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Secretory organelles and the cytoskeleton: Organization and interdependence

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Case Western Reserve University (Health Sciences), 1990
SECRETORY ORGANELLES
AND THE CYTOSKELETON:
ORGANIZATION AND INTERDEPENDENCE

by
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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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January, 1990
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Date  SEPTEMBER 21, 1989

*We also certify that written approval has been obtained for any proprietary material contained therein.
SECRETORY ORGANELLES AND THE CYTOSKELETON: ORGANIZATION AND INTERDEPENDENCE

Abstract

by

JERROLD ROSS TURNER

This study has used several systems to characterize i) the transport of membrane proteins to and from the cell surface, and ii) the maintenance and structural organization of the Golgi complex.

The transport of membrane proteins was studied using the regulated surface expression of the C3b/C4b receptor, CR1, on human neutrophils as a model. We developed a new method for quantitative immunofluorescence of intracellular antigens, and used this approach to document upregulation of cell surface CR1 and to identify ligand-independent degradation of CR1. This is the first example of acute ligand-independent receptor degradation. Light and electron microscopic immunocytochemistry resulted in the description of a previously unrecognized granule as the site of intracellular CR1 storage in neutrophils, and the identification of multivesicular bodies as a possible site of CR1 degradation.

The Golgi complex is composed of multiple cisternae assembled into stacks which form a single interconnected organelle. Thus, the Golgi
complex was investigated to study the maintenance of organelle structural organization. The first approach was biochemical and involved identification of Golgi-associated cytoskeletal material potentially important in cisternal stacking or maintenance of cisternal shape. Five proteins were isolated, but the data could not conclusively demonstrate the existence or function of a Golgi-associated cytoskeleton. Nonetheless, immunization of mice with these proteins and preparation of monoclonal antibodies resulted in the production of an antibody which recognizes epitopes on intermediate filaments, actin stress fibers, and previously unidentified cytoplasmic foci. It is possible that this antibody recognizes a structure involved in integration of these separate components of the cytoskeleton into a unified system.

The association of Golgi stacks with microtubules and the organization of these stacks into a single Golgi complex was also examined. The data demonstrate that reversible fragmentation of the coherent juxtanuclear Golgi follows microtubule depolymerization, apparently as a result of membrane traffic and other energy-dependent processes. Golgi organization and microtubule status were clearly dissociable, thereby proving that organization of individual small stacks into a single interconnected Golgi is an active process not simply determined by microtubule binding.
Acknowledgements

Credit for this work must be shared with my wife, Judy, whose love, encouragement, and patience make everything worthwhile. I am also grateful for her technical help and companionship during many late nights, and early mornings, in the lab.

Thank you would not begin to express my indebtedness to my advisor, Alan Tartakoff, who has provided me with a splendid example as mentor, scientist, and person. He has known when to advise, discuss, or leave me to struggle with a problem on my own. He has helped me to learn a great deal more than the work described here reflects.

I thank my committee members, Neil Greenspan, George Perry, Terry Rosenberry, and Martin Snider. My relationship with each of them has been unique and rewarding. They have been advisors, examiners, friends, and colleagues. My thanks also to Jeff Salisbury, who was a member of my committee while on the faculty of CWRU, and Mel Berger, in whose lab I studied complement receptor expression. I am also grateful to Michael Lamm and Leslie Webster for their encouragement and support.

Although there are too many to list individually, I am also appreciative of the friendship and cooperation of many individuals within
the Tartakoff lab group, the Institute of Pathology, and the CWRU School of Medicine.

Finally, I would be remiss if I did not acknowledge my undergraduate mentor, John Atkinson, who introduced me to laboratory research. Without his unselfish investment of time and effort on my behalf, and the nurturing lab environment he welcomed me to, I probably would not have undertaken this endeavor.
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I. Introduction

The cytoplasm of eukaryotic cells contains a multitude of membrane-bound compartments. These organelles are specialized in function, composition, and structure. In recent years, it has become apparent that many organelles are subdivided into regions of specialized composition. For example, in polarized epithelia it is critical to the functional integrity of the epithelium that basolateral and apical plasma membrane proteins be sorted to the appropriate surface domain (Simons & Fuller, 1985). Once delivered to a given surface, many proteins do not remain on the plasma membrane, but are internalized into intracellular compartments including endosomes and lysosomes. When these proteins recycle, they must be sorted back to the appropriate plasma membrane domain.

Sorting also occurs during synthesis of regulated secretory proteins. As these newly-synthesized proteins traverse the Golgi complex (GC), they are sorted into specialized secretory granules which are released upon appropriate stimulation of the cell. The best characterized example of sorting to the regulated path is that of peptide hormones in neuroendocrine cells (Burgess & Kelly, 1987). We have studied the regulated expression of the complement C3b/C4b receptor, CR1, in human neutrophils (Turner et al., 1988). As with regulated secretory proteins, in
resting cells CR1 exists in large intracellular pools, but is rapidly recruited to the surface following cell stimulation. This system offers two distinct advantages over neuroendocrine cells. First, the majority of CR1 is synthesized well in advance of the stimulus, since mature neutrophils are end-stage cells with limited biosynthetic capabilities. Second, CR1 recruited to the cell surface is not released. It is therefore possible to trace endocytosis which follows exocytosis. In studying this system, a new method for quantitative flow cytometry of intracellular antigens was developed (Turner et al., 1989). Using this approach it was possible to characterize intracellular proteolysis of CR1 in an acidic compartment in the absence of ligand (Turner et al., 1988). This is the first example of acute receptor degradation in the absence of ligand. Immunofluorescent and immuno-electron microscopic examination of CR1 distribution have identified a previously unrecognized population of granules as the CR1 storage site in resting neutrophils, and also suggest that the site of CR1 degradation is multivesicular bodies.

Sorting is also central to the maintenance of the Golgi complex (Farquhar, 1985). Not only must the GC sort away endoplasmic reticulum components if they are transported to the GC, but the distinct composition of the cisternae in different regions of the Golgi complex must be maintained (Tartakoff, 1983). It may well be that this compositional integrity is, in part, dependent on structural organization of the Golgi complex. The Golgi complex has three levels of structural organization. The first is maintenance of the flattened shape of the cisternae, since
thermodynamics would predict that a spherical conformation would be more stable. A second level of Golgi complex structural organization is the association of cisternae into stacks. The stacks are disassembled during mitosis (Robbins & Gonatas; 1964b; Lucocq & Warren, 1987), but there is little information available concerning the mechanisms of Golgi stack assembly and disassembly (Lucocq et al., 1989). Finally, the individual stacks of cisternae which constitute the Golgi complex are generally arranged as a single organelle associated with the microtubule organizing center (Thyberg & Moskalewski, 1985). Perturbation of the microtubule array causes relocation of these stacks, but the cisternae remain stacked (Robbins & Gonatas, 1964a; Sandoval et al., 1984). Other than the dependence on microtubules, little is known about the mechanisms by which the GC stacks associate to form a single organelle or associate with the microtubule organizing center (Ho et al., 1989).

To investigate Golgi-cytoskeletal relations, two complementary approaches were taken. In the first, stacked Golgi fractions were examined biochemically in an effort to find a Golgi-associated cytoskeleton involved in cisternal stacking or maintenance of cisternal shape. Five proteins were isolated and characterized to varying degrees. Nevertheless, the data could not conclusively demonstrate the existence of a Golgi-associated cytoskeleton. Immunization of mice and preparation of monoclonal antibodies using the Golgi-fraction derived cytoskeletal material resulted in the production of an antibody with unusual properties. This antibody reacts with epitopes on intermediate filaments, actin stress
fibers, and previously unidentified cytoplasmic foci (Turner & Tartakoff, 1989a). It is possible that this antibody recognizes an accessory protein involved in organization of microtubules, microfilaments, and intermediate filaments into a unified organelle, the cytoskeleton.

The second approach examined the relation of the GC to microtubules. This work resulted in the first demonstration of dispersed GC despite intact microtubules and, conversely, intact GC with depolymerized microtubules, at temperatures above 4°C (Turner & Tartakoff, 1989b). The mechanisms by which GC organization is dependent on microtubules were shown to be energy-dependent and, in some cases, dependent on membrane traffic. These observations support a model of GC structure which explains the role of GC-microtubule associations in GC organization.

A diversity of approaches in this work have permitted the analysis of several phenomena in membrane traffic and organelle structure. This work has addressed issues which further the characterization of each system specifically, and also adds to general understanding of the structure, maintenance, and function of the secretory and endocytic paths.
II. Intracellular Degradation of the Complement

C3b/C4b-Receptor in the Absence of Ligand

A. Summary

Human neutrophils (PMN) respond to various soluble stimuli by translocating intracellular complement C3b/C4b-receptors (CR1) to the cell surface. Ligand-independent internalization of surface CR1 has been demonstrated previously, but the fate of total cellular CR1 during PMN stimulation has not been determined. In order to study the fate of CR1 during neutrophil activation, we have employed a unique approach for the quantitative analysis of intracellular antigens which allows simultaneous measurement of total cellular and surface membrane antigen pools.

Stimulation of isolated PMN with N-formyl-Met-Leu-Phe or ionomycin resulted in a mean 7-fold increase in surface CR1 expression within 15 min. Total cellular CR1 decreased by as much as 45% within 15 min, with loss continuing for up to 1 hr. Inclusion of NH₄Cl during PMN stimulation inhibited the loss of total CR1 without affecting surface CR1 expression. Addition of phenyl-methyl-sulfonyl-fluoride inhibited loss of total CR1, and enhanced the stimulus-induced increases in surface CR1.

These data suggest that intracellular degradation of CR1 occurs during stimulation of PMN, and may involve proteolysis in an acidic
intracellular compartment. Immunofluorescent and immunoelectron microscopy suggest that multivesicular bodies are the site, or at least on the pathway to, CR1 degradation. Since our experiments were done with isolated PMN in the absence of serum and complement components, this degradation occurred in the absence of C3b, the ligand for CR1. To our knowledge, ligand-independent degradation of a cell surface receptor has not been previously detected.
B. Introduction

CR1, the complement C3b/C4b receptor of human neutrophils (PMN), is a membrane glycoprotein of \( \sim 205 \) kD (Fearon, 1980). In addition to regulating complement activation (Iida & Nussenzweig, 1981; Ross et al., 1982), CR1 is important in phagocytosis of soluble and particulate complexes to which C3b has been bound during complement activation (Ehlenberger & Nussenzweig, 1977; Wright & Griffin, 1985).

Circulating peripheral blood PMN express only 5500 CR1 molecules on the cell surface (Fearon & Collins, 1983), but a 6 to 10-fold increase in surface CR1 expression occurs within minutes of PMN activation by chemoattractants such as N-formyl-Met-Leu-Phe (fMLP) (Fearon & Collins, 1983; Berger et al., 1984), the complement fragment C5a (Fearon & Collins, 1983), or calcium ionophores (Berger et al., 1985). The increase in surface CR1 results from translocation of pre-existing CR1 from intracellular pools (O'Shea et al., 1984), and is not affected by protein synthesis inhibitors (Berger et al., 1984).

Although the increased level of surface CR1 on stimulated PMN persists for up to 2 hr (Berger et al., 1984), it is not known whether such surface expression results from unidirectional recruitment to the cell surface or represents a new dynamic equilibrium between surface and intracellular pools. Treatment of stimulated PMN which have already
upregulated surface CR1 with protein kinase C activators can induce ligand-independent internalization of surface CR1 (Changelian et al., 1985). The fate of the CR1 internalized in the absence of ligand has not been previously determined.

To evaluate the redistribution of CR1 during PMN stimulation, we have developed a new method using monoclonal anti-CR1 antibody and flow cytometry to quantify total detectable CR1 in fixed saponin-permeabilized PMN. Surface CR1 was also measured on similarly fixed PMN which were not permeabilized. We compared the effects of two distinct stimuli: fMLP, a synthetic chemoattractant which stimulates PMN via a specific receptor and an inositol phosphate-dependent signal transducing system (Snyderman & Pike, 1984), and ionomycin, a calcium ionophore which activates PMN independently of membrane receptors or specific signalling mechanisms.

In agreement with previous reports (Fearon & Collins, 1983; Berger et al., 1984; Berger et al., 1985), both stimuli induced 4- to 8-fold increases in surface CR1 on isolated PMN in the absence of C3b. As surface CR1 increased, the total cellular pool of CR1 decreased by as much as 60%. These data suggest that PMN stimulation results in ligand-independent intracellular degradation of internalized CR1. We have used light and electron microscopy to identify these compartments.
C. Materials and methods

**Antibodies.** Monoclonal anti-CR1 antibody of the IgG1 subclass produced by clone 3D9 was a gift of Dr. John O'Shea, National Institutes of Health (O'Shea et al., 1984). Monoclonal anti-CR1 antibody produced by clone C543 was a gift of Dr. Robert Schreiber, Washington University. Irrelevant IgG1 monoclonal antibody against rat β-casein was kindly provided by Dr. Charlotte Kaetzel, Case Western Reserve University. Leu-11a anti-PMN Fc receptor, anti-β₂-microglobulin, and nonspecific IgG1 were purchased as fluorescein isothiocyanate (FITC) conjugates from Becton Dickinson. Affinity-purified FITC-conjugated F(ab')₂ goat anti-mouse IgG (Boehringer Mannheim) was used for fixed cells in all experiments and for nonfixed cells in experiments which compared fixed and nonfixed cells. Since it gives higher nonspecific binding to fixed cells, (NH₄)₂SO₄-purified FITC-conjugated F(ab')₂ goat anti-mouse IgG (Cappel) was not used with fixed PMN, but was employed with nonfixed PMN in the assay for shed CR1. Horseradish peroxidase-conjugated sheep anti-mouse IgG (Biosys) was used for electron microscopy.

**Neutrophil isolation and stimulation.** Human PMN were isolated from peripheral blood on discontinuous density gradients of Percoll (Pharmacia) as described (Berger et al., 1984). PMN were resuspended at 1-4x10⁶/ml in Hanks’ balanced salt solution without Ca²⁺, Mg²⁺, or phenol
red (GIBCO), supplemented with 1 mg/ml gelatin. Stimuli, either $10^7$M ionomycin (Sigma) and 1.2 mM CaCl$_2$ or $10^{-8}$M fMLP (Peninsula), were added from stock solutions and incubated with PMN at 37°C for the indicated times. In some experiments, the following inhibitors or proteases were added concurrently at the indicated final concentrations: 37 mM NH$_4$Cl, 1 mM phenyl-methyl-sulfonyl-fluoride (Sigma), 0.01 mg/ml soybean trypsin inhibitor (Sigma), 0.1 mg/ml Eglin C (Ciba-Geigy), 0.01 mg/ml trypsin (Sigma). Gold was conjugated to bovine serum albumin (Sigma) as described (Roth, 1983), and the gold-albumin was included in incubations with fMLP for some experiments. Control flow cytometric experiments verified that gold-albumin did not interfere with increased upregulation of surface CR1.

**Fixation and permeabilization.** At times indicated after addition of stimuli and/or inhibitors, PMN were washed in ice-cold phosphate-buffered saline containing 0.5 mg/ml NaN$_3$, and resuspended at 1-4x$10^6$/ml in ice-cold periodate-lysine-paraformaldehyde fixative (McClellan & Nakane, 1974) for 20 min. All subsequent steps were at room temperature. PMN were washed with phosphate-buffered saline containing 1 mg/ml ovalbumin (PBS-ovalbumin), and divided into aliquots of $10^6$ cells for staining. One aliquot was permeabilized by washing in PBS-ovalbumin containing 0.4 mg/ml saponin (Sigma). For these samples, saponin was present throughout staining and subsequent washes. The other aliquot was not permeabilized, and was stained in PBS-ovalbumin without saponin.
Immunofluorescent staining and flow cytometry. After washing in PBS-ovalbumin with or without saponin, as appropriate (see above), monoclonal anti-CR1 antibodies were added in excess, 0.1 μg/ml, to each aliquot of 10⁶ cells in a total volume of 0.1 ml. for 30 min at room temperature. Cells were washed twice with 1.5 ml PBS-ovalbumin with or without saponin, then reacted with excess, 3.4 μg/ml, FITC conjugated anti-mouse IgG. After three additional washes, samples were resuspended in phosphate-buffered saline and fluorescence of 10,000 cells was quantified by flow cytometry exactly as described previously (Berger et al., 1985). Mean nonspecific background fluorescence was determined using permeabilized or nonpermeabilized cells incubated with FITC conjugate without monoclonal antibody. Control experiments using an excess of irrelevant primary antibody of the same subclass as 3D9 anti-CR1 followed by affinity-purified FITC-conjugated goat anti-mouse IgG had the same mean fluorescence as when the primary antibody was omitted. Mean specific fluorescence of each sample was calculated by subtracting nonspecific background fluorescence from the mean fluorescence of that sample. The specific fluorescence of permeabilized nonstimulated PMN from each experiment served as the maximal control and was assigned a relative value of 1.0. The specific fluorescence of each sample was normalized to this maximal control value. Staining was identical for those experiments using nonfixed PMN, but all steps were at 4°C, and PBS-ovalbumin contained 0.5 mg/ml NaN₃.
Fluorescence microscopy. PMN were incubated for 1 hr, fixed, permeabilized, and stained for CR1 as above. The cells were mounted in 20 mg/ml n-propyl galate in glycerol:phosphate-buffered saline (1:1), pH 9.0, to reduce photobleaching (Giloh & Sedat, 1982).

Electron microscopy. PMN were fixed for 2-3 hr at room temperature in periodate-lysine-paraformaldehyde fixative (McClean & Nakane, 1974). During fixation, the cells were allowed to settle onto poly-L-lysine coated 35 mm culture dishes. Endogenous myeloperoxidase was inactivated by three successive 20 min incubations in PBS containing 10 mM NaN₃ and 10 mM H₂O₂ (Ohno & Gallin, 1985). Permeabilization, antibody dilutions, and washes were with 0.2 mg/ml saponin in PBS containing 0.1% ovalbumin. A mixture of 3D9 and C543 monoclonal anti-CR1 antibodies, each at 0.08-0.10 mg/ml, was applied for 1 hr at room temperature, followed by four washes. Peroxidase-conjugated sheep anti-mouse Ig was diluted 1:100, and incubated with the cells for 1 hr at room temperature. The PMN were then washed four times, fixed with 1.5% glutaraldehyde in 10 mM sodium cacodylate-HCl, pH 7.4, containing 5% sucrose, and the peroxidase reaction product developed with diaminobenzidine and H₂O₂ as described (Brown & Farquhar, 1984). The cells were then osmicated, dehydrated, and embedded in Spurr’s resin.

Assay for shed CR1. To determine if intact CR1 was shed during PMN stimulation, a limiting dilution of anti-CR1 was prepared. A 0.1 µg/ml solution of anti-CR1 was diluted 6-fold with supernatants from various incubation conditions or with intact fMLP-stimulated PMN. These
mixtures were incubated at 4°C for 45 min, centrifuged at 10,000 x g for 3 min, and then used to stain aliquots of fresh nonfixed fMLP-stimulated PMN. Binding of anti-CR1 was determined by staining with excess (NH₄)₂SO₄-purified FITC-conjugated goat anti-mouse IgG F(ab’)₂, 250 μg/ml, and flow cytometry.
D. Results

Redistribution and loss of CR1 during stimulation of PMN. We first wished to determine if the total content of CR1 varied as the surface expression increased in response to stimulation. PMN were isolated and resuspended in media free of serum or C3b. The cells were incubated at 37°C with stimuli, and aliquots were removed for staining after 15, 30, and 60 min. The total cellular content of CR1 was measured by indirect immunofluorescence using quantitative flow cytometry of fixed saponin-permeabilized PMN. Surface expression of CR1 was measured on fixed nonpermeabilized PMN. Total CR1 was used to normalize the specific mean fluorescence of all samples within each experiment. This value was identical in freshly isolated PMN (held at 4°C) and PMN incubated for up to 60 min at 37°C without additional stimuli.

Surface expression of CR1 on PMN stimulated with 10⁻⁸M fMLP increased 6.3-fold during the first 15 min of incubation and remained stable over the following 45 min (Fig. 1). In contrast, total CR1 decreased by 15% during the first 15 min of stimulation, and fell an additional 15% by 30 min. Thus, even while surface CR1 expression increased, the total cellular content of CR1 decreased.

A similar study of CR1 modulation during ionomycin stimulation (Fig. 2) shows that surface CR1 increased 7.8-fold within the first 15 min,
but decreased thereafter, resulting in a net increase of 4.5-fold at 60 min. At 15 min, total CR1 was only slightly greater than surface CR1. After 30 min of ionomycin stimulation, total and surface CR1 were nearly equal, suggesting that the total cellular pool of CR1 was expressed on the surface of PMN as a result of ionomycin stimulation. The late decrease in surface CR1 on ionomycin-stimulated PMN, after the initial increase, may be related to depletion of the intracellular CR1 pool. In contrast, fMLP-stimulated PMN did not express all of their CR1 on the surface (Fig. 1), since total CR1 was greater than surface CR1 throughout the period studied. The magnitude of CR1 loss varied slightly between experiments, but within individual experiments the loss during ionomycin stimulation was always greater than the loss during fMLP stimulation.
Figure 1. Effect of fMLP on total and surface CR1 pools.

PMN were incubated with fMLP at 37°C, and aliquots were removed after 15, 30, and 60 min of incubation. The PMN were then analyzed for total (filled circles) and surface (open circles) CR1 as described. The mean of two experiments is shown.
Figure 2. Effect of ionomycin on total and surface CR1 pools.

PMN were incubated with ionomycin at 37°C, and aliquots were removed after 15, 30, and 60 min of incubation. The PMN were then analyzed for total (filled circles) and surface (open circles) CR1 as described. The mean of two experiments is shown.
Measured CR1 loss is not due to fixation or permeabilization.

We considered the possibility that the observed results were due to preferential destruction by the aldehyde fixative of more accessible surface CR1, relative to intracellular CR1 antigen. If this were the case, increased surface expression of CR1 would result in increased loss. To determine whether fixation resulted in loss of detectable surface CR1, we compared surface CR1 detected on fixed nonpermeabilized PMN to surface CR1 detected on identical samples which were incubated and stained in parallel, but not fixed (Fig. 3). PMN were incubated with or without stimuli for 0 to 60 min to produce samples with a wide range of surface CR1. Linear regression analysis of fixed and nonfixed aliquots from 41 samples generated a line through the origin with slope of 0.82, r=0.87, indicating an 18% loss of antigen due to fixation. Therefore, although some surface CR1 antigen is not detected after fixation, this loss is much too small to account for the 40% to 60% loss of total CR1 apparent in Figs. 1 and 2.
Figure 3. Analysis of the effect of fixation on surface CR1 detection.

PMN were incubated without stimuli at 0°C or 37°C for 0 to 60 min (open circles), with fMLP for 15 min (open triangles) or 30 to 60 min (filled triangles), or with ionomycin for 15 min (open boxes) or 30 to 60 min (filled boxes). Linear regression analysis of nonfixed and fixed nonpermeabilized aliquots stained for surface CR1 generated the solid line shown with slope of 0.82, r=0.87.
We then wished to demonstrate that the measured total CR1 was independent of surface expression. Total CR1 detected in fixed permeabilized PMN was plotted as a function of the surface CR1 detected on fixed nonpermeabilized PMN using paired aliquots, as above (Fig. 4). It is apparent that for any degree of CR1 surface expression, multiple values for total CR1 exist. The points tend to cluster according to the incubation conditions, suggesting that the extent of total CR1 loss varies according to the type and duration of stimulation. This implies that the loss of CR1 may be a function of the cellular processes activated by various stimuli, and is not due to fixation or permeabilization.

Extraction of CR1 during saponin permeabilization of fixed PMN was also considered. Fixed nonpermeabilized cells were stained by indirect immunofluorescence and analyzed by flow cytometry. These same samples were then treated with saponin and reanalyzed by flow cytometry. The specific fluorescence was not substantially altered by saponin treatment, indicating that permeabilization does not release surface CR1 from fixed PMN.
Figure 4. Analysis of the effect of fixation on intracellular CR1 detection.

PMN were incubated without stimuli at 0°C or 37°C for 0 to 60 min (open circles), with fMLP for 15 min (open triangles) or 30 to 60 min (filled triangles), or with ionomycin for 15 min (open boxes) or 30 to 60 min (filled boxes). To evaluate whether selective sensitivity to fixation might explain the loss of total CR1 observed, we estimated maximum surface CR1 loss due to fixation as 18% of surface CR1 (from Fig. 3). This loss is shown by the dotted line beginning at the theoretical point where 100% of CR1 is intracellular. Nonpermeabilized and permeabilized aliquots of fixed cells were stained for CR1. The points representing stimulated PMN all fall well below the line predicted, indicating that selective destruction of surface CR1 during fixation cannot account for the CR1 loss observed.
Comparison of CR1, Fc receptor, and $\beta_2$-microglobulin modulation during PMN stimulation. To evaluate whether redistribution and loss during PMN activation is specific to CR1, we measured total and surface $\beta_2$-microglobulin and 55-70 kD PMN Fc receptor in parallel with changes in CR1 during PMN stimulation. The results (Table I) indicate that, for all three proteins, only a fraction of the total cellular pool is expressed on the PMN surface. Stimulation with fMLP induced increases in surface CR1, while surface expression of both $\beta_2$-microglobulin and Fc receptor decreased slightly. These decreases in surface expression may reflect internalization, but not degradation, since the total amounts of $\beta_2$-microglobulin and Fc receptor detected were not altered.

Ionomycin stimulation also reduced surface expression of $\beta_2$-microglobulin and Fc receptor. Total $\beta_2$-microglobulin was not affected by ionomycin stimulation. Total Fc receptor decreased during ionomycin stimulation, though only half as much as CR1.

Therefore, the loss and redistribution of CR1 concomitant with increased surface expression represents a novel pattern of modulation of this receptor which is different from that of $\beta_2$-microglobulin and the 55-70 kD PMN Fc receptor.
Table I. Relative fluorescence of total and surface CR1, \( \beta_2 \)-microglobulin, and Fc receptor on stimulated and nonstimulated PMN.

Isolated PMN were incubated at 37°C for 1 hr in media alone, with \( 10^{-4} \text{M fMLP} \), or with \( 10^{-7} \text{M ionomycin} \) and 1.2 mM Ca\(^{2+} \). The cells were then fixed and analyzed for surface and total antigen. For each antigen, the values shown represent the specific fluorescence normalized to the nonstimulated permeabilized control for that antigen (total, in media alone), as described in the text. The mean \( \pm \text{SEM} \) is shown for the number of experiments indicated.

<table>
<thead>
<tr>
<th></th>
<th>Media Alone</th>
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<tr>
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<tr>
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<td>.13±.01</td>
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</table>
Evidence for intracellular degradation as the mechanism of CR1 loss. Surface receptors on other cell types are known to be internalized and degraded within lysosomes (Mellman et al., 1983; Beguinot et al., 1984; Krupp & Lane, 1982). Antibody-crosslinked surface CR1 may also be delivered to lysosomes (Abrahamson & Fearon, 1983). Lysosomal delivery of surface CR1, and other receptors, generally depends on ligand binding or crosslinking. However, our experiments utilized isolated PMN in the absence of ligand or any other crosslinking reagent. Despite the absence of crosslinking reagents, we investigated the possibility that the CR1 loss measured might involve internalization and subsequent intracellular degradation.

To examine the role of acidic compartments in CR1 loss, PMN were stimulated in the presence of the acidotropic weak base NH₄Cl, which is known to elevate the pH of acidic PMN organelles (Klempner & Styrt, 1983). Inclusion of NH₄Cl inhibited the loss of total CR1 in fMLP-stimulated PMN by 42% (Table II). NH₄Cl inhibited total CR1 loss from ionomycin-stimulated PMN by 33%. Surface CR1 expression of fMLP- or ionomycin-stimulated PMN was not affected by NH₄Cl addition (Table II). NH₄Cl did not alter the surface or total CR1 in nonstimulated PMN. These results suggest that CR1 degradation may occur in an acidic compartment.

To evaluate the importance of proteolytic enzymes in CR1 loss, we stimulated PMN in the presence of phenyl-methyl-sulfonyl-fluoride
(PMSF), a protease inhibitor which diffuses freely across cell membranes (Turini et al., 1969). Unlike some other protease inhibitors, PMSF does not alter fMLP binding or uptake (Niedel et al., 1980).

PMSF inhibited the loss of total CR1 in fMLP- and ionomycin-stimulated PMN by 87% and 69%, respectively (Table II). In contrast to NH₄Cl, which inhibited loss of total CR1 without affecting surface expression, inclusion of PMSF during PMN stimulation allowed additional increments in surface expression of CR1 (Table II). PMSF did not significantly affect surface or total CR1 in nonstimulated PMN. The effect of PMSF on total CR1 was greater than that on surface CR1, indicating that the inhibition of total CR1 loss was not entirely due to increases in surface CR1.

To test the possibility that membrane or secreted proteases degrade CR1 molecules exposed on the cell surface, we stimulated PMN with fMLP in the presence of the cell-impermeant protease inhibitors soybean trypsin inhibitor, or Eglin C; a peptide which blocks elastase and cathepsin G activity (Seemuller et al., 1981). PMN stimulated in the presence of trypsin were included for comparison. In contrast to PMSF, neither soybean trypsin inhibitor nor Eglin C enhanced fMLP-induced increases in CR1 surface expression. Trypsin reduced surface CR1 to less than 10% of the fMLP-stimulated control, confirming the protease sensitivity of CR1 (Berger et al., 1982). Thus, extracellular proteases do not appear to contribute to CR1 loss, suggesting that the major site of PMSF action is intracellular.
Although the results using NH₄Cl and PMSF implicate internalization and intracellular degradation as the mechanism of CR1 loss, we also investigated the possibility that shedding of CR1 from the PMN surface contributed to CR1 loss. This alternative was considered because shedding of CR1 has been demonstrated on osmotically shocked PMN (Takahashi et al., 1985), and a soluble plasma form of CR1, possibly shed from blood cells, has been described (Yoon & Fearon, 1985). Supernatants of PMN stimulated with fMLP or ionomycin, which had lost 44% and 56%, respectively, of total CR1 over 1 hr, were compared to supernatants of nonstimulated cells. These supernatants were used to make limiting dilutions of anti-CR1. If shed CR1 was present in the supernatants, it would be expected to bind to the anti-CR1 and inhibit the antibody's ability to bind surface CR1 on fresh fMLP-stimulated PMN.

When anti-CR1 was mixed with supernatant from PMN incubated at 37°C without stimuli, with fMLP, or with ionomycin, the amount bound by surface CR1 on fresh PMN were 90%, 86%, and 96%, respectively, of that bound with anti-CR1 diluted in buffer alone. Thus, binding of anti-CR1 by supernatants of stimulated PMN was negligible and not significantly different than anti-CR1 binding by supernatants of nonstimulated PMN. When an identical aliquot of anti-CR1 was adsorbed with intact fMLP-stimulated PMN, the amount of anti-CR1 subsequently bound was reduced by 76%, relative to an equivalent amount of anti-CR1 mixed with buffer alone. This verified the sensitivity of the assay. Thus, shedding of intact CR1 is not a likely mechanism of loss during PMN stimulation.
Table II. Inhibition of CR1 loss during PMN stimulation.

Isolated PMN were incubated at 37°C for 1 hr with 10^{-8}M fMLP or 10^{-7}M ionomycin and 1.2 mM Ca^{2+}, with or without the addition of 37 mM NH_{4}Cl or 1 mM PMSF. The cells were then analyzed for surface and total CR1. The data were normalized as described in the text. The mean ±SEM are shown for 9 experiments with NH_{4}Cl and 4 experiments with PMSF. NH_{4}Cl or PMSF increased total CR1 by less than 7% or 14%, respectively, in nonstimulated PMN. NH_{4}Cl or PMSF altered surface CR1 expression in nonstimulated PMN by less than 8% or 3%, respectively. The means without inhibitors are for cells in the same series of experiments which included inhibitors. Since the magnitude of CR1 loss varied slightly between experiments, the means for similar conditions in A. and B. are not identical. For a larger series without inhibitors see Table I.

A. Effect of NH_{4}Cl

<table>
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<th>Incubation Condition</th>
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<th>Ionomycin</th>
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<tr>
<td></td>
<td>-NH_{4}Cl</td>
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<td>total CR1</td>
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<td>surface CR1</td>
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B. Effect of PMSF

<table>
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<th>Incubation Condition</th>
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<th>Ionomycin</th>
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<td>-PMSF</td>
<td>+PMSF</td>
</tr>
<tr>
<td>total CR1</td>
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<td>0.89±.04</td>
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<tr>
<td>surface CR1</td>
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</table>
Light microscopic morphology of CR1 loss and redistribution.
We used immunofluorescent microscopy to compare the localization of intracellular CR1 in PMN incubated under the conditions described (Fig. 5). The pattern of CR1 staining in fixed permeabilized PMN was similar for PMN incubated at 37°C without stimuli or held at 4°C, and was granular throughout the cytoplasm. A negative image of the nuclear lobes was clearly visible, but little surface staining was identifiable (Fig. 5).

Corresponding microscopic examination of fMLP-stimulated cells showed dimmer overall fluorescence which partially outlined the periphery of the cell (Fig. 5). In some cells, faint cytoplasmic staining qualitatively similar to that of nonstimulated PMN was apparent.

Addition of NH₄Cl during PMN stimulation with fMLP significantly altered the pattern of fluorescent staining (Fig. 5). In contrast to PMN stimulated without added NH₄Cl, many bright punctate areas of staining were present in the cytoplasm. These foci seemed smaller than those in nonstimulated PMN, and were generally in a single large group which did not define a negative image of the nucleus.

PMSF also altered the distribution of CR1 in fMLP-stimulated PMN (Fig. 5). The overall surface fluorescence was much more uniform than in PMN stimulated with fMLP alone. Additionally, most cells contained a bright cytoplasmic area of staining. These roundish foci were larger than the intracellular patches seen in NH₄Cl treated or nonstimulated PMN.
Thus, it appears that the initial intracellular pool of CR1 in nonstimulated PMN was largely depleted during stimulation. Inclusion of NH₄Cl or PMSF resulted in the accumulation of intracellular pools which were morphologically distinct from each other and from those in nonstimulated PMN, suggesting that intracellular CR1 exists in at least three distinct compartments, depending on the activation state of the cell.
Figure 5. Immunofluorescence of total CR1 demonstrates loss and redistribution after fMLP stimulation and inhibition of loss by NH₄Cl and PMSF.

PMN were incubated for 1 hr without stimuli, with fMLP, fMLP and NH₄Cl, or fMLP and PMSF, as indicated. The PMN were fixed, permeabilized, and stained for total CR1. Exposure and magnification were identical for all of the images shown. Bar = 10 um.
Electron microscopic localization of CR1 in resting and activated PMN. Thus far, the data have demonstrated that an intracellular pool of CR1 is translocated to the cell surface upon PMN activation, and that the intracellular sites of degradation are likely to be distinct from the storage sites. However, intracellular sites which contain CR1 have never been identified ultrastructurally. To better characterize the processes of CR1 translocation and degradation, we sought to localize intracellular CR1 in resting and stimulated PMN.

In resting PMN, CR1 is found in small, smooth surfaced, electron-lucent vesicles (Fig. 6). The irregular borders and small size of these vesicles clearly distinguishes them from primary and secondary granules. These vesicles are found in the cell periphery as well as more central locations, and are occasionally found in association with Golgi cisternae (Fig. 6a).

In fMLP activated cells, intracellular CR1 is not found in small vesicles, but at the plasma membrane and in large multivesicular bodies (Fig. 7a, e). CR1 appears to be present on both the limiting membrane and inner membranes of the multivesicular bodies. These multivesicular bodies are infrequently seen in resting PMN, and are probably formed by endocytosis upon cell activation, since they incorporate extracellular colloidal gold-albumin (Fig. 7c). Thus, it seems likely that these multivesicular bodies retrieve CR1 from the cell surface, and are on the pathway towards CR1 degradation.
Figure 6. Ultrastructural localization of CR1 in resting PMN.

The membranes of small clear vesicles are labelled with peroxidase reaction product (arrows), but conventional granules are not labelled (g). Some Golgi cisternae are also labelled (G).
(This figure provided by Dr. M. Berger, Case Western Reserve University School of Medicine.)
Figure 7. CR1 and multivesicular bodies in activated PMN.

PMN were activated with fMLP for 1 hr.

a, e: Intracellular CR1 is localized to multivesicular bodies in activated PMN.

b: A negative control in which an irrelevant monoclonal antibody was substituted for anti-CR1.

c: Multivesicular bodies internalize gold-conjugated bovine serum albumin from the extracellular fluid during PMN activation.

d: Surface CR1 (detected with gold- rather than horseradish peroxidase-conjugated antibodies) is clustered on the surface activated PMN.

(This figure provided by Dr. M. Berger, Case Western Reserve University School of Medicine.)
E. Discussion

We have developed a new approach for the quantitative analysis of cellular antigens which allows concurrent measurement of total cellular and surface membrane antigen. This method may be applicable to the study of other proteins with large intracellular pools and critically regulated surface expression, including the adipocyte glucose transporter (Karnielei et al., 1981) and urinary epithelium H⁺ transporters (Schwartz & Al-Awqati, 1986). Quantitative analysis of surface expression and proteolysis may also be simplified for proteins like the insulin (Krupp & Lane, 1982), epidermal growth factor (Beguinot et al., 1984), and Fc receptors (Mellman et al., 1983), which are degraded following ligand-mediated internalization. Although similar information might be obtained by alternative approaches, flow cytometry has the additional advantage of measuring individual cells, thus permitting identification of subpopulations of cells which behave differently. The method we describe requires that, like CR1, the molecule studied is immunoreactive, accessible to antibody, and not extracted or excessively altered during fixation and permeabilization.

The results indicate that, even while chemical stimuli induced 4- to 8-fold increases in CR1 surface expression, the total cellular pool of CR1 decreased by as much as 60% over 1 hr. Since these studies utilized isolated PMN, which do not synthesize complement components (Berger &
Frank, 1980), and neither serum nor complement components were added, this degradation was ligand-independent. Thus, in contrast to other receptors which are recycled intact following ligand-independent internalization, CR1 appears to have been degraded.

Our initial finding is corroborated by the data of Changelian et al. who measured total cellular content of CR1 by radioimmunoassay and reported a 15-20% decrease in total cell CR1 during brief phorbol ester stimulation of PMN (Changelian et al., 1985). The loss they reported is less than the loss we observed, possibly because the duration of their experiments was only 27 min, vs. 1 hr in this study. Additionally, the cells they used may have already degraded some CR1 during the activation and increased surface expression induced by their purification procedures (Fearon & Collins, 1983).

In contrast to CR1, the total cellular β2-microglobulin and Fc receptor remained unchanged after fMLP stimulation. The small decrease in total Fc receptor following ionomycin stimulation suggests that Fc receptor may, in part, follow the same path as CR1. This hypothesis is supported by the observation that crosslinking of either CR1 or Fc receptors by anti-CR1 F(ab')2 or aggregated IgG, respectively, caused co-capping of both receptors (Jack & Fearon, 1984).

To better understand the redistribution and loss of total CR1, several possible mechanisms were investigated. Although hyperosmotic stress can cause CR1 to be shed from human PMN (Yoon & Fearon, 1985), shedding cannot account for the CR1 loss we observed. The
apparent lack of involvement of secreted and surface proteases was an expected result, since we stimulated PMN in the absence of cytochalasin B, and PMN do not release significant quantities of granular enzymes under these conditions (Goldstein et al., 1973).

Inclusion of the weak base NH₄Cl during stimulation inhibited the loss of total cellular CR1, consistent with the hypothesis that CR1 is degraded in an acidic intracellular compartment. The fact that surface CR1 expression was not affected by NH₄Cl suggests that NH₄Cl does not affect CR1 externalization or decrease internalization. Microscopic examination of CR1 in NH₄Cl-treated stimulated PMN revealed an intracellular pool which differed morphologically from that in nonstimulated cells and may represent endosomes or immature lysosomes. This would be consistent with the known effects of NH₄Cl in preventing acidification and maturation of lysosomes (Klempner & Styrt, 1983, Wileman et al., 1984).

The membrane-permeant protease inhibitor PMSF also inhibited loss of total cellular CR1 in stimulated PMN. In contrast to NH₄Cl, PMSF treatment during stimulation increased surface expression of CR1 above that of controls similarly stimulated without PMSF addition. These data suggest that in cells not treated with protease inhibitors, intracellular degradation may limit the maximum surface expression achievable. This especially seems to be the case with ionomycin stimulation. Alternatively, the activity of a protease or esterase inhibited by PMSF may be necessary for internalization. Most cells stimulated in the presence of PMSF
contained a brightly stained intracellular deposit of CR1, possibly representing lysosomes or multivesicular bodies.

The quantitative and morphological data imply that CR1 which is protected from degradation by PMSF may return to the surface membrane and/or be directed to the lysosome. In contrast, neutralization of the endosomal or lysosomal pH with NH₄Cl may prevent CR1 from returning to the cell surface. Inhibition of receptor recycling following endosomal pH neutralization has been demonstrated for both epidermal growth factor and transferrin receptors (King et al., 1980; Stein & Sussman, 1986). Since pH neutralization also reduces transport from endosomes to lysosomes (Wileman et al., 1984; Ukkonen et al., 1986), CR1 protected from degradation by NH₄Cl may remain in the endosome. Thus, the differences between the effects of NH₄Cl and PMSF are probably due to distinct mechanisms of action since NH₄Cl neutralizes the acidic environment, while PMSF inhibits proteases, such as cathepsin G and elastase (Barrett, 1981a, b), by direct covalent modification without neutralizing the surrounding pH.

The ultrastructural localization of CR1 in resting and activated PMN provides the first identification of such pools. The vesicles which contain CR1 in resting cells are morphologically similar to the storage pools of alkaline phosphatase in PMN (Rustin et al., 1979) and glucose transporters in adipocytes (Blok et al., 1988), both of which are rapidly transported to the surface upon cell activation. These structures may also be related to the labile intracellular stores of gelatinase, termed tertiary
granules, which have been characterized biochemically (Dewald et al., 1982), but not morphologically. The striking accumulation of CR1 in multivesicular bodies confirms the hypotheses made based on immunofluorescent microscopy, and suggests that these structures are either the site of intracellular CR1 degradation, or are at least on the pathway to that site.

Ligand-independent internalization of CR1 occurs in PMN stimulated with activators of protein kinase C, such as phorbol esters or synthetic diacylglycerols (Changelian et al., 1985; O'Shea et al., 1985). Since treatment of PMN with phorbol esters and fMLP each lead to activation of protein kinase C and induce CR1 phosphorylation in PMN (Changelian & Fearon, 1986), and protein kinase C activation results in phosphorylation and ligand-independent internalization of other proteins, such as the epidermal growth factor receptor in epidermoid cells (Beguinot et al., 1985; Lin et al., 1986), it is possible that the mechanism for ligand-independent internalization of CR1 is similar to that of the epidermal growth factor receptor. However, both epidermal growth factor receptors and macrophage Fc receptors internalized in the absence of ligand are recycled without degradation (Beguinot et al., 1985; Mellman et al., 1983), in contrast to our findings for CR1. CR1 degradation could, in part, be due to its protease sensitivity (Berger et al., 1982), which may limit the ability of CR1 to survive recycling through acid protease-containing compartments.
The data presented suggest that increased surface expression of CR1 during PMN activation is accompanied by internalization, and that internalized CR1 is degraded in an acidic intracellular compartment. Since isolated PMN were incubated without addition of C3b, the CR1 degradation we observed is ligand-independent. The extent to which other receptors are degraded in the absence of ligand remains to be explored.
III. A Golgi-Associated Family of Sedimentable Proteins

A. Summary

A well-regulated system to morphologically and functionally organize the Golgi complex must exist. To isolate such a macromolecular structure, we reasoned that it must remain highly organized, and sedimentable, after solubilization of associated membranes. A Golgi-enriched fraction of rat liver was isolated by flotation, and the proteins that sedimented after detergent solubilization were analyzed by SDS-PAGE. The pellet consisted primarily of proteins with Ms of 35, 43, 45, and 57 kD, and a ~200 kD triplet. No proteins were sedimented in the absence of detergent, suggesting an association with low-density membranes in the preparation. To determine whether these proteins were related to plasma membrane contaminants, we fractionated the Golgi-enriched preparation on isopycnic sucrose gradients. Peaks of plasma membrane and Golgi enzyme activities were resolved, but the four sedimentable proteins were distributed across all fractions. Immunoblot data imply that the 57 kD and 45 kD proteins may be cytokeratins, and that the 43 kD protein is actin. The 35 kD SGP may be uricase. The ~200 kD triplet remains unidentified, but it is not spectrin. Proteins of related, but different, mobilities are present in other subcellular fractions.
These results suggest that cytoskeletal structures may be associated with Golgi membranes, and that they may include the proteins described.
B. Introduction

The Golgi complex is essential to processing and sorting along the secretory path (Farquhar, 1985; Farquhar & Palade, 1981; Tartakoff, 1983). Many newly synthesized proteins leave the endoplasmic reticulum and undergo a number of post-translational modifications as they traverse the Golgi complex. During passage through the Golgi, proteins are sorted for secretion or distribution to storage granules, lysosomes, or, in polarized epithelia, distinct plasma membrane domains. Additionally, the Golgi complex may be the site of intersection of the endocytic and secretory paths (Farquhar, 1978; Snider & Rogers, 1986). Thus, an understanding of transport within and around the Golgi complex could greatly enhance our comprehension of the regulation of intracellular traffic.

The remarkably conserved anatomy of the Golgi apparatus throughout animal and plant cells (Cunningham et al., 1966; Dalton & Felix, 1956; Manton, 1967; Mollenhauer et al., 1968; Rambourg et al., 1979; Whaley, 1975) suggests that the location and organization of the Golgi is important to its function. Although not apparent in individual thin-sections, serial reconstructions demonstrate that the Golgi apparatus is a single- or few-copy organelle consisting of interconnected cisternae (Rambourg et al., 1979). Stacked Golgi cisternae may be found in nearly all cell types, and this implies that a mechanism must exist to give the
cisternae their characteristic shape and to organize the complex network of Golgi cisternae.

There may be several cytoskeletal structural elements associated with the Golgi complex, judging from the distinct consequences of perturbation of the Golgi by different agents and cell cycle events. According to these structural alterations, one could propose a variety of functional roles for cytoskeletal elements in the regulation of Golgi structure.

A regulated and reversible association between individual cisternae might explain the observation that stacked Golgi elements are not found in mitotic cells (Melmed et al., 1973; Robbins & Gonatas, 1964b). Since the Golgi apparatus is present in only a few copies, fragmentation into many vesicle clusters may ensure equal distribution of the Golgi organelle to each daughter cell (Lucocq et al., 1987). Thiamine pyrophosphatase staining, osmium staining, and galactosyl transferase immunocytochemistry have identified clusters of large and small vesicles as the Golgi equivalent during mitosis in HeLa cells (Lucocq & Warren, 1987; Lucocq et al., 1987). Thus, two distinct structural features of the Golgi complex are absent during mitosis. First, the characteristic apposition of Golgi cisternae is not present, even though the identified vesicles are organized in clusters. Second, the pancake-like cisternal shape, which is retained even in subcellular fractions containing individual cisternae, is lost. Serial-section analysis suggests that the vesicles identified by histochemistry and immunocytochemistry are spherical.
The fragmentation during mitosis is different from the Golgi dispersal following microtubule disruption, since small stacks of cisternae are found in nocodazole treated cells (see Fig. 17; Robbins & Gonatas, 1964a; Thyberg & Moskalewski, 1985). As discussed below, microtubules and microtubule-associated proteins may mediate interconnections between small stacks of cisternae and their coalescence into a unified structure.

Other types of cytoskeletal proteins may be necessary for maintenance of cisternal shape and apposition, intra-Golgi transport, and organization of the ribosome- and organelle-free region of cytoplasm surrounding the Golgi stack termed the "zone of exclusion" (Mollenhauer & Morré, 1978),

In this report, we describe detergent-insoluble proteins present on purified low density Golgi membranes, plasma membrane, and in some other subcellular fractions.
C. Materials and methods

Preparation of subcellular fractions. The Golgi-enriched fraction was prepared from rat liver by a combination of published methods (Braell et al., 1984; Bergeron et al., 1973; Bergeron et al., 1982; Ehrenreich et al., 1973; Morré et al., 1970; Morré et al., 1983) which isolate organized stacks of cisternae, rather than Golgi-derived smooth microsomes. Briefly, rats were fasted overnight, sacrificed, and the livers removed. After two washes in ice-cold TKM buffer (50 mM Tris-HCl, 25 mM KCl, 10 mM MgCl₂, pH 7.5) containing 0.25 M sucrose and 1% 225,000 Mr dextran, the livers were minced and a 25% (w/w) homogenate was prepared using a motor-driven Potter-Elvehjem homogenizer. 2.0 M sucrose-TKM was added to the homogenate to bring the sucrose concentration to ~1.0 M. The homogenate was filtered through gauze and divided among ultracentrifuge tubes. 0.9 M sucrose-TKM, 0.4 M sucrose-TKM, and TKM (no sucrose) were gently layered over the homogenate, and the gradients were centrifuged for 3.5 hr at 28,000 rpm in an SW28 rotor (Beckman) or 1 hr at 40,000 rpm in an SW41 rotor (Beckman). The lipid containing TKM (no sucrose) layer was aspirated, and the Golgi fraction was carefully collected from the 0.4 M/ 0.9 M sucrose interface. Preliminary experiments indicated that after snap-freezing and storage in liquid N₂, and thawing in a 37°C water bath, the morphology, density, and electrophoretic
patterns of the Golgi fraction were all unchanged. Therefore, frozen aliquots have been used in many experiments.

Microsomal Golgi fractions were prepared as described (Ehrenreich et al., 1973), as were plasma membrane (Hubbard et al., 1983) and classical differential centrifugation subcellular fractions (after de Duve, Beaufay & Amar-Costesec, 1976).

Galactosyl transferase (Rome et al., 1979), alkaline phosphodiesterase (Hubbard et al., 1983), 5'-nucleotidase (Widnell & Unkeless, 1968) and β-hexosaminidase (von Figura, 1978) were assayed using published procedures. Total protein was measured using the Pierce BCA protein assay kit.

Fractions were mixed gently with equal volumes of 2-fold concentrated fixative (generally 3-5% glutaraldehyde) and pelleted at 10,000 rpm for 10 min at 4°C in an SW50.1 rotor (Beckman). Fresh fixative was then layered over the pellet for 12-16 hr, and the pellets were osmicated, stained en bloc with 1% uranyl acetate, dehydrated, and embedded in Spurr's resin (Polysciences). Thin-sections were examined on a JEOL 100CX II transmission electron microscope at 60 kV.

Analytical separation of Golgi complex and plasma membrane fractions. The liver of a fasted rat was homogenized in 0.5 M sucrose-TKM, and 2 M sucrose in H₂O was added until the density was equal to that of 40% sucrose. After a 10 min centrifugation at 3000 rpm in an RT6000B with H-1000B rotor (Dupont), the supernatant was underlayed (at 4°C) below a 12 ml spacer of 40% (w/w) sucrose in 5 mM Tris-HCl.
pH 7.5, above which a 20 ml linear 15-40% sucrose gradient (also in 5 mM Tris-HCl, pH 7.5) had been poured. After 12 hr at 28,000 rpm in an SW28 rotor, 1.5 ml fractions were collected, analyzed for galactosyl transferase and alkaline phosphodiesterase, and used to purify SGPs.

**Isolation of sedimentable Golgi-associated proteins.** After solubilization with 0.5% Triton X-100, ultracentrifugation at 20,000 rpm in an SW41 for 1 hr over discontinuous sucrose step gradients or cushions of 20-60% sucrose. Fractions were collected and proteins precipitated by addition of 10% TCA at 4°C. After washes with 5% TCA, and then 80% acetone (4 hr, -20°C), protein pellets were solubilized in reducing SDS-PAGE sample buffer containing 5% β-mercaptoethanol (Laemmli, 1970). Pellets from the initial gradient were solubilized in sample buffer directly. In later experiments, it was determined that the addition of 0.75 M KCl improved the purity of pelleted proteins. Thus, to only analyze pelleted proteins, Triton X-100 and KCl were routinely added to subcellular fractions to final concentrations of 0.5% and 0.75 M, respectively. The fractions were then layered over 35-40% sucrose-TKM with 0.5% Triton X-100, centrifuged for 1.5 hr at 28,000 rpm in an SW50.1, and the pellets solubilized in sample buffer as above.

**SDS-PAGE, silver staining, and immunoblots.** SDS-PAGE analysis (Laemmli, 1970), silver staining (Guevera et al., 1982), and immunoblots onto immobilon (Millipore) (Towbin et al., 1979) were as described.
D. Results

Identification of detergent-insoluble Golgi-associated proteins. Ultrastructural examination of the isolated Golgi fraction demonstrated that lipoprotein droplets and stacked cisternae constitute the bulk of the fraction, but unstacked cisternae are also present (Fig. 8A). Plasma membrane, rough microsomes, and mitochondria may also be seen infrequently. Enzymologic analyses show that galactosyl transferase activity is enriched approximately 100-fold (per mg protein) in the Golgi fraction relative to the homogenate, with an average galactosyl transferase activity yield of 35%. The enrichment is equal to the best published values, yet the single floatation gradient isolation method is significantly less laborious than previous approaches (Bergeron et al., 1973; Morré, 1971). The fraction is only approximately 15-fold enriched in 5'-nucleotidase and alkaline phosphodiesterase, both markers of the plasma membrane, and β-hexosaminidase, a lysosomal marker, is enriched only 4-fold.
Figure 8. Ultrastructure of isolated Golgi fractions and detergent treated Golgi fractions.

A: The Golgi fraction isolated is composed primarily of stacks of cisternae (arrows). Bar = 50 nm.

B, C: When fixed in the presence of nonionic detergent, many short doublet fragments, sometimes associated with membrane ghosts, are apparent. Magnification in B is identical to A. In C, bar = 10 nm.
Solubilization of the Golgi-enriched preparation with nonionic detergent followed by ultracentrifugation over a discontinuous sucrose step gradient results in sedimentation of a small fraction of the total Golgi proteins (Fig. 9). SDS-PAGE analysis of the fractions reveals that this material includes five sedimentable Golgi proteins (SGPs) with Ms of 35 kD, 43 kD, 45 kD, 57 kD, and ∼200 kD (triplet). The 43, 45, and 57 kD SGPs sediment as a group, but the 35 kD protein has a broader distribution, and the 200 kD triplet a tighter distribution. Albumin, a major secretory protein present in the rat liver Golgi fraction, does not sediment. No proteins sediment under these conditions when detergent was omitted. Furthermore, the SGPs remain with the Golgi fraction membranes when the sucrose concentration is adjusted to 1.0 M and the Golgi membranes re-isolated by flotation, just as in the initial purification. The SGPs sediment away from the twice-purified Golgi membranes when Triton X-100 is added.
Figure 9. Sedimentation of detergent-insoluble Golgi-associated proteins.

Silver stain of a reduced 10% SDS-PAGE analysis of a 30-60% discontinuous sucrose gradient over which the Golgi fraction was loaded. Centrifugation was in an SW41 rotor for 1 hr at 20,000 rpm. The Golgi fraction contained 0.5% Triton X-100. Thirteen fractions (lanes 3-15) and a pellet (lane P) were collected, with the load region occupying the first 6 fractions. Aliquots of the total homogenate (lane 1) and the Golgi fraction (lane 2) are also shown. The fractions are shown consecutively, beginning with the top of the gradient in lane 3 and the pellet in the lane P. A doublet of 43 kD and 45 kD, a single band at 57 kD, a weak band at 35 kD, and a group of proteins with Mr ~ 200 kD are seen towards the bottom of the gradient.
Since the SGPs float with the Golgi fraction during the initial isolation, they must be associated with Golgi membranes or some other low density component. To characterize the nature of this association of SGPs with membranes, Golgi fractions were treated with graded concentrations of Triton X-100. The SGPs do not pellet in the absence of detergent or after treatment of Golgi fractions with low levels of Triton X-100 (≈0.02%) which do not solubilize (Kreibich et al., 1973; Kreibich & Sabatini, 1974), but merely permeabilize, the membranes (Fig. 10A). Higher concentrations of Triton X-100 (≥0.2%), which completely solubilize the membranes, are required to release the detergent-insoluble proteins from the low-density Golgi membranes. Thus, the SGPs are likely to be anchored to the membranes, either directly or indirectly. This analysis does not allow discrimination between integral and peripheral membrane associations. However, the observation that none of the Triton-insoluble proteins are released from the membranes at low detergent concentrations, but all are released at higher concentrations suggests that the proteins may be part of a single protein complex.

Addition of up to 2 M KCl to the detergent-solubilized Golgi fraction in TKM buffer prior to ultracentrifugation eliminates the presence of a number of proteins which are variably present in pelleted material, but does not affect recovery of the five major SGPs. Since the addition of KCl did not affect recovery of SGPs, the stability of the protein-protein interactions which form the SGP complex was examined under harsher
conditions (Fig. 10C). The 43 kD and ~200 kD SGPs are solubilized by 0.1 M Na₂CO₃, pH 11, but the 45, 57, and 35 kD SGPs are still recovered in the pellet. The effect of 2 M MgCl₂ is similar, but the 35 kD is also solubilized. Only the 35 kD SGP is recovered after treatment with 6 M urea, and all SGPs are solubilized by 1% SDS, 4 M KSCN, or 6 M guanidine hydrochloride. Phosphotungstic acid has been reported to unstack isolated Golgi fractions (Mollenhauer et al., 1973; Cunningham et al., 1974), but addition of 1% phosphotungstic acid results in the sedimentation of many proteins not normally recovered in the pellet. These results suggest that the protein-protein interactions which maintain this complex are stable, but noncovalent.

Comparison of the mobility of the SGPs on reducing and nonreducing SDS-PAGE was surprising in that the 43 kD SGP which migrated as a single band under reducing conditions is actually a triplet with two faster migrating bands of ~40 kD under nonreducing conditions. The ~200 kD SGP does not enter nonreducing gels, and the 35 kD SGP migrates slightly faster under nonreducing conditions.
Figure 10. Sedimentation characteristics of Golgi-associated proteins.

A: Golgi fractions to which no detergent (lane 1), or Triton X-100 at .02% (lane 2), .1% (lane 3), .5% (lane 4) had been added were layered over 40% sucrose-TKM containing the same Triton X-100 concentrations. After centrifugation in an SW50.1 rotor for 2 hr at 28,000 rpm, pellets were analyzed on silver stained 10% SDS-PAGE.

B: After removal of large insoluble aggregates by centrifugation, KCl was added to the cleared rat liver homogenate. Triton X-100 (0.5%) was added to the sample shown in lane 2, but omitted from that in lane 1, and the samples layered over 40% sucrose-TKM containing the same concentrations of Triton X-100. After centrifugation, the pellets were analyzed as in A. Pellets from similarly treated Golgi fractions, either with (lane 4) or without (lane 3) Triton X-100, are shown for comparison.

C: Triton X-100 and 1% SDS (lane 1), 6 M urea (lane 2), 0.1 M Na₂CO₃ (lane 3), 2 M MgCl₂ (lane 4), 1% phosphotungstic acid (lane 5), 4 M KSCN (lane 6), 6 M guanidine-HCl (lane 7), or 0.75 M KCl (lane 8) were added to Golgi fractions. The fractions were then layered over sucrose cushions containing Triton X-100 and the listed additional reagent (phosphotungstic acid, KSCN, and guanidine-HCl were not included in cushions) and analyzed as in A and B. Pellets are shown.
SGPs are present in other subcellular fractions. Although the SGPs are highly enriched in the detergent-insoluble pellet of the Golgi fraction, the bulk of protein in the Golgi fraction is in the supernatant. Since the Golgi fraction isolated is certainly impure and also includes only 35-50% of the galactosyl transferase activity present in the homogenate, we asked if SGPs could be isolated from other subcellular fractions. Proteins which comigrate with the SGPs are present in the postnuclear supernatant of crude homogenate, but are also pelleted in the absence of detergent (Fig. 10B). In the presence of detergent, proteins with similar Ms to the SGPs are the major sedimentable proteins recovered in the pellet from total homogenate. In the absence of detergent many other proteins also pellet, presumably due to the sedimentation of dense membranous organelles. The ~200 kD SGP does not comigrate exactly with high molecular weight proteins present in the homogenate, and is clearly distinct from the spectrin doublet of rat erythrocytes (Fig. 11A).

Since SGPs are abundant in the homogenate, we surveyed classical differential centrifugation subcellular fractions for SGP content. Although the relative purity varies greatly, SGPs are present in all of these fractions, including the final supernatant.
Figure 11. Sedimentable proteins in other membrane fractions.

A: Higher resolution analysis of the 200 kD SGPs on a silver stained 5% SDS-PAGE. Normally prepared SGPs (lane 1), spectrin prepared as sedimentable protein from rat erythrocytes (lane 2), sedimentable proteins from rat liver homogenate (lane 3), and sedimentable proteins from plasma membrane (lane 4) are compared. Lane 5 contains SGPs prepared from an unusual Golgi fraction. The 1500 x g supernatant of a postnuclear supernatant is normally discarded in the preparation of plasma membrane sheets (Hubbard et al., 1983). A Golgi fraction was isolated by flotation from this 1500 x g supernatant, and SGPs were prepared (lane 5).

B: Sedimentable proteins from a control stacked Golgi fraction (lane 1), an unstacked microsomal Golgi fraction (Ehrenreich et al., 1973) (lane 2), and heavy microsomes (Ehrenreich et al., 1973) (lane 3) are compared on silver stained 10% SDS-PAGE.

C: Sedimentable proteins from plasma membrane (Hubbard et al., 1983) (lane 1) and the 1500 x g supernatant derived Golgi fraction, as in A, (lane 2) are compared on silver stained 10% SDS-PAGE.
If the SGPs are involved in cisternal stacking, they might be lost during preparation of a microsomal (unstacked) Golgi fractions (Ehrenreich et al., 1973). Proteins with similar mobilities to the SGPs are isolated from these Golgi fractions, but the 57 kD SGP is remarkably absent (Fig. 11B). Rough microsomes contain an abundance of the 35 and 43 kD SGPs, but do not contain the other SGPs (Fig. 11B).

The plasma membrane is a rich source of membrane-associated cytoskeletal material in rat hepatocytes (Hubbard & Ma, 1983). Plasma membrane fractions are of low density and often contaminate Golgi fractions, so we prepared plasma membrane sheets. These methods have previously been shown to purify actin (43 kD) and intermediate filament proteins (52 and 56 kD) with the membranes (Hubbard & Ma, 1983). These proteins comigrate with the 43, 45, and 57 kD SGPs, and a protein which comigrates with the 35 kD SGP is also found in plasma membrane fractions (Fig. 11C). The high molecular weight components in Golgi and plasma membrane sedimentable proteins differ, although not reproducibly (Fig. 11A). Since plasma membrane fractions are contaminated by Golgi membranes, we developed a new analytical isopycnic separation method to clearly resolve plasma membrane and Golgi fractions. Alkaline phosphodiesterase, an enzyme localized to both apical (canalicular) and basolateral domains in rat liver, and galactosyl transferase, a trans-Golgi enzyme, were used as markers of each organelle. Peaks of plasma membrane and Golgi enzyme activities were resolved (Fig. 12), but the
SGPs distributed across all fractions (Fig. 13), making it impossible to
determine if the peak of SGPs coincided with Golgi, plasma membrane,
was bimodal and coincided with both, or coincided with neither.
Figure 12. Isopycnic resolution of Golgi from plasma membrane.

Galactosyl transferase activity (circles), alkaline phosphodiesterase activity (triangles), and sucrose concentration (line) of fractions from an isopycnic sucrose gradient, beginning with fraction 1 from the top of the tube. Fractions 1-14 comprised the gradient itself, and 15-24 were the spacer and load regions.
Figure 13. Sedimentable proteins recovered from isopycnic gradients.

Silver stain of a 10% SDS-PAGE analysis of the sedimentable proteins recovered from fractions of the gradient whose enzyme profile is shown in Fig. 12. Standards are shown (SGP), and the lanes are numbered by fraction number, beginning at the top of the tube. Fractions enriched in galactosyl transferase or alkaline phosphodiesterase are labelled GC or PM, respectively.
Immunochemical characterization of SGPs. Immunoblots of SDS-PAGE resolved SGPs (Towbin et al., 1979) demonstrated that the 43 kD is detectable by monoclonal mouse anti-actin antibody C4 (Galloway et al., 1987). A monoclonal anti-vimentin antibody V-9 (Osborn et al., 1984) did stain a 57 kD band not seen in the protein stain, and could be clearly distinguished from the 57 kD SGP. Similarly, a 50 kD protein clearly resolvable from the SGPs was detected by monoclonal anti-Type II keratin (Amersham). The 45 and 57 kD SGPs were not recognized by these specific anti-intermediate filament reagents, but were detected by a broad specificity anti-intermediate filament monoclonal antibody (Pruss et al., 1981).

Monoclonal anti-β-tubulin, anti-MAP 1A, anti-MAP 1B, anti-MAP 2, anti-vinculin, and anti-tau antibodies were non-reactive in these immunoblots. Polyclonal rabbit anti-α,β-tubulin, anti-human spectrin, anti-chicken spectrin, anti-tropomyosin, and anti-tau antisera were also non-reactive. Antibody activity was verified by staining lanes which were loaded with crude microtubule proteins. Although the 57 kD SGP roughly co-electrophoreses with tubulin, a doublet is not obvious on gels which do resolve α- and β-tubulin. In occasional gels, the 57 kD SGP is obviously of greater mobility than either α- or β-tubulin. Additionally, the persistence of the 57 kD SGP in 2 M KCl and 4°C, and the lack of reactivity with three different anti-tubulin antibodies suggest that the 57 kD SGP is not tubulin. No antibodies were reactive with the ~200 or 35 kD SGPs.
Thus, it appears that at least some of the 43 kD SGP is related to actin, and that the 45 and 57 kD SGPs are related to intermediate filaments. The putative identity of the 45 and 57 kD SGPs as intermediate filaments is not surprising, since they comigrate with "52" and "56 kD" cytokeratins that have been previously identified in rat liver plasma membrane fractions. Even though negative immunoblot data cannot be conclusive, it is likely that none of the SGPs are identical to, or share major epitopes with the other cytoskeletal proteins listed above.

Polyclonal antibodies to the SGPs were prepared in mouse and affinity-purified on immunoblots of reduced SGPs (Olmsted, 1981). Affinity-purified antibody to the 45 and 57 kD SGPs cross-reacted on immunoblots, but did not react with the ~200 kD SGP. Affinity-purified antibody to the ~200 kD SGP only reacted with that protein. All three affinity-purified antibodies were negative on immunofluorescence of rat clone 9 hepatocytes fixed in 3% formaldehyde. Monoclonal antibodies were made using a mouse which produced polyclonal antiserum that reacted with the SGPs on immunoblots, but the monoclonal antibodies did not react on immunoblots, despite being screened by dot-immunoblot of SDS-treated antigen immobilized on nitrocellulose. The monoclonal antibodies obtained are described in the next section.

**Detergent-resistant Golgi-components identified morphologically.** Although filaments were not obvious by thin-section examination of the Golgi fraction prepared from rat liver, intercisternal cytoskeletal material has been identified in other organisms (Amos & Grimstone, 1968;
Mollenhauer, 1965; Whaley, 1975). Two trivial explanations for the lack of intercisternal material in rat liver Golgi stacks might be i) dissolution by harsh fixatives, or ii) difficulty in resolution due to the presence of membranes. To evaluate if either of these were responsible for our inability to identify cytoskeletal material in rat liver Golgi fractions, Golgi fractions were fixed in 3% glutaraldehyde and 25 mM lysine, a gentle fixative known to preserve normally labile actin filaments (Boyles et al., 1985). Triton-X 100 (0.5%) was included to solubilize membranes and facilitate examination of intercisternal regions. Remarkably, many doublets of straight heavily stained (electron-dense) fibers of approximately the same length as intercisternal regions were prominent (Fig. 8B, C). These may represent detergent-resistant membrane or protein derived from intercisternal regions. Although these detergent-resistant structures are similar in appearance to tabs seen on Golgi-associated lipoprotein particles (Howell & Palade, 1982), we never saw similar structures in Golgi fractions fixed in glutaraldehyde without lysine or detergent. The detergent-resistant structures could also be identified in microsomal (unstacked) Golgi fractions (Ehrenreich et al., 1973) fixed with glutaraldehyde, lysine, and detergent. A large amount of fibrous aggregated material was present in the pellet of fractions fixed with glutaraldehyde, lysine, and detergent, and some membrane-like ghosts could also be identified.
E. Discussion

Our goal in this study was to isolate a Golgi-associated cytoskeleton, or scaffold. A large body of data suggests that such a complex must exist. Our approach was to look for a detergent-insoluble sedimentable complex specifically associated with stacked Golgi fractions. Although we identified five major proteins, three were of identical mobility to actin (43 kD) and intermediate filament proteins (45 and 57 kD) known to be present in plasma membrane fractions of rat liver (Hubbard & Ma, 1983). We also found these proteins in a variety of other subcellular fractions. Since plasma membrane and Golgi fractions are both of low density, cross contamination is a significant problem in both directions. To resolve this problem, we developed a new analytical isopycnic gradient which clearly separates plasma membrane and Golgi with better resolution than previously described methods (Bartles et al., 1987). Unfortunately, even with this sensitive separation, we were unable to determine if the proteins identified were associated with only plasma membrane or Golgi, both, or neither. Thus, we are uncertain as to the origin of the SGPs isolated from stacked Golgi fractions.

A clue as to the identity of the fourth SGP identified may come from electron microscopy of SGP preparations. Semi-crystalline structures were abundant, and may represent damaged peroxisomal cores. The
peroxisomal core is composed of a single protein, uricase, which has an Mr of 35 kD (Hruban & Swift, 1964). Porcine uricase comigrates with the 35 kD SGP from rat liver on some gels. If the 35 kD protein is uricase, it is unusual that it would be in our preparations, since peroxisomes are much more dense than Golgi membranes. However, a small number of damaged peroxisomes, which are sometimes less dense than normal peroxisomes (Alexson, et al., 1985), might account for our results. This does not imply that the other SGPs are peroxisome-derived, since the 35 kD SGP has a somewhat broader distribution than the other SGPs when sedimented into a sucrose gradient. The solubilization of the 35 kD SGP by various treatments differed from the other SGPs, suggesting that the 35 kD SGP may not be part of the same complex as the other SGPs.

The ~200 kD SGP triplet remains a mystery. It seems to be a very large complex held together, at least in part, by disulfide bonds, since it does not enter nonreducing SDS-PAGE. Its distribution in sucrose gradients is tighter than the 43, 45, and 57 kD SGPs. The ~200 kD triplet is clearly not spectrin, and sometimes migrates differently than similarly sized proteins isolated from total homogenate or plasma membrane. It was not reactive with any of the antisera used, and is much too large to be clathrin heavy chains. It is possible that this ~200 kD triplet is the Golgi-associated member of a specialized family of large proteins, of which other members are specifically associated with other membrane domains.

Since our affinity-purified polyclonal anti-SGPs were nonreactive by immunofluorescence, and the monoclonal antibodies prepared against
SGPs (described in the next section) were nonreactive on immunoblots, we have been unable to localize the SGPs in intact cells. Without this critical information, it is difficult to determine whether the SGPs are in fact Golgi-associated cytoskeletal proteins which are also associated with other membranes, or whether the SGPs result from unavoidable impurities in subcellular fractions.

Nevertheless, immunization of mice with SGPs resulted in the production of an interesting monoclonal antibody (described in the next section) which facilitate studies of the cytoskeleton as a single organelle. Furthermore, we have described a superior analytical method for separation of Golgi and plasma membrane fractions.

A. Summary

Monoclonal antibodies were generated against detergent-insoluble cytoskeletal proteins isolated from low-density membrane fractions of rat liver. By immunofluorescence, one of the antibodies stained three distinct structures in cultured rat fibroblast and hepatocyte lines as well as the PtK₂ rat-kangaroo kidney epithelial line. These structures are: i) many tangled filaments similar to intermediate filaments (IFs), ii) fewer and variable numbers of straight filaments, and iii) punctate cytoplasmic foci, often most intense around the nucleus. All three of these structures are resistant to extraction by nonionic detergent. Close examination reveals that the tangled and straight filaments are not stained uniformly, but as a series of bright patches. In cells treated with nocodazole, the antibody reacts strongly with a perinuclear filamentous cage. Very few tangled filaments are detected in these cells, however, the straight filaments and punctate cytoplasmic staining are resistant to nocodazole treatment. The distribution of the tangled filaments and their response to nocodazole is characteristic of vimentin IFs, but double-label immunofluorescence shows
only partial coincidence of staining with vimentin or cytokeratin IFs. The straight filaments coincide with some actin stress fibers, but the punctate cytoplasmic staining is not related to IFs, actin, or tubulin. Thus, this monoclonal antibody stains a novel group of three seemingly unrelated cytoskeletal structures, including a previously undescribed insoluble nonfilamentous pool.

Taken as a whole, two hypotheses are consistent with these data. i) the antigen recognized may be a protein which has a large insoluble cytoplasmic pool and binds both IFs and actin, but only binds to a subset of each class of filaments. ii) The antigen could be a shared epitope present on both classes of filaments and on nonfilamentous insoluble cytoplasmic structures.
B. Introduction

The cytoskeleton is made up of intermediate filaments (IFs), microtubules, and microfilaments. Just as microtubules and microfilaments are assembled from tubulin and actin, respectively, IFs are polymeric (reviewed in: Lazarides, 1982; Steiner & Parry, 1985). In cultured cell lines derived from non-keratinizing epithelia, IF proteins are generally of two types, cytokeratins and vimentin. The cytokeratins are a heterogeneous group of proteins classified as Type I or Type II, based on size and charge. Cytokeratin IFs contain both Type I and Type II cytokeratins, and form a network which is similar to, but clearly distinct from, vimentin IFs (Osborn et al., 1980).

The IF network is functionally related to both microtubules and microfilaments. For example, depolymerization of microtubules with colcemid or nocodazole results in collapse of vimentin, but not cytokeratin, IFs into a cage surrounding the nucleus (Osborn et al., 1980). Mitosis has similar effects on vimentin, and in some cases, cytokeratin IFs (Aubin et al., 1980; compare to Franke et al., 1982). Collapse following microtubule depolymerization is also, in part, dependent on microfilaments (Hollenbeck et al., 1989). Other evidence suggesting that the IF network is associated with microfilaments include the observations that some cytokeratin IF bundles align with microfilaments after colcemid treatment (Osborn et al., 1980) and that the combined effects of microtubule inhibitors and
cytochalasins exceed those of microtubule inhibitors alone (Knapp et al., 1983; Hollenbeck et al., 1989).

Distinctly nonfilamentous cytoplasmic vimentin and cytokeratin IF aggregates have been observed during mitosis (Horwitz et al., 1981; Franke et al., 1982). Similar granular IF aggregates also form in cells with perturbed IF due to microinjection of IF-specific antibodies (Eckert et al., 1981; Klymkowsky et al., 1983). These granular IF structures are not present in normal interphase cells, but are otherwise similar in appearance to the foci we describe. However, the foci which are stained by our antibody do not contain vimentin or cytokeratins.

In this report, we present immunochemical evidence that an IF-associated epitope is constitutively present in nonfilamentous cytoplasmic foci, despite the absence of IF proteins at these sites. In addition to IF-like filaments, the epitope is also localized along some microfilaments.
C. Materials and methods

Preparation of monoclonal antibodies. Subcellular membrane fractions were prepared from rat livers homogenized with a motor-driven Potter-Elvehjem homogenizer 20% w/w in 0.25 M sucrose-TKM (50 mM Tris-HCl, 25 mM KCl, 10 mM MgCl₂, pH 7.5) at 4°C. 2 M sucrose-TKM was added to bring the sucrose concentration to ~1.0 M, and 0.9 M sucrose-TKM and 0.4 M sucrose-TKM were layered over the filtered homogenate. After 1 hr at 40,000 rpm in an SW41 rotor (Beckman), the 0.4 M/0.9 M sucrose interface was collected. It was approximately 100-fold enriched in galactosyl transferase activity, per mg of protein, relative to the postnuclear supernatant (Rome et al., 1979), but only approximately 15-fold enriched in 5'-nucleotidase (Widnell & Unkeless, 1968) and alkaline phosphodiesterase (Hubbard et al., 1983), and 4-fold enriched in β-hexosaminidase (von Figura, 1978). Cytoskeletal material was prepared from the fraction by addition of Triton X-100 to 0.5% and KCl to 0.75 M, followed by centrifugation at 25,000 rpm in an SW41 rotor over a cushion of 30% (w/w) sucrose at 4°C. The pellet was resuspended in Tris-HCl buffered saline, pH 7.5, and used to immunize BALB/c mice intraperitoneally after emulsification in Freund’s adjuvant. Each immunization was with all of the cytoskeletal material isolated from the low-density membrane fraction prepared from one rat liver, as described above. Complete adjuvant was used to emulsify the antigen for the first
immunization, and incomplete adjuvant was used for subsequent immunizations after 3, 7, 17, and 19 weeks. Spleen cells were fused with Sp2/0-Ag14 mouse myeloma cells 3 days after the last immunization (Galfre et al., 1977; Shulman et al., 1978). Hybridomas were screened by a dot immunobinding assay (Hawkes et al., 1982) against immunogen preparations which had been boiled in SDS-PAGE sample buffer containing 5% (w/v) β-mercaptoethanol (Laemmli, 1970). Two hybridomas whose secretion product intensely stained cytoskeletal elements were studied at great length. One of these appears to react with vimentin, based on analyses similar to those presented here (Turner & Tartakoff, unpublished observations). The other stains an unusual set of structures, as described below.

The CS-1 hybridoma was maintained in RPMI (Sigma) containing 10% FBS (GIBCO) and 10 mM HEPES, pH 7.5, in 5% CO₂ at 37°C. Culture supernatants were concentrated 10-fold by 50% ammonium sulfate precipitation, and the pellets resuspended in phosphate-buffered saline for immunofluorescence. Staining patterns were similar, but of lower intensity, when untreated culture supernatant was used. CS-1 monoclonal antibody is of the IgM class, since it was reactive with heavy-chain specific anti-mouse IgM antisera, but nonreactive with heavy-chain specific anti-mouse IgG antisera, in immunofluorescence experiments (below).

Sucrose gradients for antigen characterization. Cytoskeletal material was prepared by discarding insoluble material from a 20% (w/w) rat liver homogenate in 0.25 M sucrose-TKM using five successive
centrifugations (to remove unbroken cells, nuclei, and large aggregates) for 5 min at 1100 rpm in a Sorvall RT6000B with H-1000B rotor (Dupont). The pellet was discarded after each centrifugation. After addition of Triton X-100 to 1.0% and KCl to 0.75 M, the final supernatant was layered over 30% sucrose (w/w) in 10 mM Tris-HCl, pH 8, and the pellet of a 2 hr, 30,000 rpm spin in an SW41 rotor was collected in 10 mM Tris-HCl, pH 8, and frozen in aliquots. This preparation reacted strongly with CS-1 IgM on dot immunobLOTS, even after treatment at pH 11 or with 10 M urea, but not after 1% SDS in 10 mM Tris-HCl, pH 8. Preliminary electrophoresis and centrifugation experiments demonstrated that the antigen was insoluble at pH 11 and in 10 M urea, but was solubilized by 1% SDS. To compare the sedimentation of CS-1 to vimentin and cytokeratins, the cytoskeletal preparation was adjusted to 50 mM Na₂CO₃, pH 11, incubated for 1 hr at 37°C, and layered over 150 μl layers of 5, 10, 15, and 20% sucrose (w/w) in 50 mM Na₂CO₃, pH 11 in a 5 x 41 mm #344090 ultracentrifuge tube (Beckman). After 4 hr at 30,000 rpm in an SW50.1 rotor (Beckman), 75 μl fractions were collected, and analyzed on 10% reducing SDS-PAGE (Laemmli, 1970) and dot immunobLOTS. Identical sedimentation gradients loaded with high molecular weight marker proteins (Bio-Rad) were run in parallel and analyzed by SDS-PAGE. Dot immunoblots were on Immobilon (Millipore), and used the same primary antibodies used for fluorescence at the same concentrations for 15 hr at room temperature. The cytokeratin strip used a mixture of all four anti-cytokeratin monoclonal antibodies.
After 5 washes in TBS containing 1% normal goat serum, 5 μg/ml horseradish-peroxidase conjugated affinity-purified goat anti-mouse Ig(G, A, M) (Cappel) was applied for 2 hr. The blots were then washed and developed using 3,3-diaminobenzidine as substrate. Since nonspecific staining of each fraction varied, negative controls were antigen blotted without primary antibody.

**Cell culture.** Rat-kangaroo kidney epithelial PtK₂ cells (provided by J. Salisbury, Mayo Clinic), FR 3T3 rat fibroblasts (Chicheportiche et al., 1984), and clone 9 rat hepatocytes (American Type Culture Collection) were maintained in Ham's F-12 media supplemented with 10% FBS (GIBCO) at 37°C in 5% CO₂, and subcultured 2-3 times weekly. For experiments, cells were plated onto sterile 12 mm diameter glass coverslips (Bellco) and used in subconfluent condition after 2-3 days of growth. Nocodazole (Sigma) stock was 5 mg/ml in DMSO, and aliquots were stored at -20°C. Nocodazole treatment was 0.5 μg/ml (1.6 μM) for 9 hr. Results were similar after 2.5 hr of nocodazole treatment.

**Fluorescence microscopy.** Cells were fixed for 10 min in -20°C methanol or in 3% formaldehyde in PBS at 25°C. The staining pattern was not altered by choice of fixative. For detergent treatment prior to fixation, cells were washed once in PBS, incubated in 0.15% Triton X-100 in 10 mM PIPES, 10 mM EGTA, 2 mM MgCl₂, pH 7, for 1.5 min at 25°C, fixed in 3% formaldehyde, and stained as described. Antibodies and dilutions used were: C4 anti-actin used as culture supernatant (Galloway et al., 1987; kindly provided by Dr. G. Perry, Case Western Reserve
University); V-9 anti-vimentin ascites diluted 1:50 (Osborn et al., 1984; ICN); TUB 2.1 anti-tubulin ascites diluted 1:200 (Gozes & Barnstable, 1982; ICN), and LE41, LE61, LE65, and LP1K anti-cytokeratin used as culture supernatants (Lane, 1982; kindly provided by Dr. E.B. Lane, Imperial Cancer Research Fund, England). Rhodamine-conjugated affinity-purified goat anti-mouse IgM-specific antisera and FITC-conjugated affinity-purified goat anti-mouse IgG-specific antisera (Kirkegaard & Perry) were both used at 20 µg/ml.

After fixation in formaldehyde, cells were washed in Tris-HCl buffered saline (TBS), incubated with 50 mM NH₄Cl in TBS for 5 min, and nonspecific sites blocked and membranes permeabilized by a 10 min incubation in TBS containing 5% normal goat serum (TBS-5% NGS) and 0.5% Triton X-100. After methanol fixation, cells were washed for 5 min at 25°C in Tris-HCl buffered saline, and nonspecific sites blocked for 10 min in TBS-5% NGS. Antibodies were diluted in TBS-10% NGS, and antibody incubations were 30 min at 37°C. Between incubations, coverslips were washed 3-5 times with TBS-2% NGS.

A Nikon Microphot-FX equipped with 40X and 100X DIC objectives, epi-fluorescence illuminator, and B-1H and G-2A filter cubes was used for microscopy. An additional 610 nm barrier filter was used to block rhodamine fluorescence when viewing fluorescein. Controls in which one fluorescent-tagged antibody was omitted demonstrated minimal bleed-through to the other channel.
D. Results

**Distribution of CS-1.** CS-1 IgM stains three distinct structures in rat clone 9 hepatocytes, rat FR 3T3 fibroblasts, and rat-kangaroo PtK₂ epithelial cells (Fig. 14A, B). The first is a dense tangled filamentous network which surrounds the nucleus. The filaments are either short straight segments (<10 μm) interconnected by sharp angles or slightly longer arc-like filaments. The serpentine filaments characteristic of cytokeratin and vimentin are absent (compare to Osborn et al., 1980, and Fig. 15). The less prominent second type of filaments stained are long straight filaments which may extend from the perinuclear region to the plasma membrane. Neither the first nor second set of filaments are stained uniformly; both stain as a series of irregularly spaced foci (Fig. 14B). The third structure is a variable number of cytoplasmic foci distributed most heavily in the perinuclear region. These foci may be related to the irregular staining of the filaments.
Figure 14. The distribution of CS-1 in cultured cells.

A, B: In clone 9 rat hepatocytes CS-1 is present on dense perinuclear filaments (long arrow), longer straight filaments (short arrows), and punctate cytoplasmic foci (small arrowhead). The boxed area is enlarged in B to show that the filaments are not labelled continuously, but as a series of punctate foci. The arrowheads (B) point to foci along a filament marked in A (large arrowhead). The two-headed arrow runs parallel to another filament which is faintly visible in A. Bar = 20 µm.

C: After nocodazole treatment, the perinuclear cage has formed (long arrow), presumably due to collapse of the dense perinuclear filaments. The distribution of longer straight filaments (short arrows) and punctate cytoplasmic foci (arrowheads) is similar to untreated cells.

D: A telophase cell in the process of cytokinesis shows that bright perinuclear cages form during mitosis (long arrow), punctate cytoplasmic foci remain present (small arrowhead), and the cleavage furrow (between the two large arrowheads) contains CS-1.

E: After extraction with nonionic detergent, dense perinuclear filaments (long arrow), longer straight filaments (short arrow), and punctate cytoplasmic foci (arrowheads) can be identified.
Table III. Effect of nocodazole on CS-1 containing structures.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Effect of nocodazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>tangled filaments</td>
<td>collapse</td>
</tr>
<tr>
<td>straight filaments</td>
<td>no effect</td>
</tr>
<tr>
<td>cytoplasmic foci</td>
<td>no effect</td>
</tr>
</tbody>
</table>
Effects of nocodazole, mitosis, and detergent extraction on CS-1 distribution. Collapse of vimentin IFs into a perinuclear cage is a well-known consequence of both drug-induced microtubule depolymerization and mitosis (Osborn et al., 1980). Cytokeratin filaments are not similarly affected by microtubule depolymerization (Osborn et al., 1980; Aubin et al., 1980). Thus, to aid in identifying the labelled filaments as vimentin, cytokeratin, or tubulin; CS-1 distribution in cells treated with nocodazole was examined (Fig. 14C, Table III). The dense perinuclear networks of filaments were no longer present, but thick whorl-like nuclear cages had formed, presumably due to collapse of the normal structure. Remarkably, the straight filaments and cytoplasmic foci were resistant to nocodazole treatment. The effect of mitosis on distribution of CS-1 staining (Fig. 14D) is similar to nocodazole treatment in that a perinuclear cage is formed, and the cytoplasmic foci persist. However, the straight filaments are absent, and the cleavage furrow, known to be rich in actin (Barak et al., 1981), is stained. These results are more consistent with the hypothesis that CS-1 stains vimentin rather than cytokeratin IFs, since some authors report that only vimentin IFs collapse into a perinuclear cage during mitosis (Aubin et al., 1980; compare to Franke et al., 1982).

To determine whether the cytoplasmic foci were due to staining of membranous organelles (from which the original immunogen was isolated), cells were extracted with Triton X-100 before fixation. The
punctate cytoplasmic foci and both types of filaments were resistant to
detergent extraction (Fig. 14E).

Comparison of CS-1 distribution to vimentin, cytokeratin, actin,
and tubulin. The distribution of CS-1 was not identical to that expected
for either cytokeratin or vimentin IFs, but had some features
corresponding to tubulin (e.g. perinuclear concentration) and actin (e.g.
long straight fibers). We therefore compared CS-1 staining to actin,
tubulin, vimentin, cytokeratin 8 (type II), and cytokeratin 18 (type I)
distributions by double-label immunofluorescence (Table IV).

The distribution of CS-1 filaments (Fig. 15A') was similar to
vimentin IFs (Fig. 15A) in many cases, but the filaments themselves often
did not coincide. Vimentin IFs were finer and more tortuous than the
CS-1 filaments. The cytoplasmic foci and long straight filaments were not
stained with anti-vimentin.

The distributions of CS-1 (Fig. 15B', C'), type II, and type I
cytokeratins (Fig. 15B, C) were very similar. The tangled CS-1 filaments
were close in length to cytokeratin IFs, and some corresponded exactly to
cytokeratin filaments. However, the cytokeratin filaments filled the
cytoplasm more completely and were more serpentine than CS-1 filaments.
Thus, although a subset of the tangled CS-1 filaments coincided with
cytokeratin filaments, many did not. The cytoplasmic foci and most of the
long straight CS-1 filaments contained neither type I nor type II
cytokeratin.
While the overall arrangements of CS-1 filaments (Fig. 15D') and actin stress fibers (Fig. 15D) were unrelated, the straight CS-1 filaments did coincide with a subset of microfilaments. Thus, these long straight filaments may be stress fibers, or may align along them. The tangled filaments and cytoplasmic foci were not recognized by anti-actin.

The most concentrated region of CS-1 staining often corresponded to the microtubule organizing center. However, none of the CS-1 containing structures coincided with microtubules (Fig. 15E, E').

**Attempted characterization of the size of CS-1.** Preliminary experiments demonstrated that CS-1 antigen was solubilized by 1% SDS, but not 10 M urea or pH 11 Na₂CO₃. However, CS-1 antigen was only weakly recognized by the monoclonal antibody after SDS treatment. To discriminate between CS-1, vimentin, and cytokeratin, samples of cytoskeletal material were applied to pH 11 sucrose gradients, and the distribution of these proteins throughout the gradient examined by dot immunoblot and SDS-PAGE. The proteins in the cytoskeletal fraction sedimented further than molecular weight markers, suggesting that the cytoskeletal proteins were not monodisperse (Fig. 16A, B). CS-1 had a broader distribution and sedimented further down the gradient than either vimentin or cytokeratin (Fig. 16C). Nonetheless, significant overlap in the distributions of CS-1, vimentin, and cytokeratin make it impossible to determine whether CS-1 is distinct from the other two proteins. It is also possible that CS-1 is related to plectin and other high molecular weight components (Wiche et al., 1981) of the vimentin-associated cytoskeleton.
The 200 kD SGPs present in our antigen and immunogen preparations could be related to this heterogeneous group of IF-associated proteins.
Figure 15. Double-label immunofluorescence of CS-1, vimentin, cytokeratins, actin, and tubulin in PtK$_2$ cells.

The distribution of CS-1 (A'–E') is shown next to the corresponding staining with V-9 anti-vimentin (A), LE65 anti-cytokeratin 8 (B; LE61 anti-cytokeratin 8 was similar), LP1K anti-cytokeratin 18 (C; LE41 anti-cytokeratin 18 was similar), C4 anti-actin (D), and TUB 2.1 anti-$\beta$-tubulin (E). Arrows indicate areas which emphasize differences and similarities in the pairs. Bar = 20 $\mu$m.
Table IV. Comparison of the distribution of CS-1 to vimentin, cytokeratin, actin, and tubulin.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Vimentin</th>
<th>Cytokeratin</th>
<th>Actin</th>
<th>Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>tangled filaments</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>straight filaments</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>cytoplasmic foci</td>
<td>-</td>
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Figure 16. Analysis of CS-1 sedimentation on sucrose gradients at pH 11.

A: The load (L) and fractions, starting from the top, of a sedimentation gradient loaded with cytoskeletal protein and analyzed by Coomassie blue stained 10% SDS-PAGE.

B: A gradient prepared, run, and analyzed as in A, but loaded with high molecular weight markers consisting of myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), bovine serum albumin (66 kD), and ovalbumin (43 kD).

C: Dot immunoblots of fractions from gradient shown in A.
E. Discussion

We have described the unusual immunoreactivity of a monoclonal antibody which recognizes three distinct cytoskeletal structures. The first two, tangled and straight filaments, are clearly related to IFs and microfilaments. The uncharacteristic patchy staining of these filaments may be related to the cytoplasmic foci which are also recognized by the antibody. The presence of nonfilamentous insoluble cytoskeletal material in normal interphase cells is a novel observation. Although these foci may be similar to granular IF aggregates which form during mitosis (Franke et al., 1982; Horwitz et al., 1981) or after microinjection of IF-specific antibodies (Eckert et al., 1981; Klymkowsky et al., 1983), the foci described here do not contain vimentin or cytokeratin IF proteins, and are present in normal interphase and mitotic cells.

A second novel observation is that the IF-associated CS-1 epitope is constitutively associated with microfilaments in interphase cells and with the actin-rich cleavage furrow in mitotic cells. The alignment of cytokeratin bundles along microfilaments has been previously demonstrated, but only after extended colcemid treatment (Osborn et al., 1980).

Although it is not surprising that some monoclonal antibodies label IFs in a patchy, rather than continuous, pattern, recognition of this
sort of labelling by light microscopy has been previously reported only with monoclonal antibodies against a small number of IF-associated proteins (Wang et al., 1983). An unusual monoclonal antibody which recognizes both vimentin and tropomyosin stains microfilaments in a striated pattern, but this is typical of tropomyosin distribution, and vimentin IFs are stained continuously by that monoclonal antibody (Blose et al., 1981).

The punctate distribution of CS-1 on filaments suggests the colocalization of some of the punctate cytoplasmic foci along CS-1 filaments, cytokeratin IFs, and microfilaments. Conversely, the cytoplasmic foci could represent filaments too thin to be detected or with too complex a distribution to allow identification of the underlying structure. A third alternative is that the antigen could be a shared structural epitope present on IFs, microfilaments, and in nonfilamentous insoluble cytoplasmic structures.

The unique immunofluorescent distribution of CS-1 suggests that it is not an IF structural protein. However, we cannot rule out the possibility that a peculiar epitope present on both IFs and microfilaments explains these observations. As noted above, there is a precedent for this in a single monoclonal antibody which recognizes both vimentin and tropomyosin (Blose et al., 1981). Unfortunately, immunoprecipitations, immunoblots, and a variety of other sizing approaches were unsuccessful with CS-1 monoclonal antibody.

When taken as a whole, the patchy labelling of both IFs and microfilaments, identification of an insoluble cytoplasmic pool, and
labelling of only a subset of vimentin and cytokeratin IFs, strongly argue that CS-1 is a novel IF-associated protein. This protein has a distribution which is remarkably different from recognized IF-associated proteins (Wang et al., 1983; Granger et al., 1982; Lawson, 1983; Wiche et al., 1981), since, in addition to some IFs, it is also associated with microfilaments and nonfilamentous insoluble cytoplasmic foci.

The results presented are consistent with a view of the cytoskeleton as a single organelle composed of distinct, but interdependent, filamentous networks. This hypothesis has been suggested previously (Schliwa & van Blerkom, 1981), and putative interconnecting structures have been identified by electron microscopy. These interconnecting structures may contain CS-1.
V. The Response of the Golgi Complex to Microtubule

Alterations: The Roles of Metabolic Energy and Membrane

Traffic in Golgi Complex Organization

A. Summary

A striking example of the interrelation between the Golgi complex (GC) and microtubules is the reversible fragmentation and dispersal of the GC which occurs following microtubule depolymerization. We have characterized dispersal of the GC after nocodazole treatment as well as its recovery from the dispersed state by immunofluorescent localization of β1,4-galactosyl transferase in Madin-Darby bovine kidney cells. Immunofluorescent anti-tubulin staining allowed simultaneous examination of the microtubule array.

Based on our results, dispersal can be divided into three successive steps: microtubule depolymerization, GC fragmentation, and fragment dispersal. In cells treated with metabolic inhibitors following microtubule depolymerization, neither fragmentation nor dispersal occur. Thus, fragmentation is energy dependent and not tightly linked to microtubule depolymerization. The slowing of fragmentation and dispersal by monensin or ammonium chloride, as well as progressive inhibition
below 34°C, suggest that ongoing membrane traffic is required for these processes.

Similarly, recovery may be separated into four steps: microtubule repolymerization, GC fragment centralization, fragment coalescence, and polarization of the reticular GC network. Fragment centralization and coalescence were arrested by metabolic inhibitors, despite the presence of microtubules. Neither monensin nor ammonium chloride inhibited GC recovery. Partial inhibition of recovery at reduced temperatures paralleled the extent of microtubule assembly.

These data demonstrate that dispersal and recovery are multi-step processes, and that the individual steps differ in temperature dependence, energy dependence, and sensitivity to ionic perturbation. GC distribution and microtubule status have also been clearly dissociated, thereby proving that organization of the GC is an active process that is not simply determined by microtubule binding. Furthermore, the results indicate that ongoing intra-GC membrane traffic may participate in fragmentation and dispersal.
B. Introduction

The Golgi Complex (GC) of vertebrate cells is a single-copy organelle which colocalizes with the microtubule organizing center (MTOC) (Thyberg & Moskalewski, 1985). Although the reason for this colocalization is unknown, the tight association of the GC and microtubules has been demonstrated in several systems. Examples include alterations in GC structure during reorganization of the microtubule array as myoblasts fuse to form myotubes (Tassin et al., 1985), the continued association of the GC and MTOC as both reorient towards an experimental wound in a cell monolayer (Kupfer et al., 1982), and the unusual GC distributions which occur following pharmacological manipulation of the microtubule array (Sandoval et al., 1984).

A most dramatic example of this relationship is the reversible fragmentation and dispersal of the GC following treatment with agents such as nocodazole (Fig. 17) and colchicine which bind tubulin heterodimers and inhibit their polymerization, thereby progressively depolymerizing microtubules (Robbins & Gonatas, 1964a; Rogalski & Singer, 1984). Light microscopic histochemistry and immunocytochemistry show that the GC has fragmented into hundreds of islands distributed throughout the cytoplasm, and electron microscopic examination reveals that the islands are composed of stacked cisternae, identifiable as GC by
ultrastructure and cytochemistry (Pavelka & Ellinger, 1983; Robbins & Gonatas, 1964a; Thyberg & Moskalewski, 1985). Since the GC is normally composed of interconnected stacks of cisternae (Rambourg et al., 1979), these tubular interconnections may be severed following microtubule depolymerization, thus leading to dispersal of the individual stacks (Tassin et al., 1985). Alternatively, lateral unfolding and extension of the tubular interconnections may result in GC redistribution throughout the cytoplasm without disrupting GC continuity (Rogalski & Singer, 1984). Despite its dispersed state, secretory and biosynthetic activities of the GC are remarkably unaffected (Rogalski et al., 1984; Rogalski & Singer, 1984; Salas et al., 1986; Tartakoff & Vassalli, 1977). These data suggest that each dispersed GC island is a competent functional unit.

One recent morphological study has suggested that GC islands are translocated along microtubule-based tracks during recovery from the dispersed state (Ho et al., 1989). However, the mechanisms by which the GC is fragmented, dispersed, and, after nocodazole removal, reassembled, remain unknown.

We have used double-label immunofluorescence to investigate the mechanisms of GC fragmentation and dispersal following microtubule depolymerization, as well as recovery from the dispersed state in Madin-Darby bovine kidney (MDBK) cells. Polyclonal antiserum recognizing β1,4-galactosyl transferase (GalT), an integral membrane GC-localized enzyme (Roth & Berger, 1982), and a monoclonal antibody to β-tubulin were used to label the GC and microtubules, respectively. The results
indicate that both dispersal and recovery are energy dependent, and also implicate membrane traffic in dispersal. A model of GC dispersal and recovery is presented.
Figure 17. GC and tubulin distributions in normal and nocodazole treated MDBK cells.

The compact and reticular juxtanuclear GC of normal cells labelled with anti-GaIT (A) colocalizes with the MTOC (arrow) seen in the corresponding tubulin distribution (A'). After 180 min in media containing 5 μM nocodazole, microtubules are completely depolymerized (B'), and the Golgi has fragmented into numerous of islands scattered throughout the cytoplasm. Bar = 20 μm.
C. Materials and methods

Materials. Nocodazole, NaN₃, 2-deoxy-D-glucose, 2,4-dinitrophenol, cycloheximide, monensin, ouabain, dibutyryl cyclic AMP, dibutyryl cyclic GMP, phorbol myristate acetate, cytochalasin D, cholera toxin, normal goat serum, and most other reagents were from Sigma. Pertussis toxin was from List Biological Laboratories. Stock solutions were prepared as follows and stored in small aliquots at -20°C: Nocodazole, 5 mg/ml in dimethyl sulfoxide; monensin, 50 mM in ethanol; phorbol myristate acetate, 1 mg/ml in dimethyl sulfoxide. Other reagents were freshly prepared.

Cell culture. MDBK cells (kindly provided by J. Shaper, Johns Hopkins University) were maintained in Ham’s F-12 media supplemented with 10% FBS (GIBCO) at 37°C in 5% CO₂, and subcultured 2-3 times weekly. The dissection of GC recovery presented in this work was facilitated by the slow kinetics of this process in MDBK cells, relative to some other cells (not shown).

For experiments, cells were plated onto sterile 12 mm diameter glass coverslips (Bellco) and used in subconfluent condition after 2-3 days of growth. At the start of each experiment cells were shifted into F-12 media without serum. Nocodazole was used at 5 μM. In dispersal experiments, cells were incubated at 4°C for 210 - 270 min (depending on
the experiment) to depolymerize microtubules, and nocodazole was added 15 - 30 min before warming to prevent microtubule repolymerization upon warming. To disperse the GC prior to recovery experiments, cells were incubated with nocodazole for 150 - 210 min at 37°C. Nocodazole was removed by 3-5 changes of media over 15 min. During energy depletion experiments, culture was in F-12 salts (130 mM NaCl, 3 mM KCl, 1.9 mM Na_2HPO_4, 14 mM NaHCO_3, 0.3 mM CaCl_2, 0.6 mM MgCl_2, pH 7.5). Metabolic inhibitors were added 15 - 30 min prior to warming (dispersal) or nocodazole removal (recovery). During culture after removal of metabolic inhibitors, F-12 medium was supplemented with D-glucose to 5 mg/ml. To regulate pH of the medium in temperature dependence experiments done in room air, culture was in F-12 salts, substituting 14 mM HEPES in place of NaHCO_3, supplemented with 1.8 mg/ml D-glucose. Monensin, ammonium chloride, ouabain, dibutyryl cyclic AMP, dibutyryl cyclic GMP, and phorbol myristate acetate, were added 15 min prior to warming (dispersal) or removal of nocodazole (recovery). Cells were incubated with cholera or pertussis toxin for 180 min at 37°C prior to dispersal for 180 min at 37°C in nocodazole and toxin. To assess recovery in the presence of toxins, the GC was dispersed with nocodazole for 180 min prior to the addition of toxin, toxin was added for 180 min at 37°C in the continued presence of nocodazole, and recovery examined after a 90 min incubation in nocodazole-free toxin-containing media.

**Immunofluorescence.** Cells were fixed for 10 min at -20°C in methanol, washed for 5 min at 25°C in Tris-buffered saline (TBS), and
nonspecific sites blocked for 10 min in TBS containing 5% normal goat serum (TBS-5% NGS). Antibodies were diluted in TBS-10% NGS, and antibody incubations were 30 min at 37°C. Between incubations, coverslips were washed 3-5 times with TBS-2% NGS. GC was detected using 60 μg/ml polyclonal rabbit anti-bovine β1,4-galactosyl transferase IgG (Shaper et al., 1985) purified by protein-A affinity chromatography (generously provided by J. Shaper, Johns Hopkins University) followed by 1 μg/ml affinity-purified rhodamine-conjugated goat anti-rabbit IgG (Cappel). Tubulin was subsequently stained with TUB2.1 monoclonal anti-β-tubulin (Gozes & Barnstable, 1982) ascites diluted 1:200 (ICN) followed by 20 μg/ml affinity-purified FITC-conjugated goat anti-mouse IgG pre-absorbed against rabbit and human IgG (Kirkegaard & Perry).

Fluorescence microscopy. A Nikon Microphot-FX equipped with epifluorescence illuminator, B-1H and G-2A filter cubes, and a Plan 40X DIC objective was used for microscopy.

Electron microscopy. Monolayers were fixed in 2.5% glutaraldehyde, 1% formaldehyde, 100 mM sodium cacodylate, 5 mM CaCl₂, pH 7.4 for 2-3 hr at room temperature, refixed for 1 hr with 1% OsO₄ in 100 mM sodium cacodylate, pH 7.4, at 4°C, and stained en bloc with 1% uranyl acetate for 16 hr at 4°C. Monolayers were dehydrated with graded ethanols, removed from plastic Petri dishes using propylene oxide, and embedded in Polybed 812 (Polysciences). Sections were cut, stained with uranyl acetate and lead citrate, and examined using a JEOL 100CX II at 60 kV.
D. Results

Kinetics of GC fragmentation and dispersal. We have monitored GC dispersal in cells pre-incubated at 4°C to depolymerize the microtubules and then warmed to 37°C in the presence of nocodazole. Dispersal did not begin until the cells were warmed (Moskalewski et al., 1980). For example, in Fig. 18 the microtubules were depolymerized by incubation for 240 min at 4°C followed by 30 min at 4°C in media containing 5 μM nocodazole. This treatment did not alter the distribution of GalT (Fig. 18, 0'; compare to Fig. 19, 240'), but fully depolymerized the microtubules (Fig. 18, 0't). After warming to 37°C in the continued presence of nocodazole, cells were examined after 30 - 120 min. At 30 min (Fig. 18, 30'), the reticular GalT distribution had largely fragmented to generate small islands surrounding the nucleus. The tubulin remained depolymerized (Fig. 18, 30't). By 60 min (Fig. 18, 60'), fragmentation was complete and the GalT positive islands had enlarged their distribution. By 120 min (Fig. 18, 120'), these fragments completed their outward movement, including even the most peripheral cytoplasm.
Figure 18. Kinetics of dispersal in MDBK cells.

Cells were incubated at 4°C for 240 min to depolymerize microtubules, nocodazole was added for 30 min at 4°C, and cells were fixed (0', 0't) or incubated in the same medium at 37°C for 30 - 120 min, as indicated. After fixation, the cells were stained for GalT (0', 30', 60', 120') and tubulin (0't, 30't). The arrowheads (0', 30', 60', 120') mark the plasma membrane. The inset in 0't shows tubulin staining of a control cell at the same magnification. Note the scarcity of microtubules in 0't. Although a bright juxtanuclear focus of tubulin staining can often be identified (Fig. 19, 0't), it lacks the dense network of microtubules which normally radiate from the MTOC. These differences are even more striking when compared directly through the microscope. From this type of data, dispersal can be broken into three steps consisting of i) microtubule depolymerization (0', 0't), ii) GC fragmentation (30', 30't), and iii) fragment dispersal (60', 120'). Bar = 20 µm.
Kinetics of GC recovery from nocodazole. Recovery of normal GC distribution and morphology involves microtubule reorganization, GC fragment centralization, coalescence, and polarization. Fig. 19 illustrates cells incubated 180 min at 37°C with nocodazole and then re-incubated for 0 - 240 min at 37°C without nocodazole. Just before nocodazole removal, the GalT positive fragments were found throughout the cytoplasm (Fig. 19, 0') and the microtubules were completely depolymerized (Fig. 19, 0't). Within 7.5 min after nocodazole removal, the microtubule array took on a normal appearance (Fig. 19, 7.5't, compare to Fig. 19, 240't), while the GalT distribution remained almost fully dispersed (Fig. 19, 7.5'). However, linear arrays of GalT positive fragments can already be identified (Fig. 19, 7.5'), occasionally corresponding to microtubules (Fig. 19, 7.5't). By 15 min after nocodazole washout (Fig. 19, 15'), small fragments remained, but they had centralized to form a juxtanuclear cloud. By 30 min (Fig. 19, 30') larger fragments were beginning to form, and a nearly continuous juxtanuclear rim composed entirely of very large reticular fragments had developed by 60 min after nocodazole removal (Fig. 19, 60'). At 120 min the GC had begun to polarize by moving towards the MTOC on one side of the nucleus (Fig. 19, 120'), and polarization was essentially complete within 240 min of recovery (Fig. 19, 240').
Figure 19. Kinetics of recovery in MDBK cells.

Cells were incubated at 37°C in nocodazole for 180 min, and fixed (0', 0't) or incubated after nocodazole removal at 37°C for 7.5 - 240 min, as indicated. After fixation, cells were stained for GalT (0', 7.5', 15', 30', 60', 120', 240') and tubulin (0't, 7.5't, 240't). In 0't, the arrowhead shows juxtanuclear tubulin staining which marks the MTOC, despite the absence of microtubules. In 7.5't, two of the arrowheads show juxtanuclear MTOCs, and the two which coincide with arrowheads in 7.5' show a linear array of GalT staining (7.5') and the corresponding microtubule (7.5't). These data allow recovery to be defined as four steps consisting of i) microtubule repolymerization (7.5', 7.5't), ii) fragment centralization (15', 30'), iii) fragment coalescence (30', 60'), and iv) polarization (120', 240').

Bar = 20 μm.
Temperature dependence of GC dispersal and recovery. When MDBK cells with 4°C-depolymerized microtubules were warmed to 15°C for 120 min in the presence of nocodazole, fragmentation and dispersal did not occur (Fig. 20, 15°C). There was some fragmentation in cells warmed to 20°C, but large juxtanuclear fragments persisted and dispersal was limited (Fig. 20, 20°C). Fragmentation and dispersal were progressively more complete at 25°C, 28°C, and 31°C, but dispersal was not comparable to 37°C (Fig. 20, 37°C) until the cells were warmed to 34°C (not shown).

GC and microtubule recovery after nocodazole treatment occurred at lower temperatures than dispersal. After complete fragmentation and dispersal, nocodazole was removed and the cells incubated at various temperatures for 120 min. At 15°C, microtubule reassembly was limited to aster formation (not shown), and the GalT staining remained disperse (Fig. 21, 15°C). At 20°C, microtubules appeared to be of normal length, but the network was more sparse than in control cells (not shown). Despite this partial microtubule network, fragments did centralize, and some coalescence to form reticular structures occurred (Fig. 21, 20°C). Recovery of the juxtanuclear GalT staining at 25°C and 31°C were comparable to recovery at 37°C (Fig. 21, 25°C, 31°C, 37°C).
Figure 20. Temperature dependence of dispersal.

Cells were incubated at 4°C for 240 min to depolymerize microtubules, and then warmed to 15°-37°C, as indicated, with nocodazole for 120 min. GalT distribution is shown. The arrowheads mark the plasma membrane. Bar = 20 μm.
Figure 21. Temperature dependence of recovery.

Cells were incubated with nocodazole for 180 min at 37°C, and then re-incubated without nocodazole for 120 min at 15°-37°C, as indicated. GalT distribution is shown. Bar = 20 μm.
Energy is required for both dispersal and recovery of the GC.

To determine whether GC dispersal was energy dependent, microtubules were depolymerized at 4°C and cells were subsequently treated with a metabolic inhibitor cocktail containing 10 mM NaN₃, 1 mM 2-deoxy-D-glucose, and, in some experiments, 1 mM 2,4-dinitrophenol (DNP). It was important to completely depolymerize the microtubules by incubation at 4°C prior to treatment with metabolic inhibitors, since microtubule depolymerization in energy-depleted cells is resistant to nocodazole and colchicine (DeBrabander et al., 1981; Moskalewski et al., 1980). When such cells were warmed to 37°C in the presence of nocodazole and the metabolic inhibitors, fragmentation and dispersal of the GalT distribution were completely inhibited (Fig. 22A, B), although microtubules remained depolymerized (Fig. 22A', B'). Fragmentation and dispersal resumed upon removal of the metabolic inhibitors (Fig. 22C, C'). When DNP was omitted the results were identical. Thus, dispersal does not automatically occur when microtubules are absent at 37°C.
Figure 22. Energy dependence of dispersal.

After 240 min at 4°C, cells were incubated for 15 min at 4°C in nocodazole-containing medium, and then cultured in the same medium at 37°C for 90 min (A, A'). Complete fragmentation and dispersal occurred. By contrast, inclusion of 2-deoxy-D-glucose, NaN₃, and DNP in the nocodazole-containing medium completely inhibited fragmentation (B, B'). If such cells were washed free of metabolic inhibitors and incubated with nocodazole for 210 min at 37°C, fragmentation and dispersal resumed (C, C'). Corresponding GalT (A, B, C) and tubulin (A', B', C') distributions are shown. Bar = 20 μm.
To determine the energy dependence of GC recovery, nocodazole-treated cells received metabolic inhibitors prior to nocodazole removal. When nocodazole was subsequently washed out in the continued presence of the metabolic inhibitors, an assembled microtubule array was formed (Fig. 23B'), although microtubules without relation to the MTOC were common (DeBrabander et al., 1981). Under these conditions, despite the presence of assembled microtubules, GalT positive fragments remained extensively dispersed for at least 90 min at 37°C (Fig. 23B), and were indistinguishable from those in nocodazole-treated cells which were not allowed to recover (e.g. Fig. 19, 0'). Recovery did proceed normally upon removal of the metabolic inhibitors (Fig. 23C). When DNP was omitted from the inhibitor cocktail (e.g. Fig. 24) results were identical.

In order to study the energy dependence of recovery in the presence of a more normal microtubule network, nocodazole-treated cells were allowed to recover briefly (15 min) from nocodazole prior to the addition of NaN₃ and 2-deoxy-D-glucose (Fig. 24B'). Centralization of the GalT positive fragments and coalescence to form larger fragments were arrested shortly after addition of the metabolic inhibitors (Fig. 24B). Recovery continued normally upon removal of the metabolic inhibitors (Fig. 24C, C').
Figure 23. Energy dependence of recovery: I.

After 210 min at 37°C with nocodazole, nocodazole was removed and culture continued for 90 min at 37°C (A, A'). Extensive recovery occurred. If 2-deoxy-D-glucose, NaN₃, and DNP were added 30 min prior to nocodazole removal, and continued during and after nocodazole removal, recovery was inhibited (B, B'). If such cells were washed free of metabolic inhibitors and incubated without nocodazole for 210 min at 37°C, recovery began (C, C'). Corresponding GalT (A, B, C) and tubulin (A', B', C') distributions are shown. Bar = 20 μm.
Figure 24. Energy dependence of recovery: II.

After 150 min at 37°C with nocodazole, nocodazole was removed, and recovery allowed for 90 min (A, A'). If 2-deoxy-D-glucose and NaN$_3$ were added 15 min after nocodazole removal, and incubation continued for an additional 75 min, recovery was arrested (B, B'). If such cells were washed free of metabolic inhibitors and incubated without nocodazole for 90 min at 37°C, recovery resumed (C, C'). Corresponding GalT (A, B, C) and tubulin (A', B', C') distributions are shown. Bar = 20 μm.
Protein synthesis is not required for GC dispersal or recovery. Energy depletion has numerous effects on cells, including inhibition of protein synthesis. Protein synthesis might be involved in GC dispersal or recovery if newly-synthesized structural proteins or transport of newly-synthesized secretory proteins through the GC was necessary. To investigate these hypotheses, the dispersal and recovery of the GalT distribution in cycloheximide-treated cells was examined. Two protocols were used to examine dispersal. In the first, 100 μg/ml cycloheximide was included while microtubules were depolymerized at 4°C. After warming to 37°C in medium containing cycloheximide and nocodazole, incubation was continued for 120 min (Fig. 25B). Alternatively, to drain content from the endoplasmic reticulum and GC prior to dispersal, the second protocol included a 180 min pre-incubation of cells with cycloheximide at 37°C before the addition of nocodazole at 37°C (not shown). In either case, GalT and tubulin distributions in cycloheximide-treated cells were identical to controls not treated with cycloheximide.

The effect of cycloheximide on recovery was examined in cells pre-treated with cycloheximide and nocodazole for 180 min followed by recovery in nocodazole-free cycloheximide-containing medium for 120 min. Recovery of the juxtanuclear GalT distribution was identical to control cells not treated with cycloheximide (Fig. 25D).
Figure 25. Cycloheximide does not inhibit dispersal or recovery.

**Dispersal:** After 60 min at 4°C (A, B), cycloheximide was added (B only) and incubation continued at 4°C for an additional 120 min. Nocodazole was then added for 30 min at 4°C, before warming to 37°C for 120 min in the continued presence of nocodazole (A, B) and cycloheximide (B only).

**Recovery:** Nocodazole (C, D) and cycloheximide (D only) were added for 180 min at 37°C. Nocodazole was then removed (C, D) in the continued presence of cycloheximide (D only), and incubation continued for 120 min at 37°C.
Dispersal

Recovery

A, B

+ cycloheximide \( \rightarrow \) + nocodazole

\[ \begin{array}{c}
4' \\
60' \\
120'
\end{array} \]

C, D

+ nocodazole, + cycloheximide \( \rightarrow \) - nocodazole

\[ \begin{array}{c}
37' \\
180' \\
120'
\end{array} \]

A, C - control
B, D - cycloheximide
Monensin and ammonium chloride affect GC dispersal, but not recovery. To further examine the role of membrane traffic in GC dispersal and recovery, we used the carboxylic ionophore, monensin, and the acidotropic amine, ammonium chloride, to slow transport through the GC without depleting cellular ATP (Dickson et al., 1982; Matlin, 1986; Tartakoff & Vassalli, 1977). We also attempted to approximate the Na⁺/K⁺ alterations induced by monensin using ouabain (Tartakoff, 1983), a specific inhibitor of the plasma membrane Na⁺/K⁺-ATPase. Both monensin and ammonium chloride produced marked vacuolization of the reticular GalT distribution within 15 min (Fig. 26, upper insets of B', C', D'), without altering tubulin distribution.

To examine the effects of monensin and ammonium chloride on fragmentation and dispersal, microtubules were depolymerized at 4°C, and the cells briefly (15 min) treated with the appropriate drug and nocodazole prior to shifting to 37°C. The GalT distribution of monensin- or ammonium chloride-treated cells examined 120 min after shifting to 37°C was incompletely fragmented relative to control cells (not shown). After 180 min, fragmentation had progressed and reticular GalT-positive structures were no longer present; however, dispersal was limited to relatively central, and often polar, areas of the cytoplasm (Fig. 26B, C, D). The GalT distribution remained incompletely dispersed, even after 240 min (not shown). This slowing of fragmentation and dispersal was evident in cells treated with 1 μM monensin, a low and relatively GC-
specific dose (Fig. 26B), and was more pronounced with 10 μM monensin or 50 mM ammonium chloride (Fig. 26C, D). Ouabain had no effect on GalT fragmentation or dispersal (Fig. 26E).

To examine the effects of monensin, ammonium chloride, and ouabain on GC recovery, nocodazole-treated cells were briefly treated with the appropriate drug 15 min prior to nocodazole removal. Following nocodazole withdrawal in the continued presence of monensin, ammonium chloride, or ouabain, normal microtubule arrays were generated (not shown), and extensive recovery was evident (Fig. 26B', C', D'). Although a compact GalT distribution did not reform in monensin- or ammonium chloride-treated cells, the somewhat loosened structure was indistinguishable from that seen after treatment of control cells with monensin or ammonium chloride (without nocodazole) for 120 min (Fig. 26, lower insets of B', C', D'). Thus, monensin, ammonium chloride, and ouabain (Fig. 26E') had no detectable effect on recovery.
Figure 26. Effects of ionic perturbation on dispersal and recovery.

Dispersal: After 210 min at 4°C, nocodazole alone (A), or nocodazole with the indicated ionic perturbant (B, C, D, E) was added for 15 min at 4°C. Incubation was then continued in the same medium at 37°C for 180 min prior to fixation. GalT distribution is shown, the arrowheads mark the plasma membrane. Note that the GalT distribution does not extend to the plasma membrane in monensin or ammonium chloride-treated cells (B, C, D), but does in controls (A) and ouabain-treated cells (E).

Recovery: After 180 min at 37°C with nocodazole, incubation was continued with nocodazole alone (A'), or nocodazole with the indicated ionic perturbant (B', C', D', E') for 15 min. Nocodazole was removed, and incubation at 37°C continued, with ionic perturbant, for 90 min. GalT distribution is shown. The insets show the GalT distribution in cells not treated with nocodazole (A'), but treated with ionic perturbant for 15 min (upper insets of B', C', D') or 120 min (lower insets of B', C', D').

Drugs used were: 1 μM monensin (B, B'), 10 μM monensin (C, C'), 50 mM ammonium chloride (D, D'), or 1 mM ouabain (E, E').

Bar = 20 μm.
G-proteins, kinases, and actin are not involved in GC dispersal or recovery. Several studies have suggested associations between the GC and protein kinases (Capasso et al., 1985; Cheng & Farquhar, 1976; Nigg et al., 1985), as well as GTP-binding proteins (Bourne, 1988; Melancon et al., 1987). Therefore, we examined the effects of 1 mM dibutyryl cyclic AMP (Fig. 27B, B'), 1 mM dibutyryl cyclic GMP (Fig. 27C, C'), 10 nM and 1 μM phorbol myristate acetate (Fig. 27D, D', E, E'), 1 μg/ml cholera toxin (Fig. 27G, G'), and 500 ng/ml pertussis toxin (Fig. 27H, H') on dispersal and recovery. None of these agents had any effect on dispersal or recovery (Fig. 27).

There is currently no evidence supporting a role for actin in GC organization in animal cells. Consistent with a lack of actin involvement, it has been shown that normal GC morphology and the early stages of recovery after nocodazole treatment are unaffected by cytochalasin (Ho et al., 1989; Thyberg, 1980). Transport of newly-synthesized proteins through the GC is also unaffected by cytochalasin (Salas et al., 1986). We examined the role of actin in each stage of GC dispersal and recovery using 1 μg/ml cytochalasin D. Both processes continued normally (Fig. 27F, F'), confirming that actin is not involved in GC re-organization following microtubule alterations.
Figure 27. G-proteins, kinases, and actin are not involved in GC dispersal or recovery.

**Dispersal:**

**A - F:** After 210 min at 4°C, nocodazole alone (A) or nocodazole with dibutyryl cyclic AMP (B), dibutyryl cyclic GMP (C), 10 nM PMA (D), 1 μM PMA (E), or cytochalasin D (F) was added for 15 min at 4°C. Incubation was then continued at 37°C, in the same media, for 180 min.

**G, H:** Cells were incubated for 180 min at 37°C with pertussis toxin (G) or cholera toxin (H). Nocodazole was then added, in the continued presence of toxin, for 180 min at 37°C.

**Recovery:**

**A' - F':** After 180 min at 37°C in nocodazole, nocodazole alone (A') or nocodazole with dibutyryl cyclic AMP (B'), dibutyryl cyclic GMP (C'), 10 nM PMA (D'), 1 μM PMA (E'), or cytochalasin D (F') was added for 15 min prior to nocodazole washout. Incubation was then continued for 90 min after nocodazole in the continued presence of the drug listed.

**G', H':** After 180 min at 37°C in nocodazole, nocodazole with pertussis toxin (G') or cholera toxin (H') was added for 180 min. Nocodazole was then washed out, in the continued presence of toxin, and incubation continued for 90 min.
Ultrastructure of the Golgi complex after nocodazole and other treatments. We have examined the ultrastructure of MDCK cells and confirmed that morphologically small GC stacks are distributed throughout the cytoplasm after nocodazole treatment, rather than confined to the juxtanuclear region (Fig. 28). In agreement with other reports, we noted i) that the cisternae seemed shorter than in control cells, and ii) that some of the cisternae were not as flat as controls (Pavelka & Ellinger, 1983). The GC was composed of stacks arranged in the expected distribution after 240 min at 4°C or after treatment at 37°C with metabolic inhibitors to prevent dispersal or recovery. Also consistent with the fluorescent GalT distribution, monensin or ammonium chloride induced pronounced dilation of cisternae within 15 min. After 90 min of monensin treatment, many swollen vesicles which did not appear to be Golgi-derived had accumulated in the juxtanuclear cytoplasm. This suggests that the loss of compact GalT distribution in such cells may have resulted from overcrowding of this region and intercalation of the swollen vesicles among dilated GalT-containing cisternae. Vacuolization of the GalT staining pattern in nocodazole-treated cells after 15 min of monensin treatment was not obvious by fluorescence microscopy, but electron microscopic examination confirmed that marked cisternal dilation had occurred. Since the GC stacks of nocodazole-treated cells appeared smaller than controls, it may be that they contained less membrane and, therefore, were incapable of the extensive dilation necessary for vacuolization to be visible by light microscopy.
Figure 28. Ultrastructure of the Golgi complex after nocodazole treatment.

**A:** A small region of cytoplasm adjacent to the nucleus of a normal MDBK cell. All GC stacks (arrows) were localized to the area shown. Note the flatness and length of the cisternae. Magnification is identical to B.

**B:** A section of peripheral cytoplasm (boxed area in C) from an MDBK cell treated with 5 μM nocodazole for 180 min at 37°C. The two GC stacks (arrows) are widely separated from each other and the nucleus. The cisternae are stacked, but are not as flat or as long as in control cells (A). Bar = 250 nm.

**C:** Overview of cell shown in B. In addition to the two stacks in the boxed area (B), two other stacks can be identified (arrows). The nucleus separates one stack from the other three, indicative of the loss of GC polarity in nocodazole-treated cells. (Stacks on both sides of the nucleus were never seen in control cells, e.g. A.) The cell shown is oriented with the apical surface facing up and the basolateral surface facing down. Bar = 1 μm.
E. Discussion

We have used a protein-A-purified polyclonal antiserum to β1,4-galactosyl transferase (GalT), a well-characterized integral membrane protein of trans-Golgi cisternae (Roth & Berger, 1982), as a marker for the GC. GC stacks are known to remain functionally and cytologically intact after nocodazole- or colchicine-induced microtubule depolymerization (Pavelka & Ellinger, 1983; Rogalski et al., 1984; Tartakoff & Vassalli, 1977; Thyberg & Moskalewski, 1985), and we have confirmed that, for each of the conditions explored, the GC of MDBK cells is composed of stacks of cisternae (Fig. 28). Additionally, the distribution of GalT before and after nocodazole or colchicine treatment is similar to that of thiamine pyrophosphatase (Robbins & Gonatas, 1964a) as well as cis-, medial-, and trans-Golgi localized antigens (Chicheportiche et al., 1984; Yuan et al., 1987; Turner & Tartakoff, unpublished observations). Finally, viral glycoprotein and fluorescent ceramide concentrated within the GC have distributions similar to GalT both in control cells and in cells with depolymerized microtubules (Lipsky & Pagano, 1985; Rogalski et al., 1984). We therefore believe that GalT indicates the distribution of the entire stack under the conditions studied.

**GC dispersal.** GC dispersal can be divided into three stages: i) microtubule depolymerization, ii) GC fragmentation (30 - 60 min), and iii)
fragment dispersal (120 min). Through the use of reduced temperature, metabolic inhibition, and ionic perturbation, we have been able to arrest GC dispersal at each of these stages, thereby demonstrating that they are distinct processes.

Fragmentation is energy dependent, since it is blocked by metabolic inhibitors. This effect may be due to inhibition of intra-GC transport, a process known to be energy dependent (Orci et al., 1985; Tartakoff, 1986). Arrest of protein synthesis does not account for the effects of metabolic inhibition.

Fragmentation is also inhibited at 15°-20°C, even though ATP levels should be similar to those at 37°C (Tartakoff, 1986). These results may implicate membrane traffic in GC fragmentation, since budding and fusion of membranes are inhibited at 10°-15°C, and a block in GC traversal and exit persists up to 20°-22°C (Saraste et al., 1986; Tartakoff, 1986).

The slowing of GC fragmentation and dispersal by the ionic perturbants monensin and ammonium chloride, both of which slow intra-GC membrane traffic, also suggests that membrane traffic is required for GC fragmentation. Although these two agents do not have the same effects in all cells (Oda et al., 1983), they both caused rapid vacuolization of the GC, including GalT-containing regions, in MDBK cells.

Furthermore, both agents neutralize acidic compartments, have the potential to alter cytoplasmic pH, and, most importantly, slow GC traversal and exit (Matlin, 1986; Tartakoff & Vassalli, 1977). At the dose used, ammonium chloride also causes vacuolization of other acidic
compartments, e.g. lysosomes. Monensin was used at two doses, 1 μM, which did not cause accumulation of phase-lucent vacuoles and should be relatively GC-specific, and 10 μM, which would affect other acidic compartments (Tartakoff, 1983). That ouabain had no effect does not necessarily imply that Na⁺/K⁺ balance is not involved in GC fragmentation, since MDBK cells may be relatively ouabain resistant. The effects of monensin and ammonium chloride are not related to recent work showing that alkalinization of cytoplasmic pH promotes centralization of lysosomes (Heuser, 1989), since the latter phenomenon is dependent on intact microtubules.

The arrest of fragmentation and dispersal by reduced temperature, metabolic inhibition, and ionic perturbation (Table I) clearly demonstrates that, although microtubule depolymerization is necessary, it is not sufficient for GC fragmentation and dispersal. Fragmentation is an active process which is not tightly linked to microtubule depolymerization, although under "normal" conditions (37°C, no metabolic inhibitors) GC fragmentation follows microtubule depolymerization. These observations are an important extension of previous work (Moskalewski et al., 1980), since, for the first time, they separate the processes of microtubule depolymerization and GC fragmentation, thereby proving that GC organization does not follow the microtubule distribution directly, but through energy- and temperature-dependent processes.

Since monensin and ammonium chloride inhibit membrane fission (Tartakoff, 1983), their effects, as well as the 20°C block, support two
models of GC fragmentation. In the first, a critical step involves severing of the tubular intercisternal connections between Golgi stacks (Tassin et al., 1985). Upon elimination of these interconnections, the GC would be transformed from a single organelle into a loose collection of individual stacks which would be free to disperse. The second presupposes the existence of many "stacking templates" or "Golgi complex organizing centers". If these were normally clustered in the MTOC by microtubules, they might become separated in cells with depolymerized microtubules. GC membranes would follow the templates via continual budding with peripheral reformation of multiple stacked islands.

Fragment dispersal is the final step in generating the dispersed GC. Although dispersal begins soon after fragmentation, the kinetics are slower than either microtubule depolymerization or GC fragmentation. Thus, fragment dispersal may simply reflect diffusion of the individual GC islands. Since energy depletion completely blocked fragmentation, it was difficult to evaluate the energy dependence of fragment dispersal. Fragment dispersal was progressively inhibited below 34°C or by monensin or ammonium chloride, but, since fragmentation was also slowed, these experiments do not prove that fragment dispersal was affected directly.

The appearance of linear arrays of GC fragments during dispersal (Fig. 18, 30') might be due to the random placement of a large number of islands, but could also imply that defined "tracks" exist. These tracks are not likely to be microtubules, since the vast majority of microtubules were depolymerized. Nonetheless, if a microtubule-based motor were involved
in dispersal, this would suggest that both an anterograde, kinesin-like, motor (Vale et al., 1985), for dispersal, as well as a retrograde, dynein-like, motor (Fox & Sale, 1987; McIntosh & Porter, 1989; Schroer et al., 1989), for recovery, are associated with the GC. To the extent that cytoskeletal elements are involved, tracks could be composed of intermediate filaments, but not actin, since cytochalasin D had no effect. Alternatively, the linear arrays, and the inhibitory effects of monensin and ammonium chloride on dispersal, could be due to the persistence of GalT negative membranous processes which interconnect GalT positive GC fragments.

**GC recovery.** Recovery can be divided into four steps: i) microtubule repolymerization (<7.5 min), ii) GC fragment centralization (15 - 30 min), iii) fragment coalescence (30 - 60 min), and iv) GC polarization (180 - 240 min). Microtubules assemble into a normal appearing network before GC alterations are obvious. This observation is consistent with the suggestion (Ho et al., 1989) that GC fragments travel along microtubules during recovery, since microtubules initiate reassembly from the centriole (Osborn & Weber, 1976), and, therefore, must grow outward to reach dispersed GC fragments. In fact, shortly after the microtubules have reassembled, while GC fragments are rapidly centralizing, linear arrays of GC fragments can be identified (Fig. 19, 7.5'; also see Rogalski & Singer, 1984). In some, but not all, cases, these correspond to individual microtubules. This is also consistent with microtubule-based centralization, although any of the explanations given for linear arrays seen during dispersal could apply.
Centralization is an active process, since it is rapid and energy dependent. Energy dependence was demonstrated in two ways. First, in nocodazole-treated cells exposed to metabolic inhibitors prior to nocodazole washout and then allowed to recover from nocodazole in the continued presence of metabolic inhibitors, the GC fragments did not centralize, even though assembled microtubules were present. Since some of the microtubules assembled under these conditions were oriented abnormally, a second approach in which microtubules were allowed to recover briefly from nocodazole prior to the addition of metabolic inhibitors was also employed. Under these conditions, a completely normal appearing microtubule array was formed, but movement of GC fragments towards the nucleus stopped shortly after addition of the metabolic inhibitors. These results support the hypothesis that a retrograde, dynein-like, ATP-dependent motor (McIntosh & Porter, 1989; Schroer et al., 1989) is responsible for GC fragment movement. It is not clear if the initial association of the GC with microtubules during recovery is energy dependent, since linear arrays of GC fragments which colocalize with microtubules can occasionally be identified in energy depleted cells (Fig. 23B, B’). The possibility that active microtubule cycling is essential must also be considered, since microtubules in energy-depleted cells are nocodazole-resistant and, therefore, probably do not cycle (DeBrabander et al., 1981).

The block of GC centralization at 15°C probably reflects the lack of microtubule reassembly. Nonetheless, membrane functions which are
blocked at 0°-22°C (e.g. the 20°-22°C block in GC traversal) are not involved in centralization, since recovery did occur at 20°C, despite incomplete microtubule reassembly.

The coalescence of centralized GC fragments to form a larger reticular GC is also energy dependent, since GC fragments in cells allowed to recover briefly from nocodazole prior to the addition of metabolic inhibitors appeared "ready" to coalesce, but did not. It is likely that this process involves reestablishment of interconnecting cisternal bridges severed during dispersal, and thus involves energy dependent membrane fusion. The lack of inhibition by monensin and ammonium chloride does not conflict with this explanation, since they appear to inhibit membrane fission, but not fusion (Tartakoff, 1983).

The process of recovery is completed as larger GC fragments form reticular networks which surround the nucleus. Over time, these networks coalesce and polarize to form a single GC which colocalizes with the MTOC.

Electron microscopy suggests that the loosening of normal GalT distribution after extended monensin or ammonium chloride treatment (without nocodazole) may be related to the centripetal movement of lysosomes following cytoplasmic alkalinization (Heuser, 1989). However, it could also be related to the peripheral redistribution of secretory granules following monensin treatment (Iida et al., 1988).
Table V. Effects of metabolic inhibition, reduced temperature, and ionic perturbation on Golgi complex dispersal and recovery.
Why is the Golgi complex associated with microtubules? These studies enhance our understanding of the localization of the GC and the processes of dispersal and recovery. However, in doing so they emphasize many unanswered questions. For example: i) is association of the GC with microtubules important during mitosis? One might hypothesize that the relatively constant number of GC islands which are generated by nocodazole treatment represent the smallest functional GC unit, and that their formation is the essential first step in formation of the mitotic GC (Lucocq et al., 1987). This is plausible if the GC associates preferentially with cytoplasmic microtubules, relative to mitotic spindle microtubules, as has been proposed for secretory granules (Tooze & Burke, 1987). During mitosis, the small Golgi stacks generated following the depolymerization of cytoplasmic microtubules could be disassembled into vesicles (Lucocq et al., 1989), and, once the vesicles reassemble into small stacks, the microtubule- and energy-dependent recovery process we describe may mediate reformation of an intact GC.

ii) Although the dispersed islands remain stacked, do all structures normally associated with the GC remain with the islands, or are post-cisternal elements altered or absent, as has been suggested (Thyberg et al., 1980)? Since post-cisternal elements sort proteins destined for distinct plasma membrane domains in polarized epithelia (Griffiths & Simons, 1986), such a deficiency might explain the inaccurate sorting in nocodazole-treated epithelial cells (Eilers et al., 1989; Rindler et al., 1987).
iii) Are the GC islands generated by nocodazole treatment equivalent to plant dictyosomes, which are distributed throughout the cytoplasm (Mollenhauer & Morré, 1966)? Since microtubules in many plant cells do not converge on a single juxtanuclear MTOC, but are arranged circumferentially beneath the plasma membrane (Wick et al., 1981), the dispersed dictyosome distribution, with frequent clustering near the surface, suggests that the plant GC is associated with microtubules.

Although these, and other, questions regarding the relevance of Golgi complex-microtubule interactions persist, the data presented do define the requirements for GC integrity and localization. GC distribution and microtubule status have been dissociated, for the first time, through the use of reduced temperature, metabolic inhibitors, and ionic perturbants (Table V). Thus, active temperature and energy dependent processes are responsible for GC movement. Furthermore, an essential role for membrane traffic is implicated in GC fragmentation and dispersal.
References


Abbreviations

CR1  human complement C3b/C4b receptor
DMSO  dimethyl sulfoxide
DNP  2,4-dinitrophenol
EGTA  ethyleneglycol-bis-(β-aminoethyl ether)-N,N’-tetraacetic acid
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
fMLP  N-formyl-Met-Leu-Phe
GalT  β1,4-galactosyltransferase
GC  Golgi complex
HEPES  N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid
hr  hour
IF  intermediate filament
MDBK  Madin-Darby bovine kidney
min  minute
MTOC  microtubule organizing center
PBS  phosphate-buffered saline
PBS-ovalbumin  PBS containing 1 mg/ml ovalbumin
PIPES  1,4-piperazinebis(ethane sulfonic acid)
PMN  human neutrophil
PMSF  phenyl-methyl-sulfonyl-fluoride
rpm  revolutions per minute
SDS  sodium dodecyl sulfate
SDS-PAGE  SDS polyacrylamide gel electrophoresis
SGP  sedimentable Golgi protein
TBS  Tris-HCl buffered saline
TBS-5%-NGS  TBS containing the indicated fraction (v/v) of normal goat serum
TKM  50 mM Tris-HCl, 25 mM KCl, 10 mM MgCl₂, pH 7.5
0.25 M sucrose-TKM  TKM containing the indicated concentration of sucrose