complexes occurred at the leading edge during clearance of patches. We allowed only 8 minutes for clearance of patches on this cell to minimize possible dilution by diffusion of fluorescence signal from inserted rh-anti-HA. The same result was obtained when up to 30 minutes was allowed for insertion to occur (data not shown). Also, similar results were obtained when possible reinsertion of Thy-1 and H2 Ab complexes was examined (data not shown).

Discussion

Tests of Membrane Flow Schemes

One common feature of the various source/sink mechanisms is the assumption that nonaggregated membrane components constituting the surface between moving patches are also flowing rearward. The membrane flow schemes of Abercrombie et al. (1972), and of Harris (1976) suggested that the entire complement of plasma membrane components, all proteins and lipids, are assembled at the leading cell margin and subsequently flow rearward toward a sink. Harris pointed out that simultaneous comparison of aggregated and nonaggregated surface markers could provide a test of his scheme but such experiments have not been reported until this study. Middleton (1979) reported that Thy-1 tagged with antibodies was not cleared from lamellar surfaces, and Heath (1983*b*) obtained similar results for another antigen on chick fibroblasts. However, subsequent measurements showed that Thy-1 has a lipid-like diffusion coefficient (Ishihara et al.,

1987) so that Thy-1 would not have been expected to exhibit a significantly gradated distribution. Also, in those earlier investigations the possibility that the uniform surface distribution was maintained by recycling and insertion of the tagged antigens at the leading edge was not addressed. In double-label experiments (Fig. 7), we showed that HA–pAb complexes remain dispersed on the lamellar surface during the time that Pgp-1–mAb complexes are cleared from that same surface. We also ruled out the possibility that HA–pAb might only appear not to be capped, while really being transported rearward, internalized, and reinserted at the leading edge (Fig. 8). Since two additional nonpatched Ag-Ab complexes were found not to be capped, this may illustrate the behavior of nonaggregated membrane components in general. Together, these results disprove the bulk membrane flow schemes envisioned by Abercrombie and by Harris and show that not all tagged membrane proteins present on the lamellar surface are subject to the capping mechanism.

A different membrane flow scheme was proposed by Bretscher (1976, 1981, 1984) to explain capping. He hypothesized that the membrane components flowing away from the leading edge are primarily lipids which are internalized through coated pits and subsequently returned to the leading edge to form new protrusions (see Bretscher, 1984). A basic prediction of the retrograde lipid flow hypothesis is that nonrecycling membrane components will be distributed by the lipid flow in a manner determined by their ability to overcome the flow by diffusion (Bretscher, 1982, 1984). Large patches of immune complexes were suggested to have smaller diffusion coefficients than nonpatched membrane proteins. Patches would be less able to resist the lipid flow via diffusion and so would be swept rearward along with the flow. Induction of a gradient by the binding of anti-Pgp-1 mAb is at least qualitatively consistent with the lipid flow mechanism since the mAb could bind two Pgp-1 molecules and perhaps reduce the diffusion coefficient, relative to nontagged Pgp-1 molecules, enough to alter a uniform steady-state distribution found on prefixed cells. Similarly, the diffusion coefficient of Pgp-1 patches is smaller than that measured for Ha-pAb complexes and is indeed too small to measure accurately by the FRAP technique (Table II). Thus capping of Pgp-1 patches but not tagged HA on double-labeled cells (data not shown) is also consistent with the retrograde lipid flow hypothesis. However, results of other double-label experiments are not. Pgp-1-mAb is capped to form a steep gradient, whereas HA-pAb remains dispersed throughout the surface of the same lamella. The retrograde lipid flow model (RLF) predicts that, to maintain its noncapped distribution, HA–pAb should either have a greater diffusion coefficient than Pgp-1–mAb, or it should be recycled to the leading edge and reinserted. We found that neither of these predictions hold true.

It should be noted that consideration of the immobile fraction, as obtained in photobleaching measurements, raises some questions for both the retrograde lipid flow and cytoskeletal models of capping. First, technically speaking, proteins composing the immobile fraction should have lateral diffusion coefficients nearly zero and be rapidly and completely capped according to the retrograde lipid flow model. Since for HA this is not the case, it could be argued that immobilized HA–Ab complexes simply cannot flow with the bulk lipid. Similarly, immobilized HA–Ab complexes cannot be coupled to the rearward moving cortical cytoskeleton either (see below).

In a second test of an explicit prediction of the RLF hypothesis we compared the capping behavior of patched and nonpatched Pgp-1 immune complexes on the same cell. According to the retrograde lipid flow model, these two types of immune complexes, which have significantly different diffusion coefficients (see Table II), should have different steady-state surface distributions. However, we found that Pgp-1 patches and Pgp-1–mAb complexes are cleared from the lamellar surface back to the same location and at approximately the same rate (Fig. 2).

The RLF model is not adequate to account for results obtained in comparisons of these different membrane protein–antibody complexes. Very recently several other direct tests have been reported which also demonstrate that this hypothesis to explain capping is incorrect. Video photobleaching results using a fluorescent lipid analogue embedded in the plasma membrane of locomoting leukocytes indicate that the bilayer moves forward in concert with the leading edge, not rearward (Lee et al., 1990). In addition, results from tracking colloidal gold particles bound to cell surface lectin receptors on macrophages indicate that the diffusional behavior of particles is not biased by a rearward directed flow component, as should occur if a retrograde lipid flow existed on those cells (Sheetz et al., 1989).

The selective capping results also suggest that the retraction induced spreading model to explain the capping of Pgp-1-mAb complex (Ishihara et al., 1988) is not correct. In this model, the low diffusion coefficient of membrane proteins, such as Pgp-1, is explained by transient anchorage of the proteins to immobilized peripheral structures, i.e., membrane-associated cytoskeleton or extracellular matrix. The gradated distribution of membrane proteins in locomoting cells would arise if during the forward flow of membrane postulated to accompany locomotion, molecules such as Pgp-1 were not fully carried along by the forward flow because of their transient anchorage, resulting in a higher density of Pgp-1 in the rear portion of the cell. However, if this transient anchorage mechanism applied to all slowly diffusing membrane protein-antibody complexes, such as HA-pAb in the present study, one would expect all such membrane proteins to show a gradient similar to that of Pgp-1. The double labeling results with HA and Pgp-1 show this not to be the case (Fig. 7).

The present results documenting retrograde transport of membrane components require a mechanism having a greater degree of selectivity in its action than can be provided by the retrograde lipid flow hypothesis. We suggest that interaction of capped membrane components with a flowing cortical cytoskeleton is better able to provide this selectivity (see below).

Coupling of Capped Molecules to Cortical Cytoskeletal Flow

Direct evidence for the role of a moving cortical microfilament network in the capping of patches has been provided (Heath, 1983*a*, *b*). In addition, inert particles adsorbed to the lamellipodial surface are transported rearward in concert with the observable flow of actin-based structures (Fisher et al., 1988; Forscher and Smith, 1990). In fibroblasts, the rate

of rearward particle transport is greater on ruffling motile cells than on nonruffling motile cells (Harris and Dunn, 1972). Our observations correlating efficient gradient formation with pronounced and moving phase densities in the submarginal region (Fig. 3), as well as with the occurrence of arcs in the lamellar cytoplasm, are in accord with these earlier reports.

It is generally accepted that the cortical microfilament network undergoes rearward flux in motile fibroblasts (reviewed by Bray and White, 1988), although the molecular details of this flux remain to be elucidated. At the present time, the question of the mechanism of capping consists of two parts: (*a*) how is this cortical cytoskeletal flow produced? and (*b*) how is movement of integral membrane patches coupled to movement of the cortical cytoskeleton? The results presented in this study have implications for the second of these two questions.

Any proposed coupling mechanism must explain the following features of our results: (*a*) aggregates of immune complexes of a variety of membrane antigens are capped, whereas most nonaggregated Ag–Ab complexes are not capped; (*b*) a particular nonaggregated immune complex, Pgp-1–mAb, appears to be an exception and is capped at a rate similar to that of patches while its characteristic lateral mobility is preserved; (*c*) capping of Pgp-1-mAb commonly results in a gradient of the immune complex rather than complete clearance, as occurs with patches. Several basic types of coupling can be identified in the literature and these are illustrated in Fig. 9.

Direct Coupling

Direct coupling is indicated on the left half of Fig. 9 in terms of permanent or transient coupling to the cytoskeleton. The coupling mechanism must be sufficiently general to act on a variety of different membrane proteins when they are aggregated but not, in the case of H2, HA, and Thy-1, in the absence of aggregation. Thus, binding of cytoplasmic domains of the patched antigens directly to cortical cytoskeletal elements is unlikely, since influenza HA, H2, Thy-1, and Pgp-1 do not possess conserved cytoplasmic domains. The capping of Thy-1, a cell surface protein anchored in the plasma membrane by a lipid moiety, and glycolipids such as Forssman antigen (Stern and Bretscher, 1979) and gangliosides (Revesz and Creaves, 1975; Spiegel et al., 1984), present obvious difficulties for direct linkage, as does the fact that a mutant H-2 lacking most of its cytoplasmic domain showed capping behavior indistinguishable from the native H-2 in the parent cell line (Murre et al., 1984).

An alternative to a direct specific linkage is a direct nonspecific linkage. This has been suggested to arise as a result of concentrating, by cross-linking, antigen cytoplasmic domains that interact only transiently with the cytoskeleton before patching. Translational entropy is "prequenched" helping to provide a local increased binding energy for a more stable interaction (Shiozawa et al., 1989). The aggregated cytoplasmic domains of large immune complexes might be subject to a viscous drag or shear force arising due to a proximity of the flowing cortical meshwork and the fluid plasma membrane. However, capping of nonpatched Pgp-1, as well as molecules lacking cytoplasmic peptide domains such as Thy-1, also presents a difficulty for this mode of linkage.

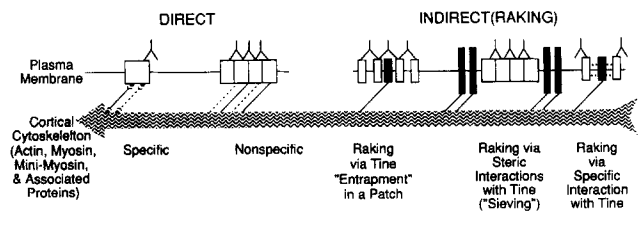

Figure 9. Possible modes of coupling plasma membrane components to subjacent cortical cytoskeleton.

Indirect Coupling (Raking)

This group of mechanisms is depicted in the right half of Fig. 9. A variety of patched antigens could be indirectly coupled to a cortical cytoskeletal flow through lateral interaction with a subset of integral membrane proteins that possess a specific, stable anchorage to moving cytoskeletal elements and that function like the tines of a rake (dePetris, 1978; Dunn, 1980; Dembo and Harris, 1981). Patches could be moved if such "tine" membrane proteins were entrapped within the patch of immunoprecipitate during its formation. If binding of antibodies, such as monoclonal anti-H-2 or anti-Thy-1, failed to form an immunoprecipitate, then no entrapment would occur and the antibody would fail to induce capping of its antigen. A similar scheme was proposed by Bourguignon and Singer (1977), who called the entrapped molecule "protein-X", and suggested that it somehow recognized aggregated membrane proteins. That membrane protein aggregates might directly incorporate tine molecules is suggested by the detergent insolubility of patched, but not nonpatched, antigens (see for example Flanagan and Koch, 1978; Braun et al., 1982). In one case, such extraction experiments with lymphocytes revealed a glycoprotein that specificially associated with patches of two different antigens (Turner and Shotton, 1987). Alignment of patches over microfilament bundles in fibroblasts is also suggestive of transmembrane anchorage (Singer et al., 1978; Heath, 1983*b*; Jacobson et al., 1984*b*).

In a similar manner, a coupling might exist in which the lateral spacing of tine molecules is sufficient to form a molecular seive that could sweep the larger patches of immune complexes along with the cortical cytoskeletal flow, while leaving behind the smaller nonpatched diffusing cell surface markers. Such a fluid coupling was proposed by Dembo and Harris (1981) to explain features of polystyrene bead motion during rearward transport.

Capping of Pgp-1-mAb Complex

A raking mechanism can explain the unusual capping of Pgp-1-mAb, and the failure of the other three primary Ab–Ag complexes to be capped, if one or more of the following situations obtain: (*a*) Pgp-1-mAb forms immune complexes larger than Thy-1-mAb or H2-mAb, and sufficiently large to be caught in the tines of a rake; (*b*) Pgp-1-mAb selectively but transiently binds to tines of the rake; (*c*) Pgp-1 is one of the tine molecules.

We suggest that the exceptional behavior of Pgp-1–mAb is not due to extensive lateral aggregation. No visible patching

accompanied capping of the Pgp-1–mAb complex and it displayed a lateral diffusion coefficient and mobile fraction characteristic for membrane protein-Ab complexes that are not capped (Table I). In addition, varying mAb incubation time (1–60 min) and concentration (0.5–1,200 μg/ml) had no effects on Pgp-1 patching or capping, while results expected of a multivalent interaction between pAb and HA were apparent after varying the pAb concentration over a much smaller range (20–50 μg/ml; Table I). However, the possibility that antibody induced dimerization or microaggregation of Pgp-1 causes capping cannot be ruled out.

Pgp-1-mAb might selectively interact with the tines of a rake. Such an interaction must be transient, permitting the immune complex to retain lateral mobility similar to HA-pAb, a noncapped immune complex (see Fig. 9, *far right*). Indeed, Sheetz and co-workers (1989) have recently shown that lectin-coated gold particles alternate between periods of random lateral diffusion and directed retrograde flow. They have suggested the capping of Pgp-1–mAb complex may proceed by such a mechanism.

Since Pgp-1-mAb and patches accumulate near the site where visible cortical actin structures (arcs) fade from view, we can infer that the raking effect ceases at that site (Holifield and Jacobson, 1991). As raking gradually ceases, the laterally mobile Pgp-1-mAb complex would "back" diffuse (against the raking action), resulting in a gradated distribution in the vicinity of lamelloplasm–endoplasm boundary as opposed to absolute clearance off the lamella.

Finally, Pgp-1 itself might be a tine molecule and interact directly with cortical cytoskeletal structures. Again, this would have to be a labile interaction to permit lateral mobility of the immune complex. Kalmiris and Bourguignon (1988) suggested that a protein sharing immunological cross-reactivity with Pgp-1 is a candidate for the tine molecule in a lymphoma cell line, based on its apparent interaction with the spectrin-binding protein, ankyrin. If Pgp-1 acts as a tine molecule in the fibroblast lines we studied, it is not required in large amounts to exert a raking effect, since its nearly complete removal by capping did not prevent subsequent capping of another antigen on the same cell. Clearly, identification and demonstration of constitutive lateral transport of tine molecules, whether Pgp-1 or other candidates, is crucial for validation of the raking model for membrane cytoskeletal interaction in the capping phenomenon.

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