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STUDIES ON THE CONSEQUENCES OF CELL SHAPE FOR MEMBRANE DYNAMICS: RELATIONSHIP TO CYTOSKELETAL ORGANIZATION

By

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ABSTRACT

STUDIES ON THE CONSEQUENCES OF CELL SHAPE FOR MEMBRANE DYNAMICS: RELATIONSHIP TO CYTOSKELETAL ORGANIZATION

By

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A large body of evidence suggests the alteration of cell shape influences the biological activity of anchorage dependent fibroblasts. The shape transition is also associated with changes in the structural organization of cytoskeletal elements. The importance of these structures to surface receptor organization, biosynthetic processes, and gene expression implies the involvement of cytoskeletal elements in an intracellular communication pathway that can be disrupted by cell shape alteration. On the basis of evidence suggesting the control of protein lateral mobility by cytoskeletal structures, protein lateral mobility was measured as a function of cell shape to determine the effect of shape induced cytoskeletal alteration. The role

of protein lateral mobility in growth factor action and the association of surface receptors with the cytoskeleton indicate the significance of using protein lateral mobility to monitor cell shape induced alterations in the association of membrane proteins with the cytoskeleton. Transformation provides a means to perturb the cytoskeleton in addition to cell shape alteration.

The major findings of this thesis include a) shape induced alteration of the cell cytoskeleton coincides with a partial release of restriction for lateral diffusion of wheat germ agglutinin (WGA) and succinyl concanavalin A receptors in untransformed BALB/c 3T3 cells; b) the observation of a bimodal population of fast and slow mobility cells after the alteration of cell shape in K-MSV transformed BALB/c 3T3 cells; c) the detection and isolation of a clone from the K-MSV BALB/c 3T3 fibroblasts exhibiting fast mobility; d) the loss of global modulation, a process affecting protein lateral mobility that requires an intact cytoskeleton, in spherical BALB/c 3T3 cells and in the "fast" mobility clone regardless of cell shape. In addition, a study done by others in the laboratory found correlative evidence that cell shape alteration affects nucleo-cytoplasmic transport in BALB/c 3T3 fibroblasts. These observations support the evidence for a role of the cytoskeleton in restriction of protein lateral mobility and support the possibility that the cytoskeleton serves as an integral element in an intracellular communication pathway.

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LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
Con A	concanavalin A
DME	Dulbecco's modified Eagle's media
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
F-actin	filamentous actin
FITC	fluorescein isothiocyanate
FRAP	fluorescence redistribution after photobleaching
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
K-MSV	Kirsten murine sarcoma virus transformed
MAPs	microtubule associated proteins
MC	methyl cellulose
mRNA	messenger RNA
NBD	4-nitrobenzo-2-oxa-1,3-diazole
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpyrrolidone

RNA	ribonucleic acid
rRNA	ribosomal RNA
S-Con A	succinyl concanavalin A
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
WGA	wheat germ agglutinin

CHAPTER I: OVERVIEW OF THE THESIS

The involvement of cell shape as a controlling influence for DNA synthesis and growth in anchorage dependent cells was vividly demonstrated by the seminal work of Folkman and Moscona (1978). The consequences of cell shape alteration were not only limited to effects on DNA synthesis, but also resulted in the inhibition of mRNA and rRNA production (Benecke et al., 1978; Ben Ze'ev et al., 1980). Translation, on the other hand, merely required cell surface contact but not extensive spreading. The processes of cell detachment, resulting in cell rounding, and reattachment followed by spreading are accompanied by disassembly and reassembly of stress fibers, microfilaments, and microtubules (Badley et al., 1980). Considering the importance of these cytoskeletal structures for protein biosynthesis (Penman et al., 1981; Cervera et al., 1981), transmembrane signal initiation (Geiger, 1983; Landreth et al., 1985), and signal transmission (Ingber & Jamieson, 1985), the possibility that the flat to spherical shape transition interrupts a cytoskeletal communication pathway from the cell surface to the nucleus, which is required for nuclear function in untransformed fibroblasts,

is plausible and has been suggested (Ben Ze'ev, 1985). A signal transduction pathway can be divided into three components: a) signal initiation, triggered by receptor/ligand binding and the resulting receptor aggregation (Schlessinger, 1980) or by alteration of cell shape (Ben Ze'ev, 1985); b) signal transmission, e.g. soluble factors (Sutherland, 1972; Joseph, 1986) or cytoskeletal organization (Ben Ze'ev, 1985; Ingber & Jamieson, 1985); and at the nuclear level, c) signal acquisition or reception as mirrored by changes in the rate of nuclear transport (Schindler & Jiang, 1985) or replication competency (Stiles et al., 1979). The role of membrane protein lateral mobility in polypeptide growth factor (PGF) signal initiation (Schlessinger, 1980) and the demonstration of the modulation and restriction of protein lateral mobility by the cytoskeleton (Edelman, 1976; Koppel et al., 1981; Tank et al., 1982) indicates the significance of the link between the control of protein lateral mobility and the cytoskeleton.

This thesis seeks to focus on events occuring at the cell surface and to examine the role of cell shape on the initial event in signal transduction, the membrane lateral diffusion of cell surface receptors. Lateral mobility in spread and spherical fibroblasts was examined for changes in the rate and extent of wheat germ agglutinin receptor lateral mobility. (I will use the term receptor for lectin binding proteins but do not necessarily imply a response

from binding as classicly inferred from the term receptor. For example, there is no immediately detectable response for WGA binding in 3T3 fibroblasts. Some lectins do initiate a response after binding and so lectin binding proteins are commonly referred to as receptors.) Other probes for membrane glycoproteins and lipids were also employed to examine the generality of potential dynamic variations in membrane lateral diffusion. A link between membrane lateral diffusion phenomenon and cytoskeletal changes was assessed by examining the potential for concanavalin A induced changes in WGA receptor lateral mobility. Such a phenomenon, termed global modulation, has been ascribed to cytoskeletal linkage of receptors. Kirsten murine sarcoma virus transformed (K-MSV) BALB/c 3T3 fibroblasts were also employed in this study to provide another form of perturbed cytoskeletal structure to examine in regard to plasma membrane lateral mobility. The state of the actin stress fiber system was analyzed and used to monitor the changes in cytoskeletal organization after alteration of cell shape and transformation state. Lateral mobility, then, is used as a dynamic, non-destructive probe to ascertain whether cell shape changes or transformation can play a role in uncoupling the putative initial event in transmembrane signaling. In addition, these experiments provide a new means to examine the suggested role of the cytoskeleton in restriction of protein lateral diffusion.

The major findings of this thesis include a) shape induced alteration of the cell cytoskeleton coincides with a partial release of restriction for lateral diffusion of wheat germ agglutinin (WGA) and succinyl concanavalin A receptors in untransformed BALB/c 3T3 cells; b) the observation of a bimodal population of cells with fast and slow protein lateral mobility after the alteration of cell shape in K-MSV BALB/c 3T3 cells; c) the detection and isolation of a clone with fast protein lateral mobility from the K-MSV BALB/c 3T3 fibroblasts; d) the loss of global modulation, a process affecting protein lateral mobility that requires an intact cytoskeleton (Edelman, 1976), in spherical BALB/c 3T3 cells and in the "fast" mobility clone regardless of cell shape.

While this project has pursued dynamic events at the plasma membrane that could be involved in transmembrane signal transduction, the work of others in the laboratory on dynamic events at the nuclear membrane provides correlative evidence for a transduction connection between plasma membrane and nuclear activity. The research on nuclear pore mediated transport gives evidence that actin and myosin may play a role in the control of nucleocytoplasmic transport (Schindler & Jiang, 1986). These mechanochemical proteins may serve as potential sites of linkage between the nucleus and the cytoplasmic/submembranous cytoskeleton. Subsequent studies paralleling my investigations of the role of shape induced

changes on plasma membrane protein lateral mobility indicate that alteration of cell shape is accompanied by an effect on the rate of nuclear transport in untransformed 3T3 cells but not in the "fast" mobility clone. The relationship of this linkage to hormone action may be inferred from investigations demonstrating that hormone pretreatment with epidermal growth factor increases the rate of nuclear transport in spread cells but not spherical cells as might be predicted by the original observations of Folkman and Moscona (1978). These studies of a dynamic linkage between the plasma membrane receptors and nuclear transport may provide the first experimental evidence for the cell cytoskeleton as the "telegraphic" mediator of plasma membrane originated signals to the nuclear surface and ultimately the chromatin.

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CHAPTER II: LITERATURE REVIEW

SIGNAL TRANSMISSION

Recently, a different pathway for communication between the plasma membrane and the nucleus via the cytoskeleton has garnered interest as indicated by the growing number of reviews on the subject. Ingber and Jamieson (1985) were intrigued by the reported effects of basal membrane substances on gene expression (Bissel et al., 1982) correlating with changes in the cytoskeleton (Sugrue and Hay, 1981; Brown et al., 1983; Ali et al., 1977), and in light of the apparent interaction of the cytoskeleton with the nucleus (Lazarides, 1980), they suggested that the cytoskeleton could act as a transmitter of signals from the outside of the cell to nucleus. Packard (1986) echoed the growing interest in a cytoskeletal pathway between the plasma membrane and the nucleus. Schindler and Jiang (in press) reviewed nuclear-cell communications: the linkages, controllers, and pathways, and discussed the evidence for the role of the cytoskeleton as a controller of nucleo-cytosplasmic transport and as a possible highway for directing a fraction of internalized

growth factor-receptor complexes to the nucleus.

The model of a signal transmission pathway via the cytoskeleton suggests that there should be three parts: a connection between plasma membrane proteins and the cytoskeleton, a cytoskeletal network spanning the cytoplasm, and an interaction between the cytoskeleton and the nucleus. The connection between plasma membrane proteins and the cytoskeleton has been demonstrated by dynamic evidence from studies on the restriction of protein lateral mobility (Edelman, 1976; Koppel et al., 1981; Tank et al., 1982); structural evidence from immunofluorescence microscopic studies (Ash et al., 1977; Gabbiani, 1977; Geiger et al., 1984; Rogalski & Singer, 1985); and biochemical evidence from studies examining the association of membrane proteins with detergent insoluble cytoskeletal structures (Painter & Ginsberg, 1982; Sheterline & Hopkins, 1981; Koch & Smith, 1978; Streuli et al., 1981). Other studies using intact cells have suggested that the binding of crosslinking agents leads to aggregation of the bound membrane receptors (referred to as patching). The coalescence of these patches into a polar cap is known as capping. The binding of Con A and other crosslinking agents such as antibodies enhanced the binding of surface receptors to a detergent insoluble cytoskeleton (Flanagan & Koch, 1978). This occurred only with divalent antibodies and not with antibody fragments that do not crosslink membrane receptors; furthermore, association of membrane

receptors with the cytoskeleton still occurred under conditions where crosslinking resulted in patching, but capping and internalization were prevented by cold treatment. Thus, aggregation appears to be a key step in enhancing the association between cell surface receptors and the cytoskeleton as well as being a key step in growth factor action (Schlessinger, 1980). The significance of these interactions between the cytoskeleton and membrane proteins to growth factor action was indicated by the report that epidermal growth factor receptors are associated with the detergent insoluble cytoskeleton (Landreth et al., 1985). These lines of evidence will be discussed further in the section on the cytoskeleton. The spanning of the cytoplasm by the cytoskeleton was most readily visualized in electron micrographs from whole mounts of cells (for example see Penman et al., 1981).

Evidence for an interaction between the cytoskeleton and the nucleus is both structural and dynamic. Electron micrographs suggested that cytoskeletal elements come, at least, in close apposition to the nuclear membrane (Franke, 1971). Even more suggestive was the report that actin and myosin play a role in the control of the rate of nucleo-cytoplasmic transport (Schindler and Jiang, 1986). In this study it was shown that an enhancement of nucleocytoplasmic transport by ATP could be blocked by treatment with microfilament disrupting drugs, anti-actin antibodies, or anti-myosin antibodies. Putting the pieces together

indicates the plausibilty of a linkage between plasma membrane proteins and the nuclear structures. In support of this, a correlation between the position of plasma membrane antigens and nuclear antigens has been noted in certain transformed cells treated with cytochalasin B (Berke and Fishelson, 1976; Otteskog et al., 1981). Cytochalasin B induced capping of membrane antigens in L cells, SV40-3T3 cells, and NRK La 334 cells. Capping did not occur in enucleated cells suggesting a requirement for the presence of the nucleus. Correlating with the capping of the plasma membrane antigens, a capping of nuclear antigens was detected on the nuclear membrane on the side of the nucleus closest to the plasma membrane cap, even though the nucleus was located on the opposite side of the cell from the cap. In another recent study a cytochalasin sensitive link between acetylcholine receptor clusters and the nucleus was observed in muscle fibers (Englander & Rubin, 1987). Nuclei of muscle fibers having a homogeneous distribution of acetylcholine receptors were observed to move through the myotube. A clustering factor reorganized the acetylcholine receptors into clusters; nuclei near the clusters became stationary. If clusters were dispersed, the nuclei in the proximity became mobile. Addition of cytochalasin with the clustering factor prevented the formation of clusters and nuclei remained mobile. Cytochalasin did not affect preformed clusters and associated nuclei. In a demonstration of the ability of nuclear structures to

affect the organization of the cytoskeleton, it has been observed that during polar body formation in mouse oocyte that chromosomal clustering induced a reorganization of subcortical microfilaments resulting in increased accumulation of actin in some areas below the membrane associated with a decrease in microvilli (Maro et al., 1986).

THE CYTOSKELETON

The cytoskeleton consists of several types of tension bearing and tension producing filaments and networks. These networks provide the structural support that gives cells their shape. The cytoskeleton is also involved in a variety of cell motile events, for example, cell movement, membrane ruffling, flagellar movement, endocytosis, exocytosis, adhesion, spreading, chromosomal separation, and cell division (Goldman and Knipe, 1972; Birchmeier, 1984; Tomasek and Hay, 1984). The cytoskeleton has even been implicated in a role of providing an organizational scaffolding to localize and regulate translation as well as many cellular enzymatic activities (Cervera et al., 1981; Clegg, 1984). The intricate cytoskeletal networks pervading the cytoplasm provide a means by which cellular proteins and activities can be localized to specific regions of the cell resulting, in effect, in compartmentalization of

cellular functions. For example, in one study it was observed that translation of mRNA occured only when the mRNA was attached to the cytoskeleton (Cervera et al., 1981). Furthermore, it has been observed that the localization resulted in the insertion of some newly synthesized cytoskeletal proteins into the cytoskeleton at the site of synthesis (Penman et al., 1981). One way that association/dissociation of enzymes and substrates to the cytoskeleton or membranes could aid or regulate cellular activities is that during the process of association and dissociation, molecules tend to remain in the vicinity for a longer period of time resulting in a quasi-two dimensional diffusion. Such hopping on and off could limit the distance of diffusion required for collision and, perhaps more importantly, allow more attempts at binding (McCloskey & Poo, 1984).

The cytoskeleton, apparently, is a strange mixture of static states and dynamic processes. One group has described it as a system in equilibrium composed of components independently in disequlibria (Ingber & Jamieson, 1985). They represented the cytoskeleton as a system of tension and compression yielding a dynamic, yet stable, structure in a model termed tensegrity. Such a model would allow cells to maintain a form, sense changes in the environment, and quickly react accordingly. In support of this model it was found that in PC12 neurites, depolymerization of microtubules results in retraction of

neurites and that retraction of the neurites can be prevented by disruption of microfilaments (Joshi et al., 1985). Microfilament disrupting drugs also increased the dose of microtubule disrupting drugs required to cause microtubule depolymerization. These results support their contention that microtubules are under compression arising from tension produced by microfilaments and suggest that variation of compression may serve as a local regulator of microtubule assembly. To better understand how the cytoskeleton works, one needs to examine the components. The cytoskeleton consists of several types of filaments and networks composed of microfilaments, microtubules, intermediate filaments, and spectrin and their associated proteins.

Microfilaments consist of actin and actin binding proteins which regulate the form of the microfilaments. Actin monomers have a polarity which can be distinguished by the possession of a barbed end and a pointed end. The monomers polymerize under appropriate conditions to form long filaments in a head to tail configuration. The steps of polymerization are: a) activation of the monomer resulting in a conformational change by cations such as magnesium; b) nucleation to form short polymers (apparently the rate limiting step); c) elongation of the nuclei by a head to tail addition of monomers; and d) longer filaments can be generated by annealing of fragments (Pollard and Craig, 1982). In solutions of purified monomer the extent

and rate of polymerization depends on the concentration of monomer present. Such filaments can, apparently, have addition of monomers at both ends simultaneously; however, the association constants appear to differ at the two ends resulting in a difference in the concentrations necessary for polymerization to occur. In vitro polymerization may occur initially at both ends until the concentration of the monomer pool drops below the critical concentration for one of the ends. At this point net depolymerization can occur at one end while net polymerization continues at the other end. Eventually, a steady state is reached where the polymerization at one end is matched by depolymerization at the other end. In such a state a phenomenon, called treadmilling, can occur (Wegner, 1976). In treadmilling a monomer is added to one end, moves through the polymer, and comes off the far end. This is the result of net polymerization at one end and net depolymerization at the far end. In vivo, however, this is believed to be a rare event since actin is usually not in a pure form but bound and regulated by many actin binding proteins (Craig and Pollard, 1982; Birchmeier, 1984). These proteins can block or cap an end of the filament resulting in polymerization or depolymerization depending on the end. Examples of these capping and length regulating actin binding proteins include α-actinin, fragmin, gelsolin, villin, and brevin. Some of these proteins, such as actinin and gelsolin, cap the filaments; others such as fragmin can fragment polymers

of actin. Actin binding proteins, profilin for example, can depolymerize polymers of actin instead of merely blocking further addition or fragmenting the longer polymers. Actin polymers can be found in various forms including long single filaments, bundles of filaments, and a meshlike network. To process actin into these various forms there is a host of actin binding proteins that can crosslink actin into bundles or networks. Membrane proteins can also be actin binding proteins, e.g. ankyrin and vinculin, and are believed to anchor actin filaments to the membrane (Birchmeier, 1984). Thus, the microfilament system can take on various forms, be stabilized, and be destabilized rapidly by interaction with a variety of actin binding proteins. Adhesion and interaction with the extracellular matrix resulting in aggregation of membrane actin binding proteins may provide another means of controlling the formation of cytoskeletal assemblies (Bissel et al., 1982).

The distribution of actin in cells generally is described in two forms, both near the membrane. Studies using electron and fluoresence microscopy suggested that actin is found mainly in stress fiber bundles and membrane ruffles (Glacy, 1983; Goldman et al., 1975; Lazarides, 1975). Often the stress fibers were observed to run the length of the cell, under the nucleus and adjacent to the portion of the plasma membrane attached to the growth substrate. Stress fibers were observed only in attached and spread cells. In the membrane ruffles the actin appeared to

be in networklike gels that could rapidly form and disappear. Other studies have suggested that actin forms a submembranous sheath entirely enclosing the membrane that may undergo rapid gel-sol transitions during membrane motile events (Davies and Stossel, 1977; Stossel et al., 1981). In one study the actin cortical sheath appeared to enclose most of the cytoplasm and the microtubule system as well (Henderson and Weber, 1979). This does not preclude the possibility of actin structures being part of the cytoplasmic cytoskeleton as most of these studies also observed weaker, general staining throughout the cytoplasm. Actin filaments are generally considered tension producing filaments through their interaction with myosin. Although it has been suggested that stress fibers might be tension bearing structures, they are usually considered to be tension producing structures (Burridge, 1981).

Microtubules are another polymeric structure of the cytoskeleton and consist of a heterodimer of α and β tubulin. GTP bound to the tubulin enhances assembly and the hydrolysis of the GTP to GDP in the microtubule stabilizes the microtubule (Bonne & Pantaloni, 1982). Assembly of microtubules is similar to that of actin in that assembly can occur at both ends with a difference in dissociation constants and critical concentrations for each end. As for microfilaments, this difference in critical concentrations at the ends could result in treadmilling of microtubules (Margolis & Wilson, 1978); however, this does not appear to

occur in vivo (Scherson et al., 1984). Microtubules also contain various microtubule associated proteins, known as MAPs, which assist and regulate microtubule polymerization. One of these MAPs, Tau, is required for microtubule assembly (Weingarten et al., 1975). It appears unlikely that microtubules exist free in the cytoplasm for any significant period of time; instead, microtubules appear to be initiated from microtubule organizing centers and grow toward the plasma membrane (Osborn & Weber, 1976; Solomon, 1980). The microtubules approach the plasma membrane but do not usually reach it; the network of microtubules appears to be encircled by and interact with a submembranous network of actin (Henderson & Weber, 1979). In support of this contention, some MAPs are also actin binding proteins (Craig & Pollard, 1982). Microtubules, however, have been demonstrated to interact with membranes in algae (Murray, 1983), hepatic cells (Reaven & Azhar, 1981), and brain and thyroid tissue (Bhattacharyya & Wolff, 1975); therefore, a role for microtubules in cell surface events can not be eliminated. The microtubules play an important role in the maintenance of cell architecture and cell motility (Goldman, 1971). Microtubules appear to be tension bearing elements in the cell, perhaps acting as the two by fours of the cell (Ingber & Jamieson, 1985; Joshi et al., 1985). There is evidence that compression of the microtubules may even play a role in depolymerization and length regulation of microtubules (Joshi et al, 1985). Microtubules also

appear to be involved in several other cellular activities including protein (Busson-Mabillot et al., 1982) and lysosomal (Hoffstein et al., 1977) secretion; localization of mitochondria (Bernier-Valentin & Rousset, 1982); organelle movement via kinesin, a microtubule locomotor equivalent of myosin in acto-myosin based filaments (Vale et al., 1985); and may even interact with DNA (Corces et al., 1978).

A third type of filamentous structures is the intermediate filament, so called because its thickness is intermediate that of microfilaments and microtubules. Intermediate filaments are composed of polymerized monomers as are microfilaments and microtubules. The monomers however differ among cell types and fall into five general classes: desmin in myogenic cells; neurofilaments in neuronal cells; glial filaments in astroglial cells; vimentin in mesenchymal cells; and a complex class of keratins from epithelial cells (Steinart et al., 1984; Lazarides, 1982). Since this project uses cultured fibroblasts, the rest of this discussion will be concerned with vimentin, the primary type of monomer found in these cells. The intermediate filaments form a highly insoluble network extending from the nucleus to out near the periphery of the cell. The intermediate filaments appear to form a meshwork of filaments that have been described as a basket or a cage. The intermediate filament system appears to contain its own arsenal of intermediate filament

associated proteins to control assembly and disassembly. This network of intermediate filaments appears to be much more static than the dynamic microfilament and microtubule systems. Even when cells round up, the intermediate filament network fails to depolymerize; instead, it collapses about the nucleus forming a cap or ring. The network appears to also be linked to the microtubule system, since the network will collapse about the nucleus when cells are treated with microtubule disrupting agents (Steinart et al., 1984). The intermediate filament network has also been suggested to interact with a cortical actin system on the ventral surface of the cell (Henderson and Weber, 1979). Because of its insolubility and close association with the nucleus, one of its major roles is considered to be centration of the nucleus in addition to other structural and organizational duties.

Spectrin is another cytoskeletal element that until recently was thought to be limited to erythrocytes. Spectrin is a large asymmetric molecule composed of a heterodimer (composed of Band 1 and Band 2) that can associate to form a tetramer (for reviews see Steck, 1974; Branton et al., 1981). Spectrin is attached to the membrane via a protein referred to as ankyrin, which attaches it to the membrane by binding to a transmembrane protein known as band 3. Spectrin also interacts with actin oligomers through band 4.1. In models of the erythrocyte membrane, spectrin is portrayed as two dimers linked head on and

interacting with actin oligomers at either end. The actin oligomers would thus appear to act as nodes for spectrin tetramers to bind to. Ankryin and band 3 serve to tack the system to the membrane. Recently, the counterparts to spectrin, ankryin, and band 4.1 have been identified in several nonerythroid cell types (Burridge et al., 1982; Moon et al., 1985; Cohen et al., 1982). Whether the structures formed by these components in nonerythroid cells are the same as in the erythroid cells is still not clear.

HISTORY OF PROTEIN LATERAL DIFFUSION

According to the Singer and Nicolson (1972) model, the plasma membrane consists of a phospholipid bilayer forming a sea where proteins can float embedded in the bilayer or associated with the bilayer. Proteins associated with only the external or cytoplasmic face of the bilayer are called peripheral membrane proteins. Proteins embedded in one half of the bilayer are considered to be integral proteins and proteins traversing the entire membrane are referred to as integral, transmembrane proteins. The intermixing of labeled antigens after cell fusion demonstrated the ability of membrane proteins to laterally diffuse (Frye and Edidin, 1970). Saffman and Delbruck (1975) developed diffusion equations to predict the rate of protein lateral diffusion. They predicted that the rate of protein lateral diffusion would be only weakly affected by the size of the molecule

and limited by the viscosity of the lipid bilayer. Subsequent measurement of protein lateral diffusion such as rhodopsin in rod outer segments (Poo and Cone, 1974) or various proteins in artificial vesicles prepared from plasma membranes (reviewed in McCloskey and Poo, 1984) supported the Saffman-Delbruck model. The measurement of protein lateral mobility in natural membranes, however, demonstrated a much slower rate of diffusion than expected by a factor of 10-100 (for reviews see Cherry, 1979; McCloskey and Poo, 1984).

The measurement of the rate of protein lateral diffusion was facilitated by the development of fluorescence redistribution of photobleaching (FRAP). In FRAP a sample is homogeneously labeled with a fluorescent probe. Diffusion is detected by photochemically destroying the fluorescent label in a small region of the sample with a focussed laser beam. Monitoring the redistribution offluorescent probe from outside the bleached region into the bleached region over a period of time allows the calculation of the rate of diffusion and extent of recovery (see appendix A). Initially, the use of intense laser light to photobleach the label caused concern over the possibility that crosslinking of membrane components could result in artifactual measurements. Several experiments have been carried out that suggest this is not the case (reviewed in Wolf et al., 1980): a) localized heating from exposure to the laser beam is less than 0.03°C; b) the

measurement of diffusion by FRAP on model membranes were independently confirmed by measurments using fluoresence correlation spectroscopy; c) FRAP results are generally consistent with estimates obtained from intermixing of surface antigens after heterokaryon fusion; d) FRAP measurements are generally independent of the degree of photobleaching; e) multiple bleaches in the same region of a cell do not affect diffusion rates and result in increases in the extent of recovery (after successive bleaches the immobile components will have been bleached and would no longer be detectable and so only mobile components are seen causing the apparent increase in the extent of recovery), significant crosslinking could be observed as an increase in immobile components replacing those bleached in the previous bleach; f) FRAP does not alter membrane morphology in scanning electron micrographs; g) free radical quenchers do not alter FRAP results; and h) dual labeling of F_c receptors with two labels demonstrated that extensive bleaching of one label does not affect FRAP measurements made with the other label. It has been demonstrated that extremely long bleaching times using low power, in the range of seconds instead of the milliseconds typically used in FRAP, can result in oxidation of lipids (Sheetz & Koppel, 1979) probably through the production of singlet oxygen. Thus caution is called for in the selection of bleaching times. Under the typical conditions of FRAP cellular damage is unlikely to occur since the fluorescent

dyes chosen for FRAP are excited at wavelengths above the region where cellular molecules typically absorb.

The development of FRAP facilitated the measurement of diffusion and as mentioned above, resulted in the discovery of the restriction of protein lateral diffusion in natural plasma membranes. This sparked a search for the cause of the restriction of protein lateral diffusion. The first clue came from studies of the effect of concanavalin A on patching and capping of membrane antigens in lymphocytes. The observation that concanavalin A restricted cap formation, followed by the finding that the restriction could be partially reversed by cytoskeletal disrupting agents (Edelman et al., 1973), led to the proposal that a Con A induced reorganization of the cytoskeleton resulted in an increased interaction of membrane proteins with the cytoskeleton (Edelman, 1976). Studies, in which FRAP was used to measure the rate of protein lateral diffusion, confirmed that concanavalin A treatment resulted in a decrease in protein mobility and found that the combination of a microtubule disrupting drug (colchicine) with a microfilament disrupting drug (cytochalasin B) completely reversed the restriction of protein lateral mobility induced by concanavalin A (Henis and Elson, 1981). On this basis the cytoskeleton became a leading candidate for the restriction of protein lateral mobility. An examination of protein lateral diffusion in spectrin deficient mouse erythrocytes (spherocytes) found that protein lateral

diffusion was 50 fold faster in the spherocytes than in the normal erythrocytes (Koppel et al., 1981). These results were interpreted in the context of spectrin interacting with membrane proteins resulting in the slow diffusion observed in normal erythrocytes. Further evidence for the role of cytoskeletal interaction in restriction of protein lateral diffusion was provided by the investigation of lateral diffusion in membrane blebs (Tank et al., 1982). Membrane blebs are regions of the plasma membrane pulled away from the underlying cytoskeleton. Protein lateral diffusion in these blebs was found to be 50 fold higher than in unaffected regions of the membraneand similar to that of lipid diffusion.

In addition to the dynamic evidence for an interaction between membrane proteins and the cytoskeleton there is physical evidence from numerous studies. Some studies using electron microscopy indicated a proximity of cytoskeletal structures to the membrane (Henderson & Weber, 1979; Stossel et al., 1981). Other studies have found an association between surface antigens and cytoskeletal elements at the fluorescent or electron microscopic level in chicken fibroblasts (Rogalski and Singer, 1985), rat mammary ascites (Carraway, et al, 1982), neutrophil leukocytes (Sheterline and Hopkins, 1981), and a murine mastocytoma (Koch and Smith, 1978) to name a few. In light of the use of wheat germ agglutinin as the probe for protein lateral mobility in this thesis, it is significant

to note that one ultrastructural study found WGA and Con A receptors localized to regions of the membrane overlying the filaments of the cortical network (Roos et al., 1985). In several of these studies it was also determined that detergent extraction of the cells had no effect on the distribution of the antigens or the cytoskeleton. Furthermore, concanavalin A has been demonstrated to increase the interaction of membrane proteins with the cytoskeleton in platelets (Painter and Ginsberg, 1982) and to recruit actin to the membrane in lymphocytes (Michaels et al., 1984). Crosslinking of Ig receptors by Ig enhanced the attachment of the Ig receptors to actin structures even in the absence of capping suggesting that aggregation is a critical step in initiating a link between the receptor and the cytoskeleton (Flanagan & Koch, 1978). While numerous studies have suggested an association between membrane proteins and actin in many cell types and between spectrin and ankyrin in erythrocytes, only a few have suggested an association between membrane proteins and other cytoskeletal elements such as microtubules and intermediate filaments. Microtubules have been shown to bind to plasma membranes (Reaven & Azhar, 1981) and a membrane glycoprotein has been found to remain with intermediate filaments upon detergent extraction (Lehto, 1983). However, ultrastructural studies would suggest that microtubules and intermediate filaments are generally excluded from the cortical submembranous region which appears to be occupied

mainly by actin networks (Henderson & Weber, 1979). Because of the effect of colchicine on global modulation, microtubules are thought to play a significant role in the interaction of the cytoskeleton with membrane proteins. In light of the evidence for interaction between the two cytoskeletal systems (Craig & Pollard, 1982; Henderson & Weber, 1979), it is possible that microtubules play a role in organizing or supporting a cortical actin system.

Interaction of membrane proteins with the cytoskeleton was not the only model proposed to explain the restriction of protein lateral diffusion. Density of protein in the membrane has been shown to affect lateral diffusion probably by increasing the viscosity of the membrane (Golan et al., 1984; McCloskey & Poo, 1984). The amount of protein in natural membranes may slightly affect lateral diffusion, but is almost certainly not enough to explain the observed slower rate of protein lateral diffusion. It has been proposed that segregation of lipids into solid and fluid domains, or even two immiscible fluid domains, could slow protein lateral diffusion as proteins diffuse through solid domains or cross boundary regions (Webb et al., 1981; Axelrod, 1983; McCloskey and Poo, 1984). While such domains have been observed in plant cells (Metcalf et al, 1986), the evidence of their presence in animal cells is still limited. A more likely candidate is the interaction of membrane proteins with the extracellular matrix. The extracellular matrix is a complex system of proteins,

proteoglycans, and glycoproteins that form a matrix outside the cell and is believed to be involved in attachment and perhaps even gene expression (Bissel et al., 1982) and differentiation (Ingber & Jamieson, 1985). Many of the components, such as fibronectin, are known to interact with membrane proteins as part of their role in attachment (Yamada & Olden, 1978) Fibronectin has even been suggested to be associated with and perhaps organize actin structures (Hynes and Destree, 1978; Burridge & Feramisco, 1980; Virtanen et al., 1982). It seems likely, then, that the extracellular matrix could play a role in the restriction of protein lateral diffusion. It is surprising how little experimental evidence exists for such a role. One component of the extracellular matrix, fibronectin, has been demonstrated to be immobile on the time scale of the experiment, but the lateral mobility of cell surface antigens was not affected by the presence or absence of fibronectin (Schlessinger et al., 1977). The main evidence for such a model was provided by a study on the effects of cell density on lateral mobility and the effects of plating cells on dishes precoated with extracellular matrix components (Weir and Edidin, 1986). Protein lateral mobility was compared on cells from sparse cultures with cells from dense cultures. No effect on the rate of protein lateral diffusion was observed; however, the extent of recovery decreased in the dense cultures. When cells were sparsely plated on dishes precoated with extracellular

matrix components and compared to cells plated onto proteolyzed extracellular matrix components, it was observed that the presence of extracellular matrix components decreased the extent of recovery, but not the rate of recovery. Thus, it seems likely that the extracellular matrix associates with its receptors so tightly that those proteins appear immobile on the time scale of the experiment, while the unbound proteins remain unaffected as detected by the lack of effect on the rate of lateral diffusion. This differs, then, from the results of experiments investigating the interaction of the cytoskeleton with membrane proteins noted above where the rate of diffusion is affected. It has been suggested that the interaction with the cytoskeleton may occur with an on/off rate fast enough to allow diffusion to be detected on the time scale measurable by FRAP (Koppel et al., 1981; Koppel, 1981; Elson and Reidler, 1979). The results from the extracellular matrix experiment could then be interpreted to mean that membrane proteins associate with the extracellular matrix with too slow an on/off rate to detect an effect on the rate of lateral diffusion, but those already bound would appear to be immobile.

THE SYSTEM: ALTERATION OF CELL SHAPE

BALB/c 3T3 fibroblasts are an aneuploid culture line derived from mouse embryos. Untransformed BALB/c 3T3 cells demonstrate cell growth regulated by the density of the cell population and a requirement for serum growth factors (Holley, 1975; Holley & Kiernan, 1968). When cells are plated sparsely onto a suitable growth substratum in the presence of serum, the cells will attach and spread into a characteristic elongated shape. As the density of the cells increase and the cells come into contact, the growth rate slows and the cells become slightly rounded until the cells form a monolayer and assume a cobblestone pattern. At confluence the cells become quiescent in a phenomenon called density dependent inhibition of cell growth. Attachment and spreading is also required for cell growth. Cells suspended in agar or methyl cellulose, a common test for transformation, become quiescent but remain viable for a few days (Otsuka & Moskowitz, 1975; MacPherson & Montagnier, 1964). Suspension of cells in methyl cellulose has been a useful means for suspending cells in way that allows recovery of the cells by simple dilution and centrifugation. The methyl cellulose is dissolved into the normal media and serum used for culturing the cells and merely acts as a thickener to suspend the cells and decrease aggregation. The cells will grow in methyl cellulose if they are provided a large enough substratum,

such as glass fibrils, indicating that the methyl cellulose is nontoxic to the cells (Stoker et al., 1968). Cell shape and adhesion was demonstrated to be vitally important to cell growth in anchorage dependent fibroblasts by Folkman and Moscona (1978) in a landmark study investigating the effect of varying substrate adhesiveness on cell shape and DNA synthesis. Fibroblasts suspended in methyl cellulose demonstrated a rapid decrease in DNA and mRNA synthesis followed by a more gradual decrease in protein synthesis (Benecke et al., 1978; Ben Ze'ev et al. 1980; Farmer et al., 1978). Reattachment of the cells where spreading was prevented, was followed by a rapid recovery of protein synthesis, but not DNA or mRNA synthesis. mRNA was still present in suspended cells but demonstrated aberrent translation properties in in vitro translation systems. Protein translation resumed shortly after attachment before mRNA synthesis reumed and even in the presence of actinomycin (blocks mRNA synthesis). From these results it was concluded that mRNA is stored in an unusable form during suspension. DNA and mRNA synthesis resumed only when extensive cell spreading was allowed to occur. Interestingly, a major protein comigrating with actin in two dimensional gel electrophoresis showed enhanced synthesis during the recovery from suspension. Clearly, cell shape is important to several key cellular metabolic processes in anchorage dependent fibroblasts. It has been shown that doses of growth factors, normally sufficient for

stimulation of cell growth in spread cells, had no effect on suspended, spherical cells (Schubert & Lacorbiere, 1976). A relation between cell shape and the mitogenic response has been noted in various systems (Ben-Ze'ev, 1985). Furthermore, a recent study has demonstrated the association of EGF-receptor complexes with the detergent insoluble cytoskeleton was decreased by the alteration of cell shape in an anchorage dependent cell line (Landreth et al, 1985). This raises the possibility that the normal signal transduction pathway has been disrupted in suspended cells.

Alteration of cell shape also has a dramatic effect upon the organization of the cytoskeleton. The cytoskeleton in attached and spread fibroblasts is a highly organized network of microfilaments encircling the cytoplasm, stress fibers running underneath the nucleus, a few microfilaments running through the cytoplasm, bundles of microtubules radiating throughout the cytoplasm and enclosed by the microfilaments, and an intermediate filament network surrounding the nucleus and extending through the cytoplasm (Henderson & Weber, 1979). When cell shape is altered by detachment of the cells, the stress fibers, microfilaments in the cytoplasm, and microtubules disappear as observed by electron microscopy (Badley et al., 1980). It seems probable that the intermediate filament network collapses about the nucleus when the microtubules disappear as happens in colchicine treated cells (Steinart et al.,

1984). The fate of the cortical actin network is unknown. In HeLa cells, alteration of cell shape does not alter the monomer to polymer actin ratio suggesting that cell shape alteration results in a reorganization of and/or possible shortening of microfilaments, but it does not seem to result in a depolymerization to monomers (Blikstad & Carlsson, 1982). The alteration of the cytoskeleton is related to the change in cell morphology and has even been suggested to be linked to the change in gene expression that occurs upon cell shape change (Ben Ze'ev, 1985).

The use of anchorage dependent fibroblasts, such as BALB/c 3T3 cells, provides a convenient system in which to study phenomena accompanying cytoskeletal alteration upon changes in cell shape including putative effects on protein lateral mobility, communication pathways, and nuclear activities. True, the cytoskeleton can be disrupted in other ways, such as the use of cytoskeletal disrupting drugs. The cytoskeletal disrupting drugs, colchicine (microtubule disrupting) and cytochalasin B (microfilament disrupting) were in fact used to demonstrate the involvement of the cytoskeleton with the restriction of membrane protein lateral diffusion via global modulation, as discussed above. The effects of the cytoskeletal disrupting drugs, however, produce conflicting results. Attempts to detect an effect of cytoskeletal disrupting drugs on protein lateral mobility without global modulation, i.e. cells not treated with concanavalin A, has

produced mixed results. In some instances the drugs appeared to enhance lateral mobility only slightly or not at all; in others it seemed to even decrease mobility to some extent (Elson & Reidler, 1979; Elson & Schlessinger, 1980; Eldridge et al., 1980; McCloskey & Poo, 1984; Webb et al., 1981). The effects on the cytoskeleton also appear to vary. Usually considered to cause microfilament break down, cytochalasin B has been observed to cause local increases in microfilament concentration underneath the membrane in some cells (Miranda et al., 1974). Even the effects of cytoskeletal disruption by drugs on cellular metabolism varies. Microtubule disruption by colchicine initiates DNA synthesis (Crossin & Carney, 1981); whereas, microfilament disruption by cytochalasin blocks initiation of DNA synthesis (Maness & Walsh, 1982) but can also enhance the effect of growth factors in initiation of DNA synthesis (Otto et al., 1979). In addition, the cytoskeletal disrupting drugs have some side effects such as the inhibition of glucose uptake by cytochalasin B (Estensen & Plagemann, 1972).

Transformation of fibroblasts is another factor that alters the organization of the cytoskeleton. Transformed fibroblasts lack the thick bundles of stress fibers readily apparent in untransformed cells. Instead, the transformed cells contain fewer, thinner stress fibers and in some instances no apparent stress fibers (Ambrose et al., 1970; Pollack et al., 1975). Filamentous actin aggregated into

patches described as rosettes near the ventral surface associated with adhesion plaques has also been observed (David-Pfeuty & Singer, 1980; Carley et al. 1981). It is intriguing that these F-actin aggregates are insensitive to actin disrupting drugs (Carley et al., 1983). As with the microfilaments, microtubules are scarce in the cytoplasm of transformed cells (Brinkley et al., 1975). Despite these differences in the organization of the cytoskeleton, the lack of effect on the lateral mobility of membrane proteins has been noted (Eldridge et al., 1980). While this lack of effect is difficult to understand, it must be noted that transformed cells do appear to have some functional form of cytoskeleton to maintain cell shape and mobility. In addition, cytochalasin B in some transformed cells, but not untransformed cells, has been observed to initiate a co-capping of plasma membrane and nuclear membrane antigens and therefore hinting at the possibility of a linkage between the plasma membrane and the nuclear membrane in these treated, transformed cells (Otteskog et al., 1981). Another example shows that transformation can affect a dynamic process in which the cytoskeleton plays an active role. An examination of the effect of transformation on global modulation found that transformation inhibited the ability of concanavalin A to restrict protein lateral mobility (Edelman & Yahara, 1976). The story behind the effects of transformation does seem to be complicated, as seen for the cytoskeletal disrupting drugs; however, the

ability of the transformed cell to grow in suspension (Freedman & Shin, 1974) suggests that the comparison of the effects of the alteration of cell shape in transformed cells to untransformed cells could prove useful.

The experiments discussed in my thesis refer to studies done on fibroblastic cells and thus the conclusions drawn from these studies are limited to fibroblastic cells. Other cell types, such as endothelial cells, have a different shape and cytoskeletal organization and so may have different responses to the conditions of these experiments which has not been tested.

In summary, the alteration of cell shape in anchorage dependent fibroblasts provides a system with known effects on gene expression and the cytoskeleton. The development of such a system would be useful in examining the role of the cytoskeleton in restriction of protein lateral mobility and putative communication pathways. The availability of transformed cells from the same cell line provides another means of examining lateral diffusion and putative communication pathways in cells with a perturbed cytoskeleton as well as an opportunity for the comparison of the effects of cell shape alteration in a cell system in which cell growth is unaffected by suspension.

Summary of Objectives

1. Examine the role of cell shape in the putative initial event in transmembrane signaling, the lateral mobility of membrane receptors and its link to the cytoskeleton by:
   a. the investigation of WGA receptor lateral mobility in spread and spherical cells
   b. monitoring the state of the actin stress fiber system
   c. correlating these events with studies by others in this laboratory on nucleocytoplasmic transport

2. Test for differences in receptors and cell surface morphology
   a. characterize lectin receptor binding
   b. examine glycoprotein and glycolipid receptors
   c. scanning electron microscopy

3. Monitor the consequences of shape change on glucose utilization and DNA Synthesis

4. Determine the extent of the increased lateral mobility in spherical cells by extending the measurements to
   a. lipid mobility

b. other glycoproteins that bind succinyl concanavalin A receptors.

5. Examine the effect of transformation as another means of perturbing the cytoskeleton on
   a. WGA receptor lateral diffusion
   b. the actin stress fiber system in conjunction with the alteration of cell shape

6. Test the relationship between the increased protein lateral diffusion after cell shape alteration and the cytoskeleton by
   a. determining the effect of cell shape alteration on global modulation
   b. examining the relation between the altered stress fiber patterns and global modulation in the E7G1 clone.

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CHAPTER III

PROTEIN LATERAL DIFFUSION IN SPREAD AND SPHERICAL FIBROBLASTS

INTRODUCTION

The involvement of cell shape as a controlling influence for DNA synthesis and growth in anchorage dependent cells was originally demonstrated by the work of Folkman and Moscona (1978). Further efforts showed that while mRNA production, rRNA and DNA synthesis, and cell growth require an extensively spread cell, protein synthesis occurs upon cell attachment (Benecke et al., 1978; Ben Ze'ev et al., 1980). These results and other work (Otsuka & Moskowitz, 1975; MacPherson & Montagnier, 1964) suggest that the spherical to spread transition of anchorage dependent cells which follows cell attachment to growth surfaces, influences the subsequent biological activity of these cells. The changes in cell architecture associated with the spherical to flat structural transition have been correlated with the reassembly of stress fibers, microfilaments, and microtubules (Badley et al., 1980). In light of the importance of these cytoskeletal structures for protein biosynthesis (Penman et al., 1981; Cervera et al., 1981), and transmembrane signal aquisition (Geiger, 1983; Landreth et al., 1985) and transmission (Ingber & Jamieson, 1985), the possibility that the flat to spherical

shape transition interrupts a cytoskeletal communication pathway from the cell surface to the nucleus has been suggested (Ben Ze'ev, 1985). Since the lateral mobility and topology of membrane receptors has been demonstrated to be modulated by the cell cytoskeleton with consequences for transmembrane signaling (Edelman, 1976; Koppel et al., 1981; Tank et al., 1982), we have attempted to determine the role of shape induced cytoskeletal changes in the lateral mobility of BALB/c 3T3 fibroblast plasma membrane phospholipids and glycoprotein receptors. In an effort to examine whether shape changes can play a role in uncoupling receptor mediated signaling, changes in receptor lateral mobility are monitored to serve
event. Transformed cells, then, represent a system in which the normal communication pathway is bypassed and this is reflected in the cells ability to grow in suspension. The experiments to be discussed were performed on spherical and spread untransformed, and Kirsten murine sarcoma virus transformed (K-MSV) BALB/c 3T3 fibroblasts. The major result of this work is that the apparent diffusion coefficient for both spherical untransformed and transformed cells was 12-fold greater than for spread cells. This suggests that in adhering, flat cells the membrane receptors were associated more closely with assembled cytoskeletal elements which may serve to transmit transmembrane signals from the cell membrane to the interior, particularly the nucleus.

MATERIALS AND METHODS

Cell Culture

BALB/c A31 3T3 (3T3) and Kirsten mouse sarcoma virus transformed (K-MSV) BALB/c 3T3 fibroblasts were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DME) plus 10% calf serum. Spread cells were plated on tissue culture dishes on the day before use. Spherical cells were prepared by trypsinization and suspension in DME/10% calf serum containing methyl cellulose (MC, 1.5% final concentration) for one hour, one day, or two days. DME/MC was prepared in a fashion similar to that described elsewhere (Rheinwald and Green, 1974). Briefly, methyl cellulose was autoclaved and dissolved in sterile water at 60°C to a concentration of 2.3% and stirred at room temperature for 20 minutes. The methyl cellulose was diluted to 1.6% by the addition of 2x concentrated DME and calf serum (10% final concentration). The DME/MC was stirred overnight at 4°C before use. Spherical cells were also prepared for some studies by EDTA removal and suspension in methyl cellulose for one hour.

Fluorescence Redistribution After Photobleaching (FRAP)

The instrument used was similar to that described by Koppel (1979). Diffusion measurements on spread cells were obtained using a point bleach and multipoint analysis

described elsewhere (Koppel, 1979) and reviewed in the appendix A. Lateral diffusion was measured on spherical cells using an edge bleach and normal mode analysis as presented previously (Koppel et al., 1980). Protein mobility was monitored using rhodamine-labeled wheat germ agglutinin (WGA) (Vector, Burlingame, CA). Lipid mobility was monitored using NBD-phosphatidylcholine.

Fluorescence Microscopy

Actin filaments in cells were labeled with rhodamine-phalloidin (Molecular Probes, Junction City, OR) and fixed using the one step method described in the package insert. The distribution of WGA receptors was examined on cells labeled with 100 µg/ml rhodamine-WGA and fixed with 3.7% formaldehyde. Control samples contained 0.2 M N-acetylglucosamine in the incubation and wash buffers. Fibronectin patterns were determined by fixing the cells with 3.7% formaldehyde for 30 minutes at room temperature, washing the cells with phosphate buffered saline (PBS) for 20 minutes at room temperature, and labeling the cells with anti-fibronectin antibodies (1:100 dilution). Control samples were incubated with normal rabbit serum. Samples were washed with PBS and incubated with rhodamine-labeled goat anti-rabbit antibodies (1:30 dilution) for 30 minutes at 37°C. Antibodies were obtained from Miles Scientific (Naperville, IL). Samples were washed 3 times with PBS/5% goat serum. All samples were mounted in PBS/90% glycerol

(v/v)/5% N-propylgallate (w/v).

Cell Population Histograms

Cells, labeled with fluorescein-WGA and fixed, were scanned with a laser beam in 2 dimensions using the ACAS 470 Fluoresence Workstation (Meridian Instruments, Okemos, MI). The resulting fluorescence was collected, measured by a photomultiplier tube, and digitized to produce 2-dimensional images of the labeled cells. Cell population histograms were obtained through computer analysis of the digital images.

WGA Binding

The amount of WGA bound to the cells was estimated using fluorescein-WGA. Various concentrations of FITC-WGA were incubated with the cells for 30 minutes at 4°C. After washing, the cells were lysed in HEPES (12.5 mM) buffered Hank's Balanced Salt Solution (HBSS/HEPES) containing 1% sodium dodecyl sulfate (SDS). The fluorescence was determined in a Perkin-Elmer spectrofluorimeter using an excitation wavelength of 460 nm, an emission wavelength of 520 nm, and 5 nm slit widths. The amount of fluorescein-WGA bound to the cells was estimated by comparison to a standard curve of FITC-WGA in HBSS/HEPES/1% SDS and was normalized to the amount of protein. The amount of protein was determined using a modified Lowry procedure (Markwell et al., 1978). The standard curve could also be prepared by

incrementally adding FITC-WGA to previously measured cell samples with no noticeable effect which suggested that the fluorescence was not absorbed by the sample.

Comparison of WGA Receptors

Equal number of cells were lysed in 1% SDS/1 mM PMSF. Equal amounts were loaded and separated on duplicate 10% polyacrylamide gels. Proteins were transferred onto nitrocellulose sheets by the procedure of Towbin et al. (1979) for 3-4 hours at 400 mA. The blots were incubated overnight in PBS/2%PVP (phosphate buffered saline, pH 7.4, polyvinylpyrrolidone) as suggested by Bartles and Hubbard (1984) to block excess protein binding sites. The blots were incubated for 3-5 hours at 4°C, shaking with 2 µg/ml WGA-horse radish peroxidase in PBS/PVP. The blots were washed 5 times for 5 minutes with PBS/PVP and rinsed briefly with Tris buffered saline. Buffers used for replicate blots included 0.2 M N-acetylglucosamine plus or minus 0.2 M AMP as controls. Color development was carried out as described in the Bio-Rad procedure using Biorad color development reagent.

Characterization of Glycolipid Receptors for WGA

Spherical cells were prepared by overnight suspension in DME/MC. Immediately before use, spread cells were removed from their substrate and counted. Spherical cells were removed from the methyl cellulose and counted. 5×10^6

cells were placed into centrifuge tubes and centrifuged for 5 minutes at low speed to pellet the cells. The cells were extracted as described by Folch et al. (1957). Briefly, 3 mls of methanol were added to the cell pellets and mixed extensively. 6 mls of chloroform were added to the samples and again mixed extensively. To the methanol:chloroform mixture was added 0.5 mls of 4 M potassium chloride and 2.5 mls of water followed by mixing. Phases were separated by low speed centrifugation. The chloroform phase was removed to a tared tube. The chloroform extraction was repeated with a second 6 mls and added to the first 6 mls. The organic phases were evaporated under nitrogen, redissolved in 2:1 chloroform:methanol, and stored at -20°C until use in sealed ampules under nitrogen. The aqueous phase was lyophilized in a tared tube and redissolved in 2:1 chloroform:methanol at time of use. Glycolipids were separated by thin-layer chromatography (TLC) using 5:4:1 chloroform:methanol:water. WGA receptors were probed with radioiodinated WGA as described previously (Smith, 1983). WGA binding could be inhibited by addition of 0.2 M N-acetylglucosamine. WGA was labeled using chloramine T as described elsewhere (Greenwood et al., 1963).

Characterization of Cell Growth

Thymidine uptake:

Spread cells were plated on 12 well plates (4×10^5 cells/well). Spherical cells were grown in 150 cm^2 flasks until 0 hour. To synchronize the cells, all cells were blocked twice with 3 mM thymidine as described elsewhere (Adams, 1980). Cells were released by washing and adding fresh media. Cells to be made spherical were removed with trypsin and placed in DME/MC; this was considered 0 hour. At 1, 5, 9, 13, 17, 21, 25, and 33 hours, samples were removed and incubated for one hour with 10 µCi ^{3}H-thymidine/sample (spherical cells were removed from DME/MC before labeling). Cells were washed twice with PBS, lysed in 0.5 ml 0.05 N NaOH/1% SDS, and counted by liquid scintillation methodology.

Glucose utilization:

The utilization of glucose was determined using a novel technique described by Pollard *et al*. (1981). The method uses the exchange of tritiated hydrogen with water from the 2 position of D-glyceraldehyde-3-phosphate (originally the 5 position of glucose) during its isomerization to dihydroxyacetone, catalyzed by triosephosphateisomerase. In this way, tritiated water is essentially the only radioactive product of the metabolism of D-[5-^{3}H]glucose. The tritium is extracted from the water

into an organic fraction containing scintillants. The tritium from the water exchanges with hydrogen on isoamyl alcohol in the scintillation fluid, while the unmetabolized radioactive glucose remains in the aqueous fraction and immiscible with the scintillation fluid. Briefly, the cells were incubated with various concentrations of glucose spiked with 1.5 µCi/ml D-[5-^{3}H]glucose in glucose-free HBSS (1 ml/sample). At 0 hour and at 4 hours triplicate 50 µl aliquots were removed to scintillation vials containing 200 µl of Benedict's reagent (43.25 g sodium citrate mixed with 25.0 g anhydrous sodium carbonate in 200 ml of water; add 4.325 g cupric sulfate in 25 ml water and adjust volume to 250 ml with water). 80 µl of 1 M carrier glucose was added and samples were boiled for 30 minutes in a water bath. After cooling for 10 minutes at room temperature, 5 ml of scintillation fluid (750 ml toluene, 250 ml isoamyl alcohol, 18.95 g PPO (2,5-diphenyloxazole), and 1.14 g POPOP (p-bis[2-(5-phenyloxazoyl)]benzene) was added and mixed. Phases were allowed to separate for at least 5 minutes before scintillation counting. Aliquots were also analyzed for protein concentration by the method of Markwell et al. (1978).

Determination of ATP levels

The amount of ATP in spread untransformed and transformed fibroblasts was determined using an enzyme assay with Luciferin/Luciferase (Sigma, St. Louis) based on

that described elsewhere (package insert and Webster et al., 1981). Cells were plated on 60 mm dishes at 3 x 10^5 cells/dish. Cells were removed with EDTA, pelleted, suspended in 300 µl PBS, and injected into 700 µl boiling water for 15 minutes to prevent degradation of ATP and cooled on ice. To 200 µl sample was added 75 µl Luciferin/Luciferase (40 mg/ml) and buffer (0.025 M Tricine, 5 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM dithiothreitol, pH 7.8) to 1 ml. After rapid mixing, the amount of light emitted over 1 minute was recorded on a chart recorder with a spectrofluorimeter set at emission wavelength 555 nm and 20 nm slit width. Extrapolation to 0 time allowed estimation of the initial emission. Comparison of light emitted from samples to that from known concentrations of ATP allowed estimation of ATP levels. Protein concentration was determined from aliquots using a modified Lowry procedure described by Markwell et al. (1978).

Scanning Electron Microscopy

Spread cells were fixed with 4% glutaraldehyde for two hours, post-fixed with 0.5% osmium tetraoxide for 15 minutes, dehydrated through a series of ethanol washes, and critical point dried. Spherical cells were removed from DME/MC, fixed and post-fixed as for spread cells. Fixed spherical cells were attached to SEM grids or disks with poly-L-lysine, dehydrated through a series of ethanol washes, and critical point dried. SEM was performed on a

JEOL 35C at 15 kV.

RESULTS

Consequence of Shape and Transformation on the Number of WGA Receptors in 3T3 Fibroblasts

Glycoprotein receptors for WGA served as a general probe to monitor lateral mobility of plasma membrane proteins in both untransformed and transformed cells. To assure appropriate comparisons between different cell shapes and transformation states, a series of investigations were directed to characterizing the number and type of WGA receptors under all measuring conditions. These experiments were performed both in bulk and on a single cell basis. It was of particular importance to insure that the techniques required to remove cells for spherical analysis did not greatly alter the number or type of WGA receptors on the cell surface. In this context, we determined the binding of WGA to spread cells, EDTA removed cells, and trypsin removed cells. The binding to trypsin removed cells was determined after one hour, one day, and two days in suspension. The removal of 3T3 cells (untransformed) with EDTA or trypsin had little effect on the number of WGA receptors (see figure 1A, differences were within standard deviations). As seen in figure 1A, cells suspended in DME/MC for one or two days also bound an amount of WGA similar to that of the spread cells. The binding of WGA to transformed K-MSV 3T3 cells appeared to

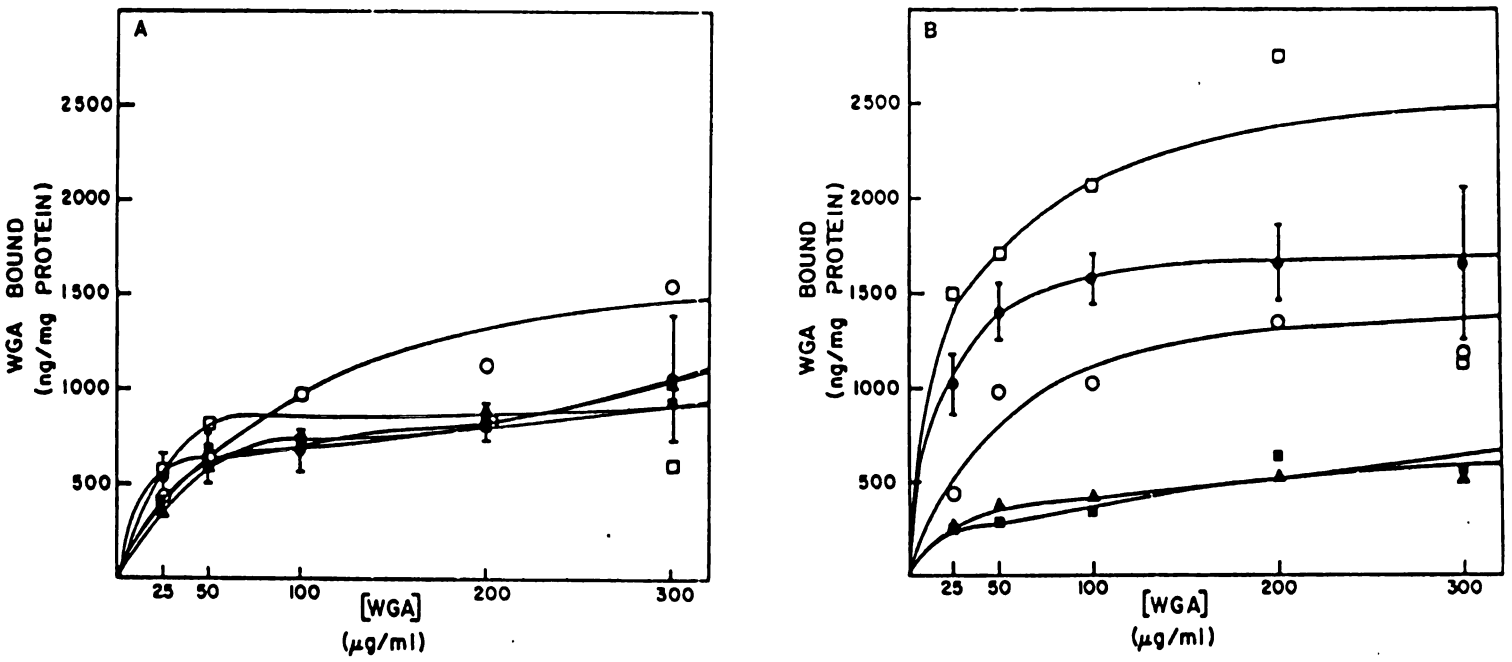

Figure 1. The binding of FITC-WGA to untransformed 3T3 fibroblasts (A) and K-MSV transformed 3T3 fibroblasts (B) in the spread and spherical state was determined at various concentrations of FITC-WGA. The binding of WGA to spread cells is denoted by (●). The binding to spherical cells was determined after trypsinization and suspension in methyl cellulose for one hour (▲), one day (○), and two days (□). WGA binding to spherical cells that had been removed with EDTA and suspended for one hour (■) was also determined. The error bars denote the standard deviation for the spread cells which was similar in the spherical cells.

be more sensitive to the removal process, as shown in figure 1B. However, after one or two days of suspension, the spherical cells bound an amount of WGA similar to that found on spread cells. Similar results were obtained when radioiodinated WGA was used instead of FITC-WGA to determine binding (see Figure 2). These results demonstrate that the number of WGA receptors was similar in the different cell states for diffusion measurements.

Additional support for the results of the bulk cell measurements observed in figure 1 was obtained by using an ACAS 470 Fluorescence Workstation to quantitate the amount of fluorescently derivatized WGA on a single cell basis. Using digital imaging techniques, a large number of cells were scanned and the amount of fluorescence per cell was determined. Frequency histograms showing the distribution of fluorescence per cell are presented in Figure 3. The binding to individual cells showed some variations, but the populations of WGA binding cells had a similar intensity distribution, as well as a similar average fluorescence per cell, regardless of cell shape. These results are in agreement with the bulk binding studies shown in Figure 1 and indicate that the number of WGA receptors is not affected by transformation or altered cell shape. Using epi-fluorescent microscopy, the aggregation state of the WGA receptors was examined. Figure 4 shows fluorescence micrographs of FITC-WGA bound to spread and spherical 3T3 and K-MSV 3T3 cells. The WGA receptors appeared to be

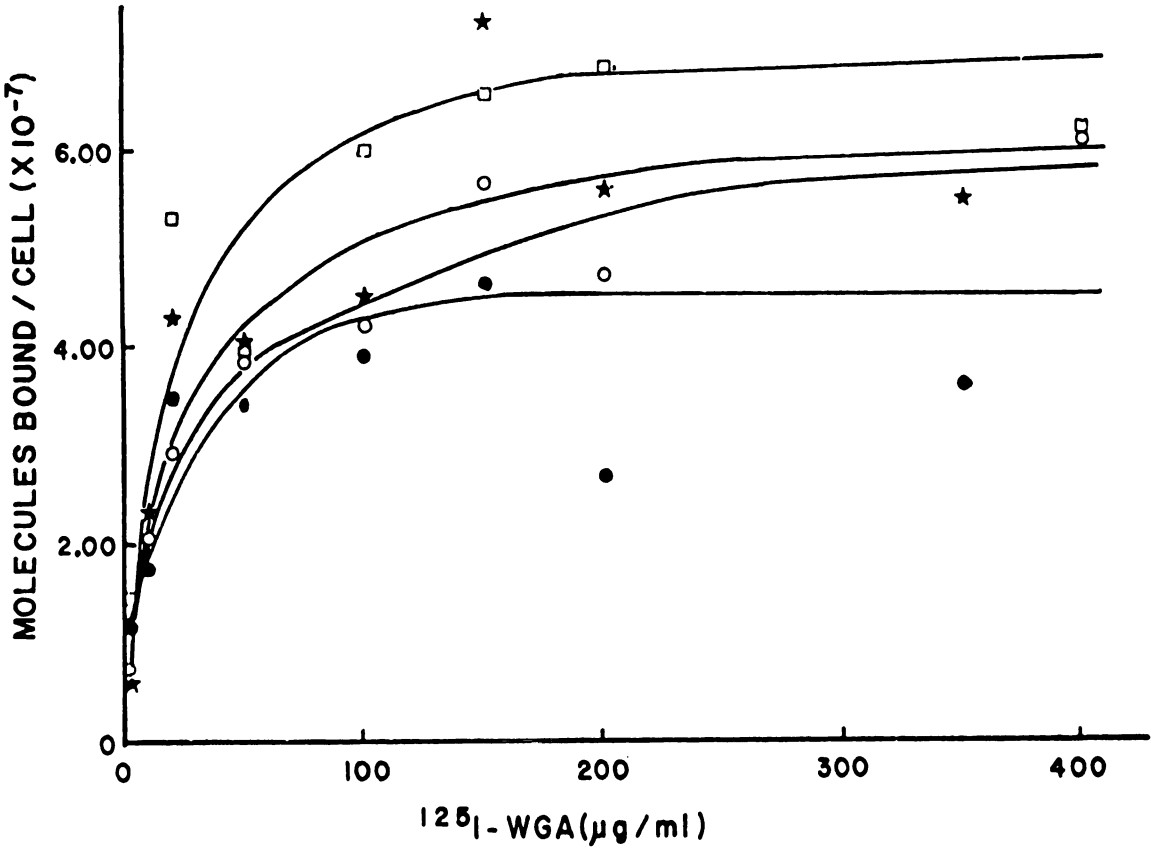

Figure 2. The binding of radio-iodinated WGA to untransformed and K-MSV transformed 3T3 fibroblasts in the spread and spherical state was determined at various concentrations of WGA. The binding of WGA to spread untransformed (★) and transformed (○) fibroblasts was similar to WGA binding in spherical untransformed (●) and transformed (□) 3T3 fibroblasts.

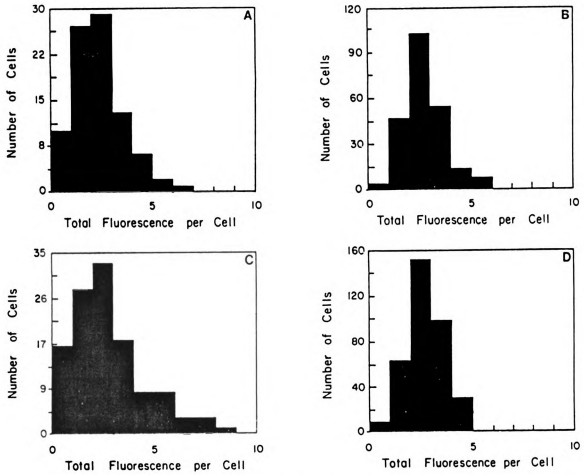

Figure 3. Population analysis of the amount of fluorescence per cell which is related to the amount of FITC-WGA bound by the cell. The amount of fluorescence from each cell scanned was determined by digital imaging methodology and is presented as frequency histograms. The distribution of binding for spread (A) and spherical (B) 3T3 cells as well as spread (C) and spherical (D) K-NSV transformed 3T3 cells is shown.

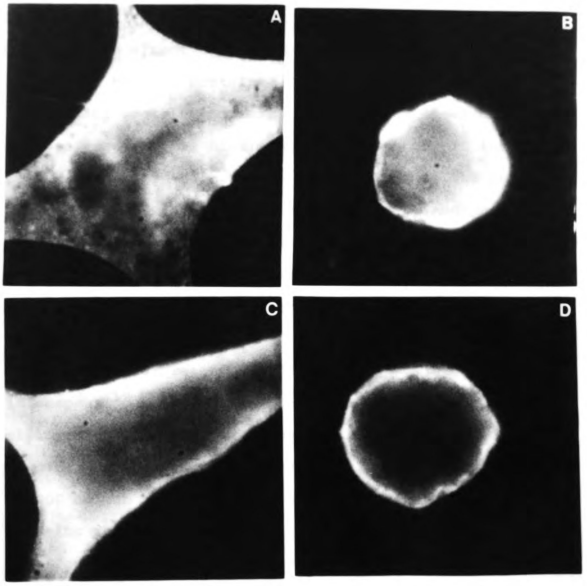

Figure 4. Fluorescence micrographs of spread and spherical 3T3 fibroblasts labeled with FITC-WGA. (A) spread BALB/c 3T3 cells; (B) spherical BALB/c 3T3 cells; (C) spread K-MSV BALB/c 3T3 cells; (D) spherical K-MSV BALB/c 3T3 cells

homogeneously distributed over the cell surface in all cell types.

A comparison of the WGA receptors from whole cell lysates from spread and spherical cells shown in Figure 5 examined the effect of the removal and suspension process on the WGA binding proteins. The majority of the specific WGA binding proteins appeared to be in the upper half of the Western transfer blot since many of those in the bottom half were not removed by N-acetylglucosamine (the hapten inhibitor for WGA) as shown in lane I. The nonspecific binding appears to be a charge dependent phenomenon that can be blocked by charged species such as AMP (this lab, unpublished observations) as shown in lane J. In general the major bands on the transfer blot specific for WGA appeared similar regardless of cell shape, length of time in suspension, or transformation. One of the major glycoproteins on murine fibroblasts is an 80,000 dalton WGA binding glycoprotein (Jacobson et al., 1984). A band in the 80,000 dalton region in Figure 5 is one of the WGA binding proteins that appeared to be unaffected by suspension or transformation. Since FRAP measures an ensemble diffusion coefficient it would require major differences in the WGA surface receptors to account for the increase in protein lateral mobility and such differences were not apparent in the WGA receptor number or whole cell WGA binding glycoproteins.

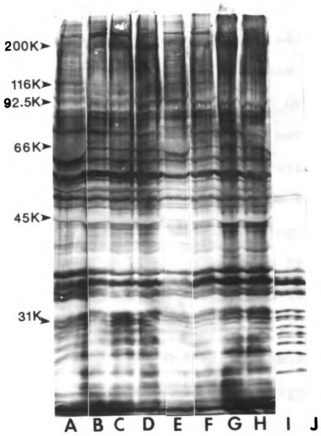

Figure 5. Western transfer blot of WGA glycoprotein receptors from spread and spherical 3T3 fibroblasts. WGA receptors from spread BALB/c and K-MSV BALB/c 3T3 cells are shown in lanes A and E, respectively; from spherical 3T3 cells suspended for 1 hour in methyl cellulose are shown in lanes B (BALB/c) and F (K-MSV BALB/c); 3T3 cells suspended for 1 day are shown in lanes C (BALB/c) and G (K-MSV BALB/c); 3T3 cells suspended for 2 days are shown in lanes D (BALB/c) and H (K-MSV BALB/c); controls for blots incubated with 0.2 M N-acetylglucosamine (I) and 0.2 M N-acetylglucosamine plus 0.2 M AMP (J). Molecular weight markers are as indicated.

The possibility of WGA binding to glycolipids was also considered. The autoradiogram from thin-layer chromatographs probed with ^{125}I-WGA is presented in Figure 6. The most apparent bands on the autoradiogram were found in both the aqueous and organic fractions and are most likely protein contaminants. There were only faint bands observed in the organic fraction which had a relative mobility similar to that of the ganglioside standards (which bound WGA and acted as a positive control) despite the fact that five times as much starting material was used for these TLC plates as for the western transfer blots shown in Figure 5. These bands appeared in approximately equal amounts in 3T3 and K-MSV 3T3 cells, regardless of cell shape. Of particular importance for the interpretation of our results was that the amount of glycolipid was considerably less than that of the protein contaminants seen in both the organic and aqueous fractions. Furthermore, in the aqueous fractions no bands were detected in the region of the gangliosides standards indicating all the detectable glycolipid receptors had been extracted with the organic phase. Thus, it seems unlikely that the binding of WGA to glycolipids is significant in our diffusion measurements.

These results suggest that WGA binding glycoproteins are sufficiently similar in amount and type under the differing shape and transformation conditions so that they may be used as comparative probes of lateral mobility.

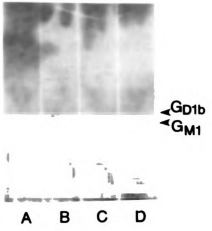

Figure 6. Autoradiogram of glycolipids separated by TLC and probed with radio-iodinated WGA. WGA glycolipid receptors from spread BALB/c and K-MSV BALB/c cells are shown in lanes A and C, respectively; receptors from spherical BALB/c and K-MSV BALB/c cells are shown in lanes B and D, respectively.

Lateral Mobility in 3T3 Fibroblasts

Lateral mobility of lipids and WGA receptors was measured in spread and spherical untransformed and K-MSV transformed 3T3 fibroblasts. Table I is a compilation of those results. Using NBD-phosphatidylcholine as a probe for phospholipid mobility, no major differences could be observed as a function of cell shape or transformation. The values observed for NBD-phosphatidylcholine in the untransformed (about 2 x 10^{-9} cm^2/s)and transformed (about 4 x 10^{-9} cm^2/s) 3T3 cells were similar to those reported for FDB cells (1 x 10^{-9} cm^2/s by Tank et al. (1982) and various transformed cells (about 4 x 10^{-9} cm^2/s) reported by Aroeti & Henis (1986). The factor of two observed between spread cells and spherical cells for untransformed and transformed cells may reflect the kind of cell surface irregularities such as small microvillar structures that were suggested by Aizenbud and Gershon (1982) to be able to affect diffusion measurements by no more than a factor of two. Scanning electron micrographs of the fibroblasts demonstrated the differences in the amount of microvilli (Figure 7). In contrast, WGA receptor mobility appeared to be markedly affected by the alteration of cell shape.

WGA binds to glycoproteins containing N-acetylglucosamine and sialic acid residues and so binds to an ensemble of proteins in the membrane. The diffusion coefficient measured by FRAP will therefore be an average

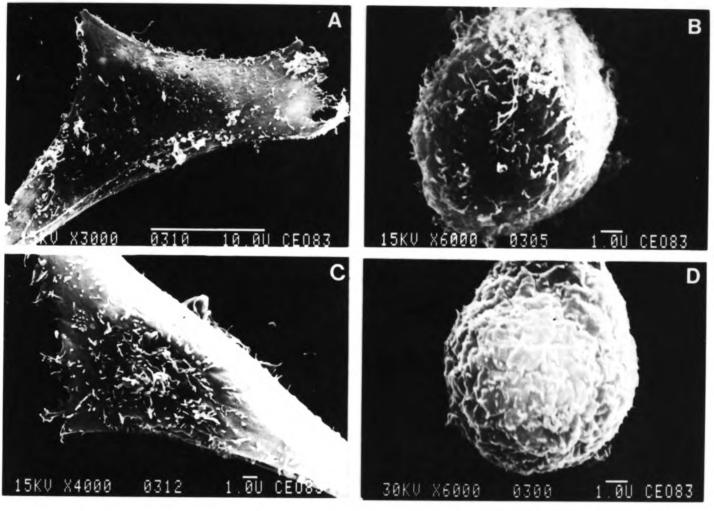

Figure 7. Scanning electron micrographs of spread and spherical 3T3 fibroblasts. Spread untransformed and transformed 3T3 fibroblasts are shown in A and C, respectively. Spherical untransformed 3T3 fibroblasts and K-MSV transformed 3T3 fibroblasts are shown in B and D, respectively.

of the diffusion coefficients of all the WGA receptors. The use of the term diffusion coefficient refers to an apparent diffusion coefficient measured in a biological system and therefore is not a constant in the classical physical sense. It is, however, a useful measure of the mobility of membrane components and I will continue to use the term diffusion coefficient for the measured lateral diffusion as has been commonly used in the literature for FRAP studies.

The measured WGA receptor lateral diffusion coefficient for spread 3T3 cells was slow at 0.33×10^{-10} cm^2/s (Table I). The restriction of WGA receptor lateral diffusion is obvious when the diffusion coefficient for WGA receptors in spread 3T3 cells is compared to the diffusion coefficient for lipids in the same cells. WGA receptor mobility was 50- to 100-fold slower than lipid mobility in spread 3T3 cells. Alteration of cell shape by suspension in DME/MC for one or two days was accompanied by a dramatic increase in WGA receptor lateral mobility. The lateral diffusion coefficient for WGA receptors was about 12-fold faster in spherical 3T3 cells than in spread 3T3 cells. Although the diffusion coefficient (3.9×10^{-10} cm^2/s) for spherical 3T3 cells was an order of magnitude faster than in spread cells, it was still about 10-fold slower than lipid diffusion coefficients. The restriction of WGA receptor diffusion appeared to have been partially eased in spherical cells, yet some shape-independent means of restricting protein lateral mobility remained.

TABLE 1. PHOSPHOLIPID AND PROTEIN LATERAL MOBILITY

		SPREAD CELLS			SPHERICAL CELLS		
CELL TYPE	PROBE	D [a]	%R [b]		D	%R	
3T3-B	NBDPC	19±11	54±9	(14)	38±23	46±23	(16) [c]
3T3-K	NBDPC	35±13	70±6	(14)	60±45	59±24	(14)
3T3-B	WGA	0.33±0.35	54±20	(26)	3.9±2.1	45±24	(32)
3T3-K	WGA	0.29±0.20	58±16	(18)	8.3±7.3	45±19	(30)

[a] D is the diffusion coefficient ($\times 10^{-10}$) in cm^2/s;
[b] %R is the percent recovery;
[c] Number of experiments

Transformation of the 3T3 cells was not associated with a change in WGA receptor diffusion in spread cells. In agreement with the results of Eldridge et al. (1980) for SV40 transformed cells, there was no difference in the diffusion coefficient for WGA receptors in 3T3 and K-MSV 3T3 cells (see Table I). Again, alteration of cell shape in K-MSV 3T3 cells from spread to spherical was accompanied by an increase in WGA receptor lateral diffusion. Spherical K-MSV 3T3 cells had an increased rate of protein lateral diffusion similar to that seen in untransformed 3T3 cells. An important observation related to this result was the significant standard deviation found for the measured diffusion coefficient of spherical transformed cells. This was suggestive of the existence of a population of transformed cells that may, in fact, have significantly different diffusion rates in the spherical state. Indeed, a clone was isolated from the K-MSV 3T3 cells with faster diffusion rates. This is further characterized in Chapter IV.

To insure that these results did not represent physical alterations induced by cell manipulation, the lateral mobility of WGA receptors was measured on 3T3 cells removed with EDTA and suspended in DME/MC for one hour. The lateral mobility of WGA receptors on EDTA removed cells was compared to that on cells removed with trypsin and suspended in DME/MC for one hour or one to two days. Although there was a slight increase in the measured

diffusion coefficient in EDTA and trypsin removed cells suspended for one hour in comparison to cells suspended for one or two days, all values were within standard deviation and within a factor of two (see Table II). The effect of methyl cellulose on diffusion measurements was tested by plating cells on dishes, allowing them to attach and spread, and covering them overnight with DME/MC before doing the FRAP measurements. Methyl cellulose was removed by washing before labeling as in the spherical cells. As seen in Table II, there was no difference from the values obtained for spread cells shown in Table I.

Cell Growth as a Function of Shape

Thymidine uptake studies were initiated to demonstrate that in 3T3 fibroblasts, shape, indeed, could alter DNA metabolism in untransformed but not transformed cells (Folkman & Moscona, 1978). These measurements were necessary to show that the FRAP experiments were performed under the reported shape dependent metabolic conditions. The pattern of thymidine uptake was similar in the spread cells. Release of cells blocked at the G_1/S interphase of the cell cycle resulted in a rapid uptake of thymidine 1 hour later (see Figure 8). The next peak of thymidine uptake (S phase) was at 21 hours for spread 3T3 cells and at 17 hours for spread K-MSV 3T3 cells. The doubling time was 20 hours for 3T3 cells and 16 hours for K-MSV 3T3 cells. Transformed fibroblasts are known to grow in

TABLE 2. EFFECT OF PREPARING 3T3 CELLS FOR SUSPENSION

TREATMENT	D [a]	%R [b]
EDTA REMOVED	6.3±2.3	48±24 (11) [c]
TRYPSIN REMOVED	8.2±2.9	46±17 (12)
1 or 2 DAYS IN MEDIA/MC	3.9±2.1	45±24 (32)
MEDIA/MC ON SPREAD CELLS	0.22±0.15	45±21 (8)

[a] D is the diffusion coefficient ($X10^{-10}$) in cm^2/s;
[b] %R is the percent recovery;
[c] Number of experiments

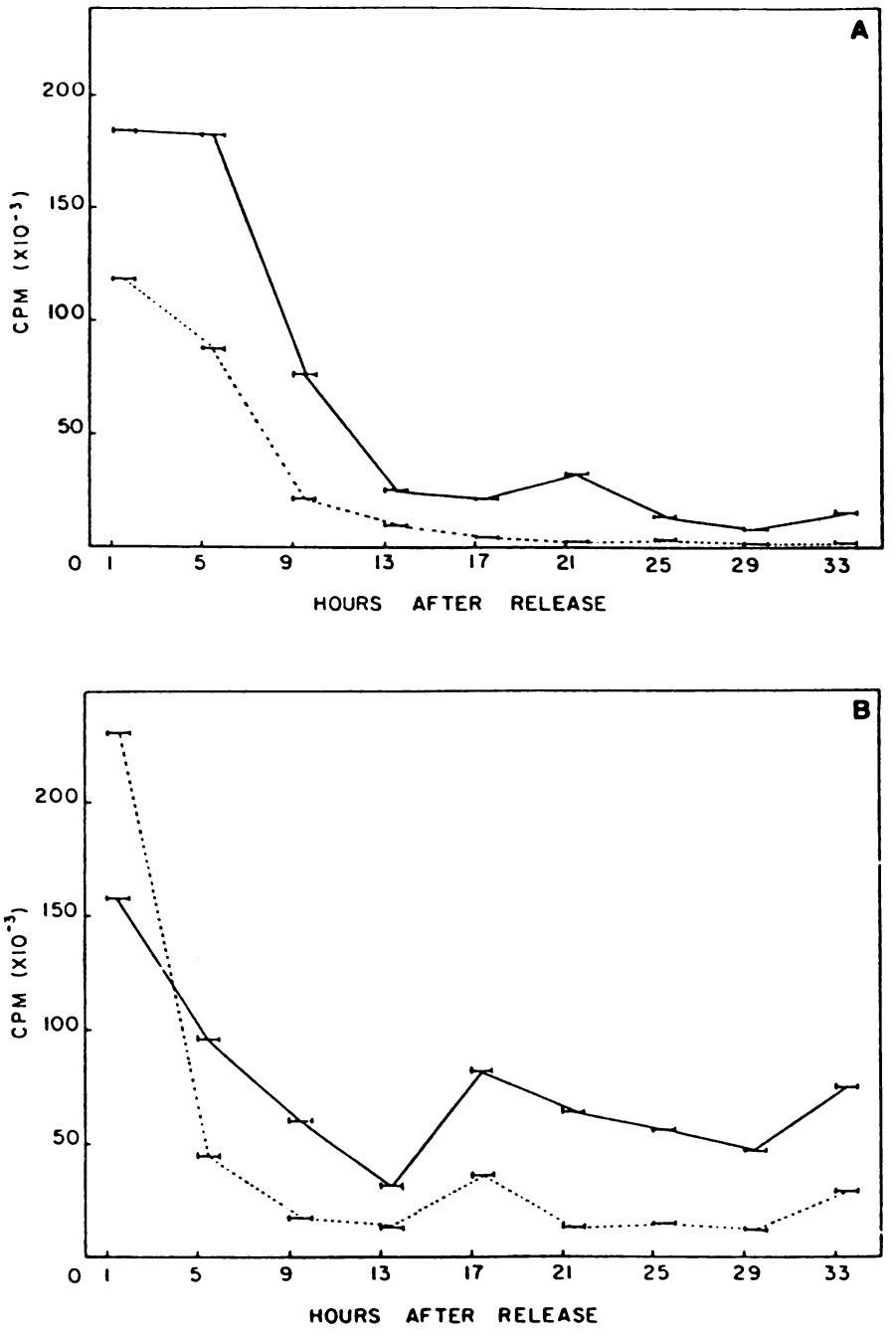

Figure 8. Uptake of ^{3}H-thymidine into 3T3 (A) and K-MSV transformed 3T3 (B) fibroblasts. Uptake was measured in spread (solid line) and spherical (dashed line) cells. Horizontal bars mark the uptake during the one hour periods in which cells were pulsed with labeled thymidine and are the average of duplicate samples.

suspension, whereas untransformed fibroblasts do not (MacPherson & Montagnier, 1964; Stoker et al., 1968; Freedman & Shin, 1974; Otsuka & Moskowitz, 1975). As expected, spherical K-MSV 3T3 cells continued through the cell cycle and showed a second peak of thymidine uptake at 17 hours, similar to that of the spread cells. The untransformed 3T3 cells, blocked at the G_1/S interphase and suspended in DME/MC, took up thymidine at the first time point 1 hour later but did not take up thymidine at 20 hours, apparently remaining in G_1 phase (Otsuka & Moskowitz, 1975). These results demonstrated that the transformed cells were proliferating in suspension at the time FRAP experiments were carried out; whereas the untransformed 3T3 cells in suspension were not.

The consequences of cell shape alteration on metabolic processes, such as RNA, DNA, and protein synthesis have been investigated as detailed above; however, little work has been done on the role of cell shape in carbohydrate metabolism. One study reported that suspension of chick embryo fibroblasts resulted in a decrease in hexose transport (Bissell et al., 1977). Hexose transport has also been reported to be enhanced by transformation (Eliam & Vinkler, 1976; Bissell et al., 1977); however, other studies suggested that the apparent enhancement in glucose transport by transformation is a reflection of the increased phosphorylation of glucose and that it is not a specific function of the viral genome, rather it reflects

the overall growth rate of the transformed cells which are not affected by cell density or suspension (Romano & Colby, 1973; Romano, 1976). Furthermore, the rate of glucose uptake is not a primary determinant of cell growth rate under the usual conditions of cell culture (Romano & Connell, 1982). Instead of monitoring the uptake of glucose, the utilization of glucose was monitored as a function of cell shape. The method used provides an estimate of the apparent K_M and V_{max} for the overall process of glucose metabolism from uptake to the isomerization of D-glyceraldehyde-3-phosphate to dihydroxyacetone phosphate in the glycolysis pathway. As shown in Table III, neither alteration of cell shape nor transformation had much effect on the metabolism of glucose in 3T3 fibroblasts under conditions where neither cell density nor serum would limit cell growth in the untransformed cells. Since glucose is not the only metabolic means of producing energy for cellular use, the level of ATP in untransformed and transformed 3T3 fibroblasts was determined. Using a Luciferin/Luciferase assay the amount of ATP was found to be similar in EDTA removed untransformed 3T3 (9.77$\pm$2.90 µg ATP/mg protein) and K-MSV transformed 3T3 (7.02$\pm$1.30 µg ATP/mg protein) cells. According to these assays, then, transformation does not affect glucose metabolism or the levels of ATP significantly in these cells.

TABLE 3. GLUCOSE UTILIZATION IN 3T3 FIBROBLASTS

Cell Type	K_m [a]	V_{max} [b]	
SPREAD 3T3	1.1±0.6	0.5±0.3 x 10^{-6}	(6) [c]
SPHERICAL 3T3	1.8±0.8	1.1±0.2 x 10^{-6}	(3)
SPREAD K-MSV 3T3	1.8±0.7	1.3±0.6 x 10^{-6}	(3)
SPHERICAL K-MSV 3T3	2.7±1.7	1.9±0.6 x 10^{-6}	(4)

[a] mM
[b] mmol/10^6 cells/min
[c] Number of experiments

Actin and Fibronectin Distribution as a Function of Cell Shape and Transformation

The state of the actin stress fiber system was monitored using rhodamine-labeled phalloidin, an actin specific probe. Alteration of cell shape in 3T3 cells was accompanied by a disappearance of stress fibers as reported previously (Badley et al., 1980) (see Figures 9A and 9B). Transformation was also associated with microfilament reorganization (see Figures 9D and 9E) as reported previously (McNutt et al., 1973; Pollack et al., 1975; Carley et al., 1981). Stress fibers were thick and numerous in the spread untransformed 3T3 cells. The spread transformed K-MSV 3T3 cells were of two types. About 30% had a few wispy stress fibers and rosette structures resembling those described by David-Pfeuty and Singer (1980). These rosette structures appear to contain F-actin aggregates near the ventral surface (Carley et al., 1981); whereas the remainder had no apparent stress fibers only the rosettes.

Fibronectin patterns on 3T3 fibroblasts were examined by immunofluorescence microscopy. Fibronectin is a major attachment protein in the extracellular matrix, important for cell adhesion and spreading. Transformation of fibroblasts has been demonstrated to affect fibronectin associated with the cell surface (Yamada et al., 1976; Ali et al., 1977; Hynes et al., 1978). As shown in Figure 9, there was less fibronectin associated with spread K-MSV 3T3

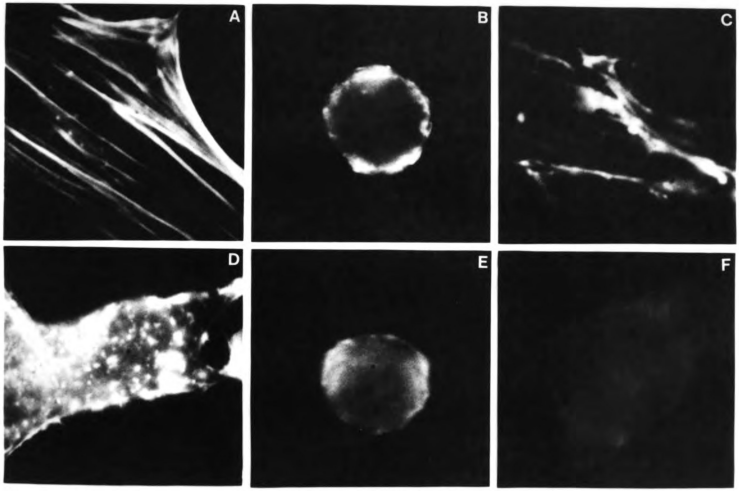

Figure 9. Fluorescence micrographs of spread and spherical 3T3 fibroblasts labeled with rhodamine-phalloidin, an F-actin specific probe, and spread 3T3 fibroblasts incubated with anti-fibronectin antibodies and then labeled with rhodamine goat anti-rabbit antibodies. Spread BALB/c and K-MSV BALB/c 3T3 cells labeled for F-actin are shown in A and D, respectively; spherical BALB/c and K-MSV BALB/c 3T3 cells labeled for F-actin are shown in B and E, respectively; spread BALB/c and K-MSV BALB/c 3T3 cells labeled for fibronectin are shown in C and F, respectively.

cells (9F) than untransformed 3T3 cells (9C). These results indicate that the transformed cells differ from the untransformed cells in at least one extracellular matrix component. Despite this difference, WGA receptor lateral diffusion was similar in spread 3T3 and K-MSV 3T3 cells, suggesting that at least fibronectin of the extracellular matrix does not control WGA receptor lateral mobility under the conditions of our experiment in agreement with the results of Schlessinger et al. (1977a).

DISCUSSION

Cell shape in anchorage dependent cells has been demonstrated to have a dominant influence on nuclear events such as DNA, rRNA, and mRNA production, while protein synthesis requires cell-surface contact. In both cellular activities, nucleoplasmic and cytoplasmic, the organization of the "cytoskeletal framework" has been implicated; an extended spread morphology is necessary for nuclear activity, while a point attachment appears to be sufficient to trigger translation of mRNA attached to detergent insoluble structures (Farmer et al., 1978; Cervera et al., 1981). Considering the recent attempts to examine the organization of the cytoskeleton and its influence on the lateral mobility of plasma membrane proteins, and the rheology of the cell, we have pursued a series of investigations to examine potential links between the metabolic consequences of cell shape change and the concomitant changes in the mobility of plasma membrane glycoprotein receptors. The cytoskeleton, in this regard, may serve to integrate and transmit signals acquired at the cell surface to intracellular sites where the initiation of biosynthetic processes may require specific types of cytoskeletal interactions or organization. In this context, the major observation to be reported is that the dynamic properties of a class of plasma membrane glycoproteins is

dramatically altered in a shape transition from flat and adherent to spherical and detached (a 12-fold increase in the ensemble diffusion coefficient). Under these conditions, phospholipid mobility in 3T3 fibroblasts was not significantly affected.

That these changes were a result of shape change and not significant differences in attachment to extracellular matrix may be inferred from diffusion results in adhering transformed fibroblasts which secrete considerably less fibronectin. This is noticeable in the decreased degree of spreading and in the ease of detaching the K-MSV 3T3 fibroblasts. A comparison of the lateral diffusion data for the WGA receptors in untransformed and transformed adherent, spread cells demonstrated no significant differences. This has also been observed by Eldridge et al. (1980) in a comparison of SV40 transformed and untransformed cells. The observed shape dependent changes in lateral mobility are reminiscent of the phenomenon of global modulation. As presented by Edelman (1976) and characterized by Schlessinger et al. (1977b), Henis & Elson (1981), Edelman et al. (1976), Michaels et al. (1984), and Pasternak & Elson, (1983); cells exposed to tetravalent Con A, even when only a fraction of the cell surface was bound by Con A-platelets, have a 6- to 8-fold decrease in the diffusion coefficient for a number of classes of cell surface receptors, do not form a cap, demonstrate recruitment of actin to the plasma membrane, and are

significantly more resistant to deformation. That these properties are related to the cytoskeletal state or reorganization is suggested by the reversal of these Con A induced changes by cytochalasins or colchicine. We believe our results provide evidence that the fibroblasts in the adherent, spread state are analogous to the Con A attached lymphocytes in that the aggregation of cell surface proteins by Con A or surface attachment initiates changes in the cytoskeleton that increase cortical cytoskeleton/membrane interactions detected by Pasternak & Elson (1983) as enhanced resistance to deformation. A cortical cytoskeleton, by adherence or Con A binding, would be under greater tension provided through recruitment and contraction of cytoskeletal elements at points of attachment. The increased interaction with the membrane and increased tension on the membrane could affect the diffusion of membrane proteins by either passively slowing down diffusion through a more resistant polymeric network or varying the on/off rates of proteins capable of associating with the cortical cytoskeleton as has been suggested previously (Koppel et al., 1981). Upon release from spreading and significant attachment, the microfilament, microtubule, and intermediate filament networks are affected and the diffusion of membrane receptors is increased as observed for cytochalasin/colchicine reversal of global modulation in lymphocytes. This is concomitant with a

cytochalasin/colchicine mediated loss of resistance to deformation reported to occur in lymphocytes (Pasternak & Elson, 1983). More support for these contentions is provided in Chapter V which examines global modulation in spread and spherical cells.

Medullary and Cortical Cytoskeleton

Although a major enhancement was observed in the diffusion coefficient of spherical cells, the rates observed were still approximately 10-fold less than reported for membrane proteins in which the membrane associated cytoskeletal network has been either removed or disorganized (Koppel et al., 1981; Tank et al., 1982). This suggests that in the spherical cells, a constraint to unhindered lateral diffusion still exists. In consideration of the global modulation results (Henis & Elson, 1981), the deformation studies which suggest biphasic characteristics of the force-compression curves for lymphocytes, and cytochalasin B sensitive resistance to compression (Petersen et al., 1982), it may be appropiate to suggest at least two controlling actin based networks for membrane surface receptor movement. A cytoskeleton anchored to the membrane in the manner found in mammalian erythrocytes (cortical), and a cytoskeleton that exists in the cytoplasm and may, in fact, be anchored or be organized from the nucleus (medullary). In this linked system, tension may be altered in the cortical cytoskeleton by changes in the

organization or structure of medullary cytoskeletal elements and vice versa. Tension itself, on the other hand, may be a local mediator or regulator of cytoskeletal reorganization as has been suggested for PC12 neurites (Joshi, et al., 1985). These changes are induced by contact mediated recruitment of actin, myosin, and other cytoskeletal molecules to membrane/surface contact sites or by reorganization of these structural elements at the plasma membrane initiated by surface receptor aggregation as reported to occur in lymphocytes after Con A treatment (Michaels et al., 1984). In this manner, a bi-directional communication system may be modulated by changes at its surface which are transmitted to the cytoplasm where medullary cytoskeletal reorganizations then cause rearrangements at the cell surface, modifying the physical properties of the membrane and perhaps relaying a signal to the nucleus.

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CHAPTER IV

IDENTIFICATION AND ISOLATION OF A FAST MOBILITY POPULATION FROM TRANSFORMED 3T3 FIBROBLASTS

INTRODUCTION

In an examination of the effect of cell shape on the lateral mobility of membrane proteins, it was observed that a bimodal distribution existed with respect to measured diffusion coefficients for Kirsten murine sarcoma virus transformed BALB/c 3T3 fibroblasts (K-MSV 3T3) maintained in the spherical state. An analysis of the data suggested the existence of a subpopulation of cells having an abnormally high rate of diffusion for wheat germ agglutinin (WGA) glycoprotein receptors. Using the technique of fluorescence redistribution after photobleaching (FRAP), an effort was initiated to identify and then isolate a clonal population of "fast" mobility cells. These cells would be employed to examine a number of cellular properties and ascertain the cortical and medullary cytoskeletal elements that are potentially involved in the control of protein lateral mobility. In addition, this mutant could be used to examine a number of mechanisms requiring cytoskeletal links between cell surface receptors and nuclear structures.

This communcation discusses the isolation of a clonal population of transformed 3T3 fibroblasts that demonstrate unhindered lateral mobility for membrane proteins ((2.1 $\pm$

1.6) x 10^{-9} cm^2/s) when cell shape is altered from flat and adherent to spherical and detached. The observed "fast" mobility is consistent with the fastest rates reported for proteins in cytoskeletal perturbed blebs from muscle cells ((4.0 ± 1.0) x 10^{-9} cm^2/s) (Tank et al., 1982), spectrin deficient spherocytes ((2.5 ± 0.5) x 10^{-9} cm^2/s) (Koppel et al., 1981), and rod outer segment localized rhodopsin ((2-4) x 19^{-9} cm^2/s) (Poo & Cone, 1974). The actin stress fiber patterns in the "fast" mobility population were similar to the parent K-MSV 3T3 cells in that the majority had no visible stress fibers and the rest had fewer and thinner stress fiber cables than the untransformed BALB/c 3T3 cells. About half of the K-MSV 3T3 cells contained rosette structures as described in the preceding paper; however, only a few percent of the clonal cells contained the rosette structures. The increased rate of protein lateral diffusion in spherical cells of the "fast" mobility population and the subtle differences in the actin stress fiber structures correlated with a difference in the control of the rate of nucleo-cytoplasmic transport in these cells (Jiang and Schindler, unpublished observations). Significantly, the "fast" mobility trait appeared to be stably inherited, at least for 23 passages.

MATERIALS AND METHODS

Cells: BALB/c 3T3 and Kirsten murine sarcoma virus transformed BALB/c 3T3 cells were obtained and cared for as noted in the preceding chapter. A "fast" mobility population was derived through a series of subclonings on 96 well tissue culture plates and from colonies grown in soft agar from single cells. Clones having fast protein lateral diffusion were further subcloned. After a total of 3 subclonings on 96 well plates and 2 selections from soft agar, the "fast" mobility population (designated E7G1) was obtained and maintained through common tissue culture methods.

Lateral Mobility Measurements: Lateral mobility of lipids and glycoproteins was determined in the plasma membrane using the technique of Fluorescence Redistribution After Photobleaching (FRAP) as described in the preceding chapter. NBD-phosphatidylcholine was used to monitor lipid mobility. Rhodamine-labeled wheat germ agglutinin (WGA) was used to monitor protein lateral mobility. Cells were labeled for 30 minutes at 4°C with 100 µg/ml rhodamine-WGA in HBSS/HEPES.

Fluorescence Microscopy: Stress fibers in cells were labeled with rhodamine-phalloidin and photographed as described in the preceding chapter.

RESULTS

In the preceding chapter, we characterized the number and type of WGA receptors in BALB/c 3T3 and K-MSV BALB/c 3T3 cells (parent population for the E7G1 clone cells) since rhodamine-WGA was used to monitor protein lateral diffusion. Similar experiments, done with E7G1 clone cells, found no significant differences between the various cell populations. A comparison of WGA binding by bulk and single cell methods of analysis found no significant differences between E7G1 clone cells (Figures 1 & 2) and BALB/c 3T3 and parent K-MSV BALB/c 3T3 cells (Chapter III, Figures 1-3) in the spread and spherical state. As found for the parent population (Chapter III, Figure 4), E7G1 clone cells (Figure 3) had WGA homogeneously bound to the cell surface. Glycoprotein and glycolipid receptors from E7G1 clone cells (Figures 4 & 5) also were similar to those in the parent population (Chapter III, Figures 5 & 6) regardless of cell shape. It was concluded that the E7G1 clone cells do not have an anomalous WGA receptor population which account for the differences in protein lateral diffusion.

The lateral mobility of phospholipids was monitored with NBD-phosphatidylcholine. Again, little difference in lipid mobility was found when cell shape was altered from spread and adherent to spherical and detached in E7G1 clone cells despite the large increase in protein lateral

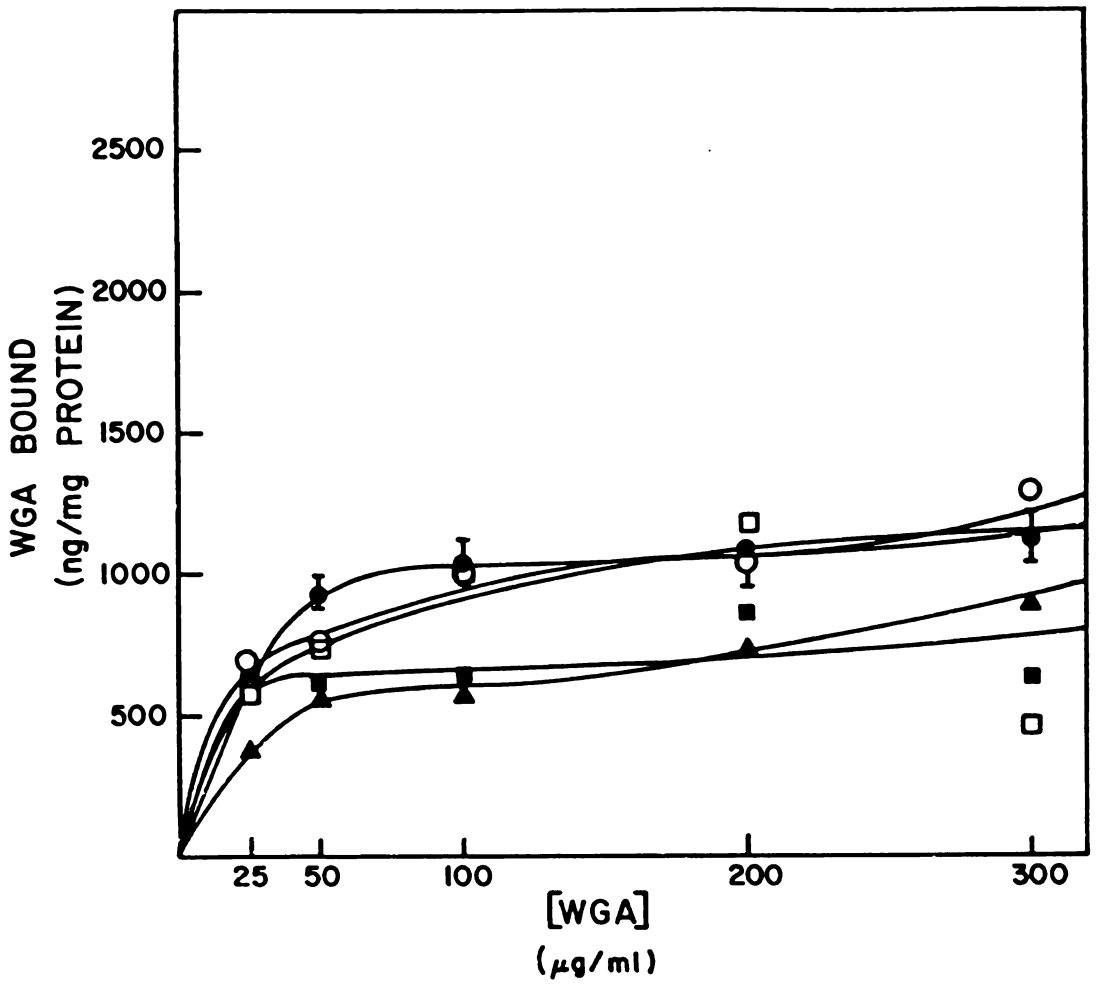

Figure 1. The binding of FITC-WGA to spread E7G1 clone cells (●) and spherical E7G1 clone cells trypsinized and suspended in methyl cellulose for one hour (▲), one day (O), and two days (□). WGA binding to spherical cells that had been removed with EDTA and suspended in methyl cellulose for one hour (■) was also determined. The error bars denote the standard deviation for the spread cells which was similar in the spherical cells.

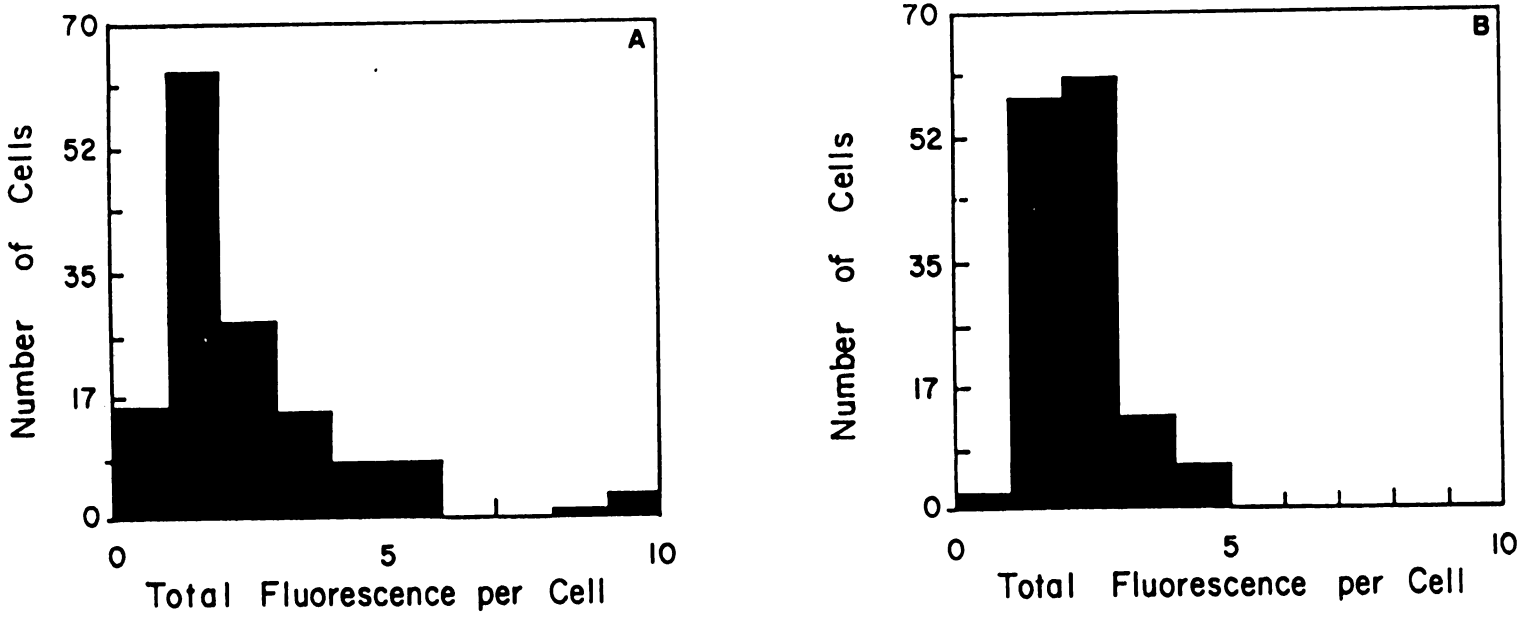

Figure 2. Population analysis of the amount of fluorescence per cell shown in the form of frequency histograms. The distribution of binding for spread (A) and spherical (B) E7G1 clone cells is shown.

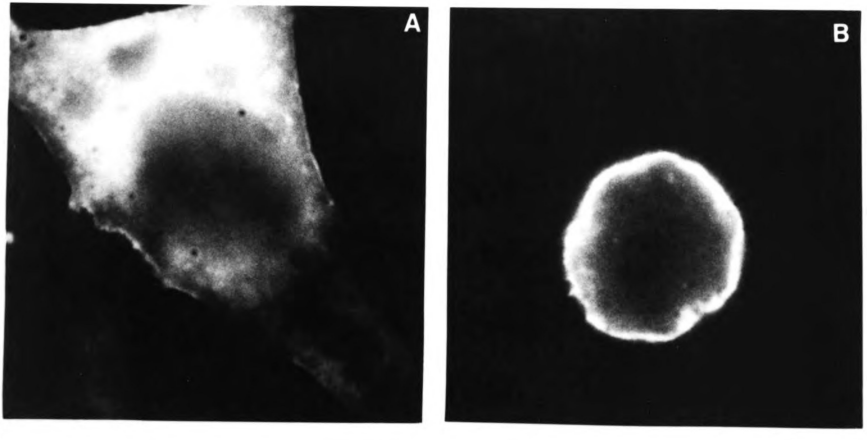

Figure 3. Fluorescence micrographs of spread (A) and spherical (B) E7G1 clone cells.

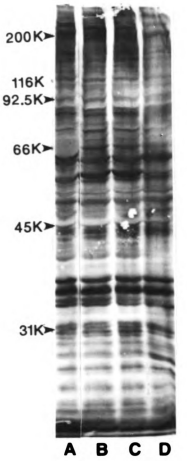

Figure 4. Western transfer blot of WGA glycoprotein receptors from spread (lane A) and spherical (lane B, 1 hour suspension in methyl cellulose; lane C, 1 day suspension; lane D, 2 days suspension) E7G1 clone cells.

Figure 5. Autoradiogram of WGA glycolipid receptors obtained from spread (lane A) and spherical (lane B) E7G1 clone cells, separated by TLC, and probed with radio-iodinated WGA.

diffusion (see Table I). Lipid mobility in the E7G1 clone cells was simlar to that observed in untransformed and parent transformed BALB/c 3T3 fibroblasts. As reported for the untransformed cells and parent transformed cells, the alteration of cell shape was accompanied by an increase in the lateral diffusion of wheat germ agglutinin receptors (see Table I). In the untransformed 3T3 cells however, the increase was by an order of magnitude and lateral diffusion in the spherical cells remained slower than lipid diffusion. In contrast, the "fast" population of cells (E7G1) demonstrated an increase in WGA receptor lateral diffusion by about two orders of magnitude. The increase was from about 1- or 2 x 10^{-11} cm^2/s, similar to spread BALB/c 3T3 cells, to about 2 x 10^{-9} cm^2/s, similar to that of lipids. Apparently, the E7G1 clone cells retain restriction of protein lateral diffusion in the spread state, which is completely lost after alteration of cell shape. In the past, such changes in membrane dynamic properties have been linked to alterations in the interaction of membrane proteins with the cytoskeleton. These results most closely parallel the results of Koppel et al. (1981), who demonstrated that a deficiency of spectrin in association with the membrane in mouse spherocytes resulted in a 50-fold increase in protein lateral diffusion. Unlike the terminally differentiated spherocyte system, the E7G1 clone cells can transmit the membrane changes to daughter cells resulting in a "fast"

TABLE 1. PHOSPHOLIPID AND PROTEIN LATERAL MOBILITY

		SPREAD CELLS		SPHERICAL CELLS	
CELL TYPE	PROBE	D [a]	%R [b]	D	%R
3T3-B	WGA	0.33±0.35	54±20 (26)	3.9±2.1	45±24 (32)[c]
3T3-K	WGA	0.29±0.20	58±16 (18)	8.3±7.3	45±19 (30)
E7G1	WGA	0.19±0.19	77±16 (11)	21±16	45±23 (53)
E7G1	NBDPC	28±19	60±9 (14)	53±19	39±15 (14)

[a] D is the diffusion coefficient ($X10^{-10}$) in cm^2/s;
[b] %R is the percent recovery;
[c] Number of experiments

mobility phenotype.

The status of the cytoskeletal element, actin, which has been demonstrated to be linked to membrane proteins (for a review see Geiger, 1983) was also investigated. The stress fiber actin (stained with the F actin specific probe, rhodamine phalloidin) of the clonal population in the spread state resembled that of the parent transformed BALB/c 3T3 cells in some ways (see Figure 6). As in the parent population there were two types observed. One type (about 17%) had some visible stress fibers, but again fewer and thinner than in the untransformed cells; the other type (about 83%) had no visible stress fibers. As noted in the preceding chapter, the parent K-MSV 3T3 cells displayed rosette structures, especially in cells lacking visible stress fibers. In contrast, only 5% of the E7G1 clone cells contained visible rosette structures. E7G1 clone cells in the spherical state had no visible stress fibers as seen for the untransformed and parent populations. Fibronectin in the E7G1 clone cells (Figure 6C) resembled that of the parent population (Chapter III, Figure 9F) i.e. has little fibronectin on the cell surface.

The consequence of such dramatic changes in cells' dynamic and structural properties on cell growth was also examined. To determine cell growth rates, the synthesis of DNA was monitored for a period of 36 hours. The pattern of thymidine uptake in spread and spherical E7G1 clone cells (Figure 7) was similar to that observed in the parent

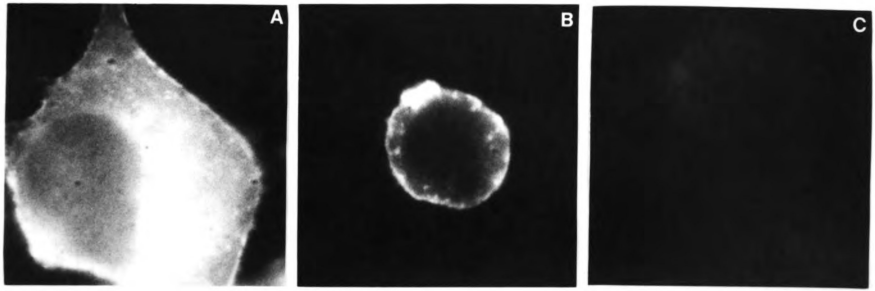

Figure 6. Fluorescence micrographs of spread (A) and spherical (B) E7G1 clone cells labeled with rhodamine-phalloidin, an F-actin specific probe. Spread E7G1 clone cells labeled for fibronectin are shown in (C).

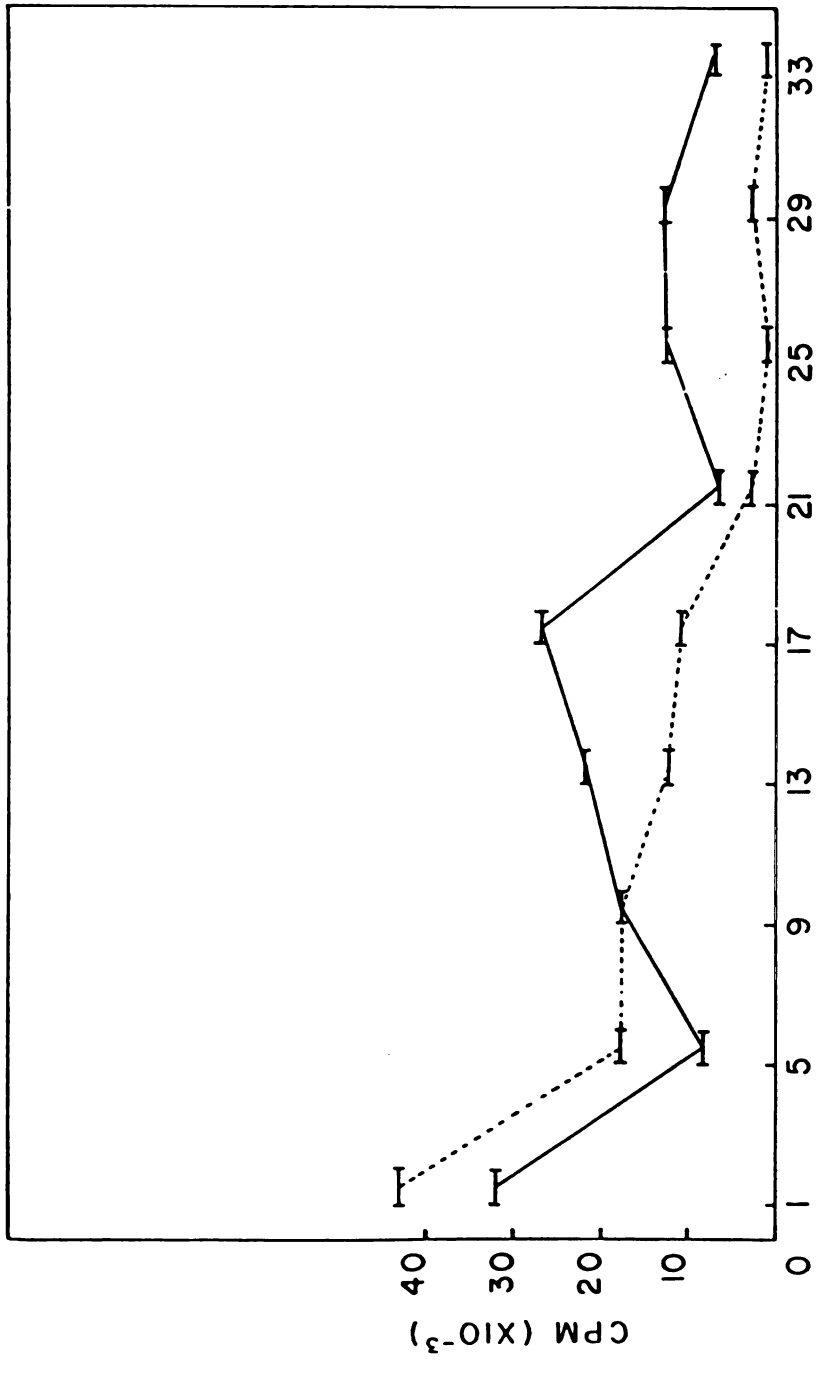

Figure 7. Uptake of ^{3}H-thymidine into spread (solid line) and spherical (dashed line) E7G1 clone cells. Horizontal bars mark the uptake during the one hour periods in which cells were pulsed with the labeled thymidine and are the average of duplicate samples.

population (Chapter III, Figure 8). DNA synthesis occured about every 16 hours. While the E7G1 population was not synchronized as well as the parent population, DNA synthesis continued in the E7G1 clone cells after cell shape alteration. The "fast" mobility population, therefore, appears to be transformed as was the parent population.

DISCUSSION

In the preceding chapter a model was proposed for a communication pathway between the plasma membrane and the nucleus via the cytoskeleton. Evidence was presented to suggest that this pathway could be cell shape sensitive and its status could be monitored through measurement of protein lateral diffusion in the plasma membrane. Based on the observation that release of the restriction on protein lateral diffusion in untransformed BALB/c 3T3 cells was only partial and on Pasternak and Elson's (1983) finding of a biphasic compression curve for lymphocytes, it was suggested that the pathway consisted of two interconnected cytoskeletal systems: a cortical and a medullary cytoskeleton. In this chapter we report the isolation of a subpopulation of cells from Kirsten murine sarcoma virus transformed BALB/c 3T3 fibroblasts in which WGA receptors are completely released from lateral diffusion restrictions when cell shape is altered from flat and adherent to spherical and detached.

A simple way to integrate these results would be to suggest that in untransformed BALB/c 3T3 cells the cortical cytoskeleton is reorganized during attachment and spreading as a result of recruitment of cytoskeletal elements to the membrane via cytoskeletal organizing proteins activated by attachment and spreading. This process may be analogous to

the binding of concanavalin A to lymphocytes and 3T3 fibroblasts which has been demonstrated to restrict protein lateral diffusion (Schlessinger et al., 1977; Henis & Elson, 1981), recruit actin to the plasma membrane (Michaels et al., 1984), and increase the resistance of cells to deformation (Pasternak & Elson, 1983). Several membrane proteins have been shown to bind actin and might serve to seed filament formation. Other membrane proteins or, perhaps, the reorganization of the cortical cytoskeleton might initiate the formation of a medullary cytoskeleton completing the link between the plasma membrane and the nucleus. During detachment and the process of rounding, a reorganization or partial dissolution of the cortical cytoskeleton could account for the partial easing of the restriction of protein lateral diffusion. In the "fast" mobility population from the transformed cells, membrane proteins in spherical cells appear to be uncoupled from cytoskeletal interactions allowing unrestricted lateral diffusion to occur. Attachment and spreading seems to be accompanied by reassociation of membrane proteins and cytoskeletal elements as detected by the observation of restricted protein lateral diffusion in spread E7G1 clone cells. This suggests that the cortical cytoskeleton is functional in spread E7G1 clone cells if not in the spherical cells. We would suggest, however, that the medullary cytoskeleton is not functional in E7G1 clone cells, or at least not connected with the cortical

cytoskeleton, based on evidence presented in Chapter V which examines global modulation in spread and spherical cells.

A key question yet to be addressed concerns the finding of a change in nuclear function or structure correlating with the change in protein lateral diffusion and cytoskeletal organization that occurs concomitant with cell shape alteration. The model proposes a communication pathway from the plasma membrane to the nucleus via the cytoskeleton. Schindler and Jiang (1986) reported that the cytoskeletal elments, actin and myosin, play a role controlling the rate of nucleo-cytoplasmic transport. The rate of nucleo-cytoplasmic transport decreased in 3T3 and K-MSV 3T3 cells after alteration of cell shape (Jiang and Schindler, manuscript in preparation), but not in the clonal population demonstrating the "fast" protein lateral diffusion. These results suggest a loss of control of a nuclear activity correlating with a loss of control of protein lateral diffusion in the plasma membrane. The biological significance of these studies becomes further apparent in consideration of the finding that while insulin or EGF can enhance nucleo-cytoplasmic transport in untransformed spread, adherent BALB/c 3T3 cells, there is no hormone effect upon transport in spherical BALB/c 3T3 cells (Jiang and Schindler, unpublished observations).

In summary, we have proposed a model for communication between the cell surface and the nucleus via the cytoskeleton similar to those suggested by Ingber and Jamieson (1985), Ben Ze'ev (1985), and Packard (1986). In this model it is suggested that adhesion or receptor mediated stimulation could affect the interaction of membrane proteins with cortical cytoskeletal elements and relay positional or stimulatory information to the nucleus via a medullary cytoskeletal system. Consistent with a functionally divided cytoskeleton, we observed a partial increase in protein lateral mobility after perturbing the cytoskeleton by alteration of cell shape in BALB/c 3T3 cells; furthermore, we found a complete release of diffusional constraints on membrane proteins in the "fast" mobility clone. The coincidence of the changes in protein lateral mobility, in cytoskeletal organization, in the control of nucleo-cytoplasmic transport, and the relation to cell proliferation suggests that altering cell shape in anchorage dependent fibroblasts provides a model system in which to examine the proposed communication pathway, especially when used in conjunction with the "fast" mobility population.

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CHAPTER V

GLOBAL MODULATION AND CELL SHAPE: EVIDENCE FOR CYTOSKELETAL INVOLVEMENT IN THE CONTROL OF PROTEIN LATERAL MOBILITY

INTRODUCTION

The model for a signal transmission pathway discussed in the previous chapters suggests that the cytoskeleton transmits messages between membrane receptors and the nucleus. The cytoskeleton is a likely candidate for such a pathway, since it spans the region between the plasma and nuclear membranes and is capable of conformational changes as well as translocation of vesicles and organelles along its length (Spudich et al., 1985; Vale et al., 1985). In the Chapters III and IV, it was suggested that lateral mobility could be used as an indicator for alterations in the cytoskeleton when cell shape was altered in anchorage-dependent fibroblasts. In this chapter further evidence is presented for a link between the change in protein lateral mobility that occurs concomitant with a change in cell shape and a change in the association of membrane proteins and the cytoskeleton.

Initial evidence suggesting a link between the restriction of protein lateral mobility and the cytoskeleton first came from studies on the effect of concanavalin A (Con A) on lymphocytes (Edelman et al., 1973). Binding of Con A restricted protein lateral mobility

in an effect called global modulation (Edelman, 1976), because binding in even a small localized region of the membrane resulted in propagation of the effect over the entire surface. Both soluble factors and cytoskeletal elements were proposed initally to be the propagators of the effect. It was found that colchicine or cytochalasin B could partially reverse the effect. When used in combination, cytochalasin B and colchicine completely reversed the effect (Henis & Elson,1981) suggesting that the cytoskeleton played a major role in regulation of protein lateral diffusion. It is clear that cytoskeletal integrity is necessary for global modulation.

The consequence of alteration of cell shape, known to alter cytoskeletal organization, for Con A induced global modulation was examined. Global modulation was found to occur in flat, adherent BALB/c 3T3 fibroblasts, but not in spherical, detached fibroblasts. Furthermore, global modulation did not occur in flat, adherent or spherical, detached cells from the "fast" clone (E7G1) discussed earlier. The preceding chapters examined the changes in wheat germ agglutinin receptor lateral diffusion upon cell shape alteration. In this chapter, the phenomenon is shown to also occur with succinyl Con A receptors indicating the general nature of the phenomenon.

MATERIALS AND METHODS

Cells: BALB/c 3T3 fibroblasts were obtained and maintained as stated previously. E7G1 clone cells are a "fast" mobility population of fibroblasts derived from Kirsten murine sarcoma virus transformed BALB/c 3T3 cells as detailed in Chapter IV. Spherical cells were suspended in media containing methyl cellulose as discussed in Chapter III.

Con A-platelets: Platelets were obtained from the American Red Cross, washed, fixed, and bound with Con A as described elsewhere (Henis and Elson, 1981).

Measurement of lateral mobility: The technique of fluorescence redistribution after photobleaching was used to monitor the lateral mobility of wheat germ agglutinin (WGA) receptors on the plasma membrane as described in Chapter III. To assess the effect of Con A on lateral mobility, cells were pretreated with Con A (100 µg/ml) or Con A-platelets for 15 minutes at 37°C and then incubated with rhodamine WGA (100 µg/ml) for 15 minutes at 4°C. Cells were washed three times before use. Control samples were incubated with succinyl concanavalin A (S-Con A) instead of Con A. In addition, the measurement of protein lateral mobility was extended to include S-Con A receptors. Cells were incubated with 100 µg rhodamine labeled S-Con A/ml HBSS-HEPES for 15 minutes at 37°C, washed, and measured as

for cells labeled with WGA.

Binding of S-Con A: The amount of S-Con A was measured on cells incubated for 15 minutes at 37°C with 100 µg/ml FITC labeled S-Con A and washed. The amount of fluorescence was determined using an excitation wavelength of 460 nm and an emission wavelength of 520 nm (5 nm slit widths) and compared to a standard curve.

RESULTS

The monitoring of protein lateral mobility with a lectin, such as wheat germ agglutinin, provides an average diffusion coefficient for the variety of membrane proteins it binds. We extended the measurements of protein lateral mobility to glycoproteins that bind succinyl Con A. S-Con A has a mannose specificity as does Con A but does not induce global modulation. As seen in Table I, the results for the lateral diffusion of S-Con A receptors paralleled the results for WGA receptors. A slower, restricted S-Con A receptor mobility was seen in flat, adherent BALB/c 3T3 and E7G1 ("fast" clone) cells. When the cell shape was altered from flat and adherent to spherical and detached; the S-Con A receptor mobility increased an order of magnitude for the BALB/c 3T3 and two orders of magnitude for the E7G1 clone cells. As for the WGA receptors, there was a similar amount of S-Con A bound to flat, adherent cells and spherical, detached cells suspended in methyl cellulose for one day. After a 15 minute incubation, BALB/c 3T3 cells bound about 4 μg S-Con A/10^6 cells and E7G1 clone cells bound about 3 μg/10^6 cells, regardless of cell shape. These results suggest that the increase in WGA and S-Con A receptor lateral mobility after cell shape alteration is a general surface phenomenon involving a broad range of membrane proteins.

TABLE 1. PROTEIN LATERAL MOBILITY

CELL TYPE	PROBE	PRETREATMENT	SPREAD CELLS D [a]	SPREAD CELLS %R [b]	SPHERICAL CELLS D	SPHERICAL CELLS %R
3T3-B	WGA	NONE	0.033±0.035	54±20 (26)	0.39±0.21	45±24 (32) [c]
3T3-B	S-CONA	NONE	0.029±0.033	62±22 (6)	0.51±0.35	64±23 (11)
E7G1	WGA	NONE	0.019±0.019	77±16 (11)	2.10±1.60	45±23 (53)
E7G1	S-CONA	NONE	0.027±0.022	45±23 (12)	2.70±2.30	44±25 (7)
3T3-B	WGA	S-CONA	0.020±0.012	65±18 (20)	0.34±0.18	45±18 (14)
E7G1	WGA	S-CONA	0.040±0.034	62±13 (10)	2.20±1.80	53±21 (14)
3T3-B	WGA	CONA	0.0065±0.0043	63±18 (15)	0.74±0.50	61±24 (16)
3T3-B	WGA	CONA-PL	0.0062±0.0033	29±14 (6)	0.51±0.21	37±22 (7)
E7G1	WGA	CONA	0.026±0.029	55±24 (16)	2.20±1.20	47±22 (14)

[a] D is the diffusion coefficient ($X10^{-9}$) in cm^2/s;
[b] %R is the percent recovery;
[c] Number of experiments;
CONA-PL = Con A-Platelets

The requirement of an intact cytoskeleton for global modulation suggests that global modulation may be used to demonstrate the role of the cytoskeleton in restriction of protein lateral mobility. Global modulation occured in spread BALB/c 3T3 fibroblasts where pretreatment with Con A resulted in a 5-fold decrease in the rate of WGA receptor lateral diffusion (see Table I) similar to that reported elsewhere (Schlessinger, et al., 1977; Henis & Elson, 1981). No significant effect was observed when cells were pretreated with S-Con A as a control. Con A bound to platelets has been used previously to demonstrate that local binding of Con A is sufficient to propagate the modulatory effect over the entire membrane. Con A platelets bound to a localized region of the cell membrane was sufficient to induce global modulation in spread BALB/c 3T3 cells as has been reported by others (Schlessinger et al., 1977; Henis & Elson, 1981). This demonstrated the effect can be propagated and was not simply the result of crosslinking the entire surface of the membrane. In contrast, Con A or Con A-platelets did not induce global modulation in spherical BALB/c 3T3 cells. These results paralleled the reported effects of cytochalasin B plus colchicine on global modulation (Henis & Elson, 1981) and coincided with the known change in cytoskeletal organization after cell shape alteration (Badley et al., 1980). Especially intriguing was the finding that no Con A induced global modulation occurred in the "fast" mobility

clone, E7G1, regardless of cell shape. The E7G1 clone cells, derived from a transformed cell line, have a pattern of stress fibers differing from that of the untransformed BALB/c 3T3 cells. The E7G1 clone cells had few, if any, visible stress fibers as noted in the preceding chapter characterizing the clone. While no correlation could be detected between stress fiber organization and restriction of protein lateral mobility in spread cells, a coincidence of stress fiber disruption and absence of global modulation was noted.

DISCUSSION

A signal transmission pathway from the plasma membrane to the nucleus via the cytoskeleton requires demonstration of the importance of cytoskeletal integrity to the sending of the message. The studies of Folkman and Moscona (1978), Benecke et al. (1978), and Cervera et al., (1981) suggest the importance of cytoskeletal integrity to cell growth in untransformed cells. These studies demonstrated the effect of cell shape alteration on DNA and RNA synthesis and showed in HeLa cells that mRNA being actively translated were attached to the cytoskeleton. Schlessinger (1980) suggested the importance of lateral mobility to epidermal growth factor and insulin stimulation. Furthermore, Landreth et al. (1985) reported a shape sensitive association of epidermal growth factor receptor with the cytoskeleton. These observations indicate the importance of cytoskeletal associations with membrane receptors in cellular function and suggest the significance of monitoring changes in lateral mobility as a means of detecting changes in the interaction of the receptors with the cytoskeleton. The preceding chapters contained evidence for an increase in protein lateral diffusion coinciding with a shape induced alteration of the cytoskeleton. The results presented in this chapter provide evidence that the increase in lateral mobility after cell shape alteration is

a general phenomenon and that alteration of cell shape coincides with the inhibition of a cytoskeletal dependent process (global modulation) that controls protein lateral mobility.

The theory of global modulation is based on evidence that Con A reorganizes cytoskeletal elements thereby further restricting plasma membrane protein lateral diffusion. The disruption of actin and microtubules prevents the occurence of global modulation. Alteration of cell shape is another means of disrupting the cytoskeleton; therefore, inhibition of global modulation by alteration of cell shape would provide evidence that the increase in protein lateral mobility after cell shape alteration (reported in the preceding chapters) is related to the disruption of the cytoskeleton. The lateral diffusion of WGA receptors on flat, adherent BALB/c 3T3 fibroblasts was restricted by pretreatment with Con A as has been reported for other proteins on lymphocytes (Henis & Elson, 1981) and fibroblasts (Schlessinger et al., 1977). WGA receptor lateral diffusion was not affected by pretreatment with succinyl Con A, which has the same binding specificity as Con A, in agreement with the literature (Schlessinger et al., 1977). This suggests that the effect on protein lateral diffusion is specific to Con A and is not the result of an effect of Con A binding on WGA binding.

The Con A induced global modulation observed in lymphocytes and fibroblasts may, in fact, use part of the

signal transmission pathway. There are a few intriguing similarities between Con A induced global modulation and hormone action of EGF which suggests a relavence for the use of global modulation in the study of the putative cytoskeletal mediated signal transduction pathway. Con A shows mitogenic activity (Edelman et al., 1973), caused aggregation of surface receptors, reorganizes the cortical cytoskeleton (Edelman, 1976), increases the association between the cytoskeleton and surface receptors (Painter & Ginsberg, 1982), and is inhibited by alteration of cell shape in anchorage dependent fibroblasts. Similarly, EGF action requires microaggregation of EGF receptors (Schlessinger, 1980), affects cytoskeletal organization (Schlessinger & Geiger, 1981), and EGF receptors are associated with the cytoskeleton in a shape sensitive fashion (Landreth et al., 1985). This is plausible in consideration of the following observations. One, global modulation requires a functional cytoskeleton (Edelman, 1976); two, Con A binding is followed by recruitment of actin to the membrane (Michaels et al,1984); and three, low doses of Con A, which do not restrict lateral mobility, are mitogenic (Edelman et al., 1973). The monitoring, then, of the state of the proposed signal transmission pathway by testing for global modulation may be especially appropriate. In untransformed flat adherent BALB/c 3T3 cells the restricted protein lateral diffusion, highly structured cytoskeleton, global modulation, and cell growth

all coincided. In contrast, alteration of cell shape was accompanied by an increase in protein lateral diffusion by a factor of 12, disruption of cytoskeletal elements, inhibition of global modulation, and inhibition of cell growth.

Transformation, however, results in different reponses to cell shape alteration. The transformed, flat, adherent E7G1 clone, a "fast" mobility population, also demonstrated restricted protein lateral mobility but had a disorganized pattern of stress fibers indicating that stress fibers do not play a significant role in the restriction of protein lateral mobility in spread cells. The E7G1 clone cells, however, demonstrated a loss of global modulation correlating with the lack of stress fibers. In conjunction with the observations of Pasternak & Elson (1983) demonstrating a biphasic curve for the resistance of cell to deformation, these observations suggest that the cytoskeleton may be divided into at least two functional domains. One domain would be under the plasma membrane in association with membrane proteins and would function more or less independently of a medullary cytoskeleton. Another domain would be in the medullary region of the cell would be necessary for global modulation to occur. The alteration of cell shape in the E7G1 clone cells also coincided with an increase in protein lateral diffusion; however, the increase was 5-10 fold greater than in the untransformed cells and similar to that of lipid lateral diffusion. This

suggests that the E7G1 clone cells have a functional cortical cytoskeleton when attached and spread, but which becomes nonfunctional in regard to restriction of protein lateral diffusion when cell shape is altered. Finally, the alteration of cell shape was not accompanied by an inhibition of cell growth in spite of the increased protein lateral mobility and the disruption of the cytoskeleton.

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CHAPTER VI: SUMMARY

Protein lateral mobility decreased in the plasma membrane of BALB/c 3T3 fibroblasts following cell attachment and spreading. In contrast to the spread cells which demonstrated a further decrease in protein lateral mobility after Con A treatment, no global modulation was observed in spherical cells. The apparent shape dependency of plasma membrane protein lateral mobility was also observed in Kirsten murine sarcoma virus transformed BALB/c 3T3 fibroblasts which demonstrated two subpopulations of protein lateral mobility in the spherical state. In one subpopulation lateral mobility was approximately equal to that of the untransformed cells, while the other had a fast, apparently unhindered protein lateral mobility (approximately equal to lipid mobility; this is termed the "fast" mobility clone). In contrast to the untransformed cells, Con A mediated global modulation was absent in E7G1 spread cells as well as spherical cells. Other studies have indeed suggested that the absence of global modulation is characteristic of transformed cells. Investigation of the different cell states (shape and transformation), using an F-actin specific probe to detect actin based cytoskeletal

rearrangements, indicated a strong correlation between loss of global modulation and disruption of stress fibers. An interpretation of these results was presented that views plasma membrane protein receptor lateral diffusion under the control of at least two interacting cytoskeletal organizations, a cortical (submembranous) and a medullary (cytoplasmic) cytoskeleton (see Figure 1). In this model spherical cells have a disassembled medullary cytoskeleton and a loose cortical network which interacts with membrane receptors partially restricting their diffusion. Diffusionial control is mediated by either direct or indirect interactions with the cortical cytoskeleton. During cell attachment membrane proteins involved in adhesion are crosslinked, tension is generated, and the cortical cytoskeleton is reorganized into a tighter network. In the process, the rate of protein lateral diffusion decreases. Global modulation results as Con A crosslinks its receptors and triggers increased medullary/cortical interaction. The enhanced cortical density and tension is detected as a further decrease in protein lateral mobility and an increase in membrane stiffness. We believe the crosslinking of membrane receptors and increase in tension are the driving forces in the assembly of actin based cortical structures that result in enhanced interactions with membrane receptors. The "fast" mobility clone would be an example of cells with a loose cortical network that has an insignificant

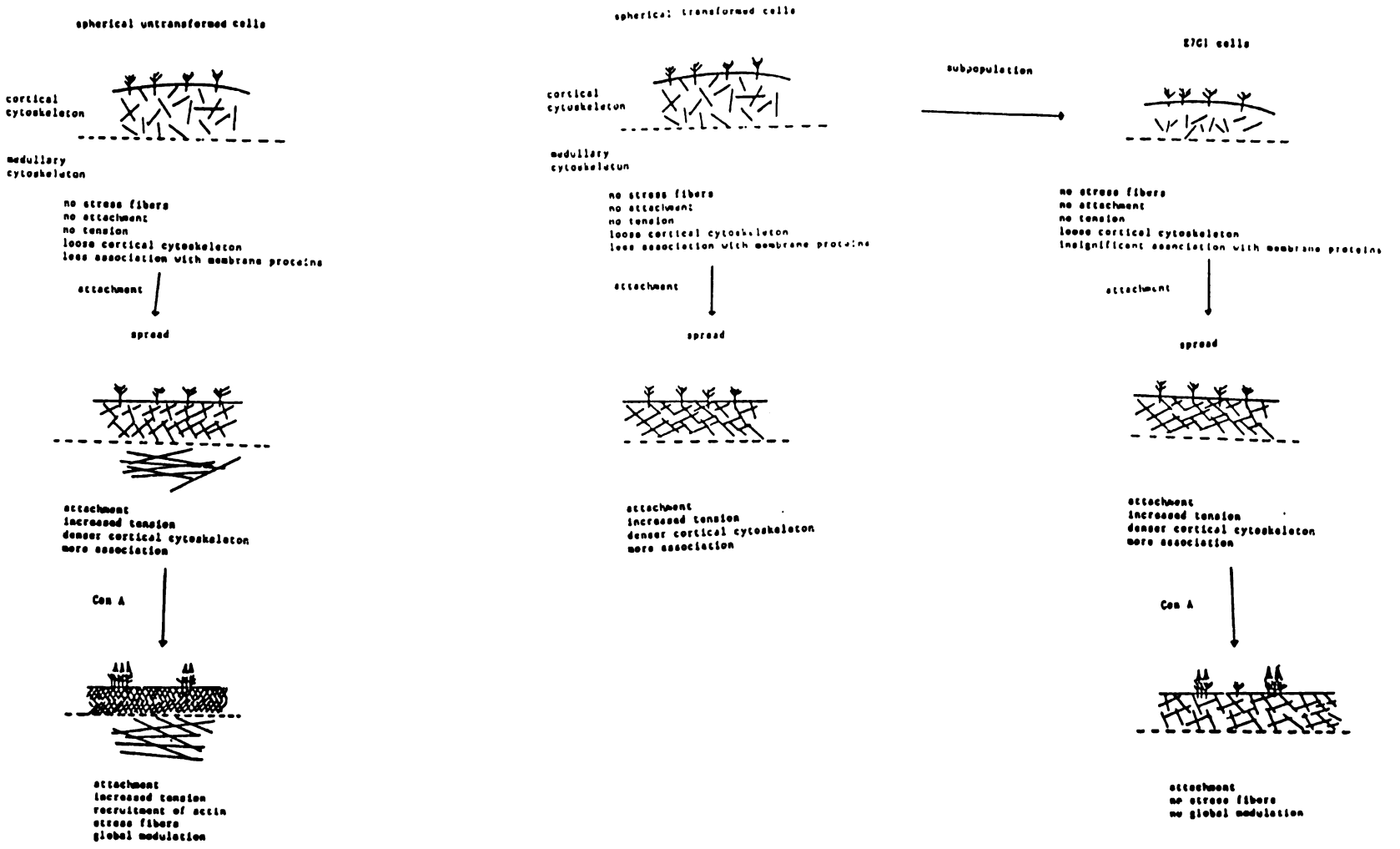

Figure 1. A model for the interaction of membrane proteins with the cytoskeleton as a function of cell shape and transformation.

interaction with membrane receptors. In this fashion the "fast" clone is reminiscent of spectrin deficient erythrocytes.

The changes in protein lateral diffusion concomitant with the changes in the cytoskeleton and cellular metabolic activities take on new significance in light of the studies by others in the laboratory demonstrating shape induced alterations in nucleocytoplasmic transport. The role of actin and myosin in the control of nucleocytoplasmic transport provides suggestive evidence for a relationship between cell shape alteration and the decreased rate of nucleocytoplasmic transport in spherical cells. In conclusion, this thesis presents a first step in connecting plasma membrane activation to nuclear response through the cytoskeleton.

APPENDIX A

The difference in the geometry of spread and spherical cells resulted in the development of diffusion equations and methods to detect diffusion in cells of planar or spherical geometries. For the measurement of lateral diffusion on planar cells such as spread fibroblasts, a solution of the diffusion equation for an infinite plane was derived. Such equations can be used for a point bleach or a multipoint method as described previously (Axelrod, et al., 1976; Koppel, 1979). In the multipoint bleach method the laser beam is scanned by an optical mirror in one direction (x) over the cell. The advantages of the multipoint method is that flow versus diffusion can be more easily detected and measurement of the bleach width necessary for the calculation of diffusion can be determined for each experiment instead of assuming it remains constant. The assumptions of the diffusion equations are that diffusion is occuring in an infinite plane and the labeled molecules have a uniform distribution, i.e. the prebleach fluorescence intensity, F(-), is independent of position. c($\underline{r}$,t) denotes the concentration of fluorescence molecules at position $\underline{r}$ at

time t. It is assumed that the bleach is centered at $\underline{r}=0$ and that redistribution, not chemical recovery, accounts for the changes in $c(\underline{r},t)$. The fluorescence intensity at time t and at position $\underline{\Delta}r$ relative to the bleaching point is described by the equation:

$$F(\underline{\Delta}r,t) = q\int I(\underline{r}-\underline{\Delta}r)c(\underline{r},t)d^2\underline{r} \qquad (1)$$

where q is the product of all the quantum efficiencies of light absorption, emission, and detection, and $I(\underline{r}-\underline{\Delta}r)$ describes the monitoring beam profile which is assumed to be Gaussian. The solution of this equation for a circularly symmetric Gaussian beam results in the equation:

$$F(\underline{\Delta}r,t)/F(-) = 1-\alpha(t)\exp[-|\underline{\Delta}r-\underline{\nu}t|^2/\omega^2(t)] \qquad (2)$$

where ν is flow velocity

ω_o is the effective laser beam size, the 1/e radius

$$\omega^2(t) = \omega_o^2(1+t/T_D) \qquad (3)$$

$$\alpha(t) = \alpha_o/(1+t/T_D) \qquad (4)$$

$$T_D = \omega_o/4D \qquad (5)$$

The basic components of a FRAP instrument are shown in Figure 1. Light from the laser is attenuated by neutral density filters (ND) and exposure times are controlled by a shutter (S). The laser beam is directed into the

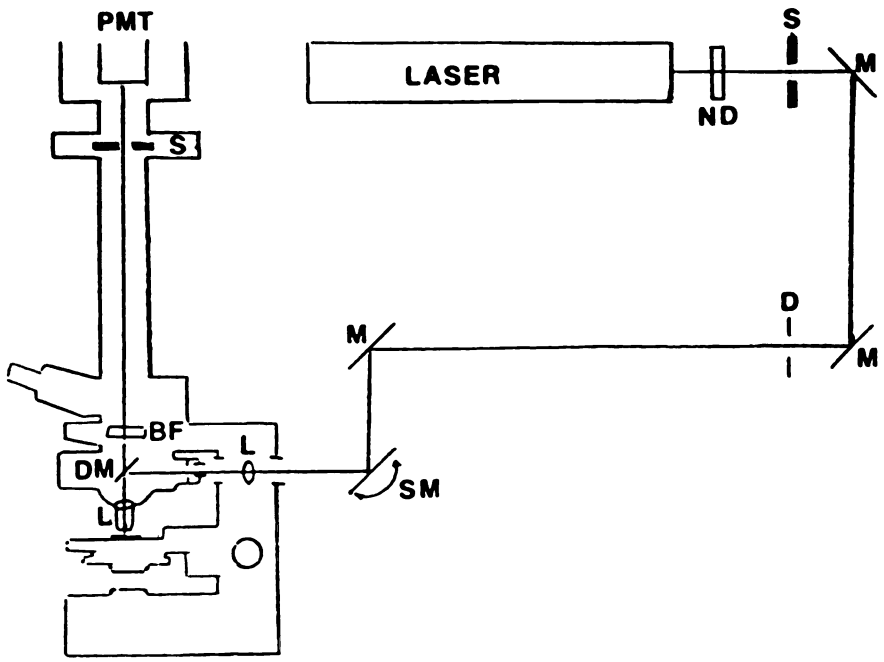

Figure 1. Diagram of the basic components of the FRAP instrument: neutral density filters (ND), shutter (S), mirror (M), diaphragm (D), scanning mirror (SM), lenses (L), dichroic mirror (DM), barrier filter (BF), photomultiplier tube (PMT).

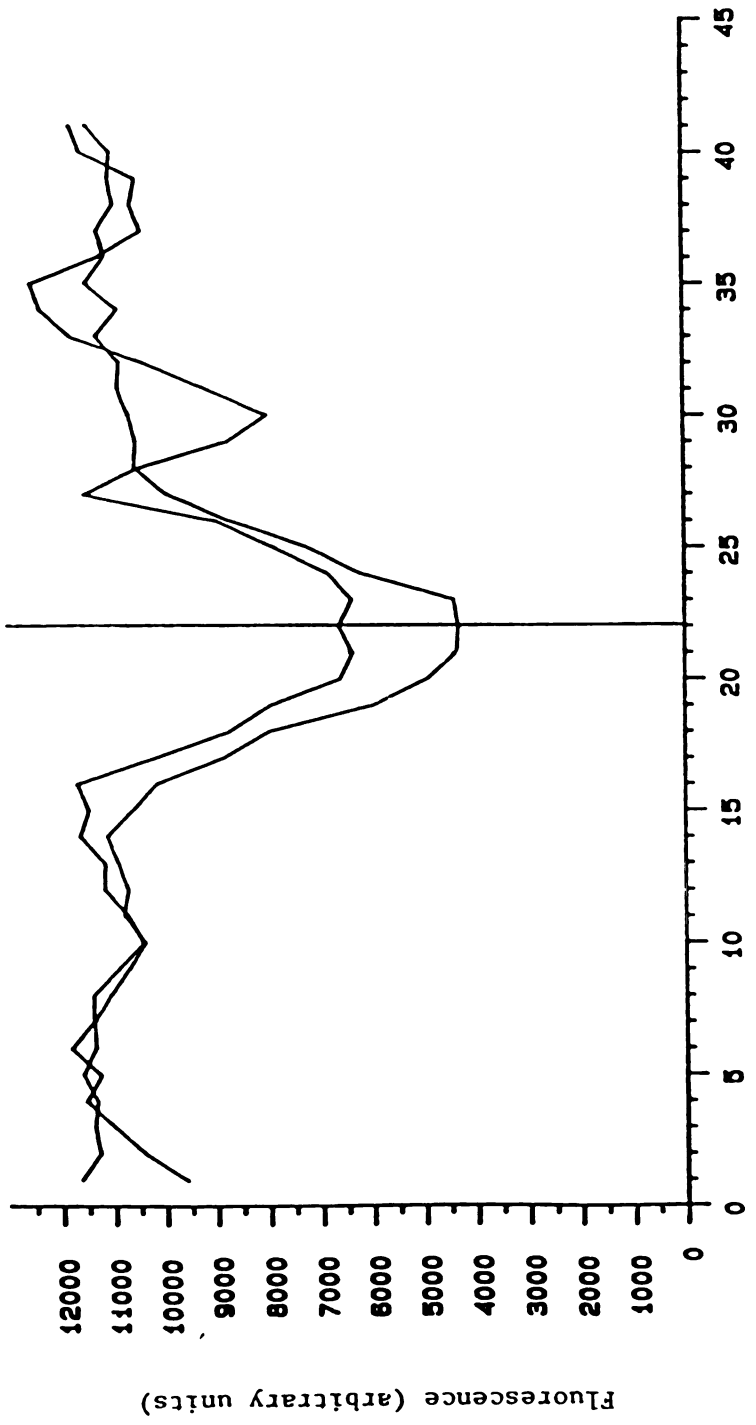

Figure 2. Representative postbleach scans of spread fibroblasts labeled with rhodamine WGA. The vertical line indicates the bleach center (x = 0).

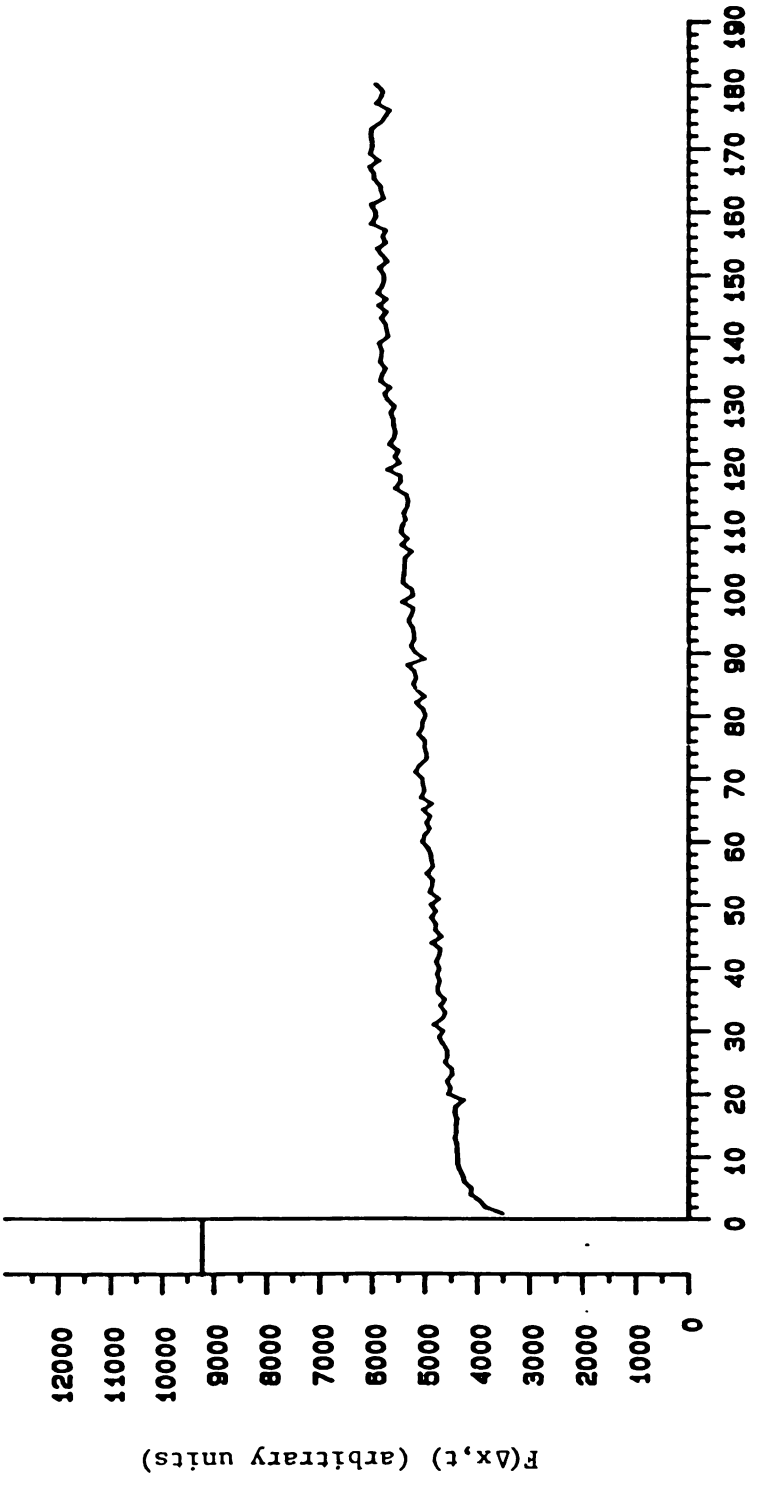

Figure 3. Representative recovery curve after a photobleach on a spread fibroblast. The bar on the ordinate at about 9000 indicates the prebleach level.

fluorescent microscope by a series of mirrors (M). The last mirror before entering the microscope is mounted on a motor permitting scanning of the laser beam (SM). The beam is focused on the samples by two lenses (L). Fluorescence from the sample passes through the dichroic mirror (DM), scattered light is filtered out with a barrier filter (BF), and the fluorescence is measured by a photomultiplier tube (PMT). The fluorescence from several points along the scan of the sample is quantitated and a representative plot of the fluorescence versus position, x, is shown in Figure 2. The two scans shown superimposed in Figure 2 are postbleach scans normalized to the prebleach scan and demonstrate the recovery process. The vertical line represents the x=0 position of an arbitrary scale centered on the bleach. F(Δ x,t) plotted versus time is shown in Figure 3 for 180 postbleach scans and demonstrates the recovery of fluorescence due to redistribution of labeled molecules. A curve fitting algorithm is used to fit the data on the curve to a form of equation 2:

$$F(\Delta x,t) = F(-)\{1-\beta\alpha(t)\ \exp[-(\Delta x)^2/\omega^2(t)]-(1-\beta)\ \omega_o\exp[-(\Delta x)^2/\omega_o^2]\} \qquad (6)$$

where β gives the mobile fraction or extent of recovery. Using the curve fitting routine for equation (6) and equations (3), (4), and (5) T_D and D can be computed.

To test the validity of the equations and the capability of the instrument, the diffusion of fluorescent dextrans in a thin layer of aqueous solution was measured. The diffusion coefficients obtained by the FRAP system for various molecular weight dextrans are shown in Figure 4 compared to diffusion coefficients obtained for various molecular weight dextrans obtained by chromatographic methods (Granath and Kvist, 1967). The values obtained by FRAP closely follow those from chromatographic methodology suggesting that the instrument and method of analysis are capable of producing reasonable results. Furthermore, the values obtained for lipid lateral diffusion and protein lateral diffusion fall within the values reported in the literature (Tank et al., 1982; Aroeti & Henis, 1986; Cherry, 1979).

The equations shown above are appropiate for adherent, spread cells where the membrane approximates a plane. Koppel et al., (1980) presented equations for analysis of diffusion on spheres such as lymphocytes, detached fibroblasts, and vesicles. The normal mode of analysis has two main advantages. It works for a general class of concentration distributions, with its only requirement being an initial azimuthal symmetry, and the relative ease of analyzing the exponential decays occuring from these initial distributions. The general solution for diffusion on a sphere with radius r, is:

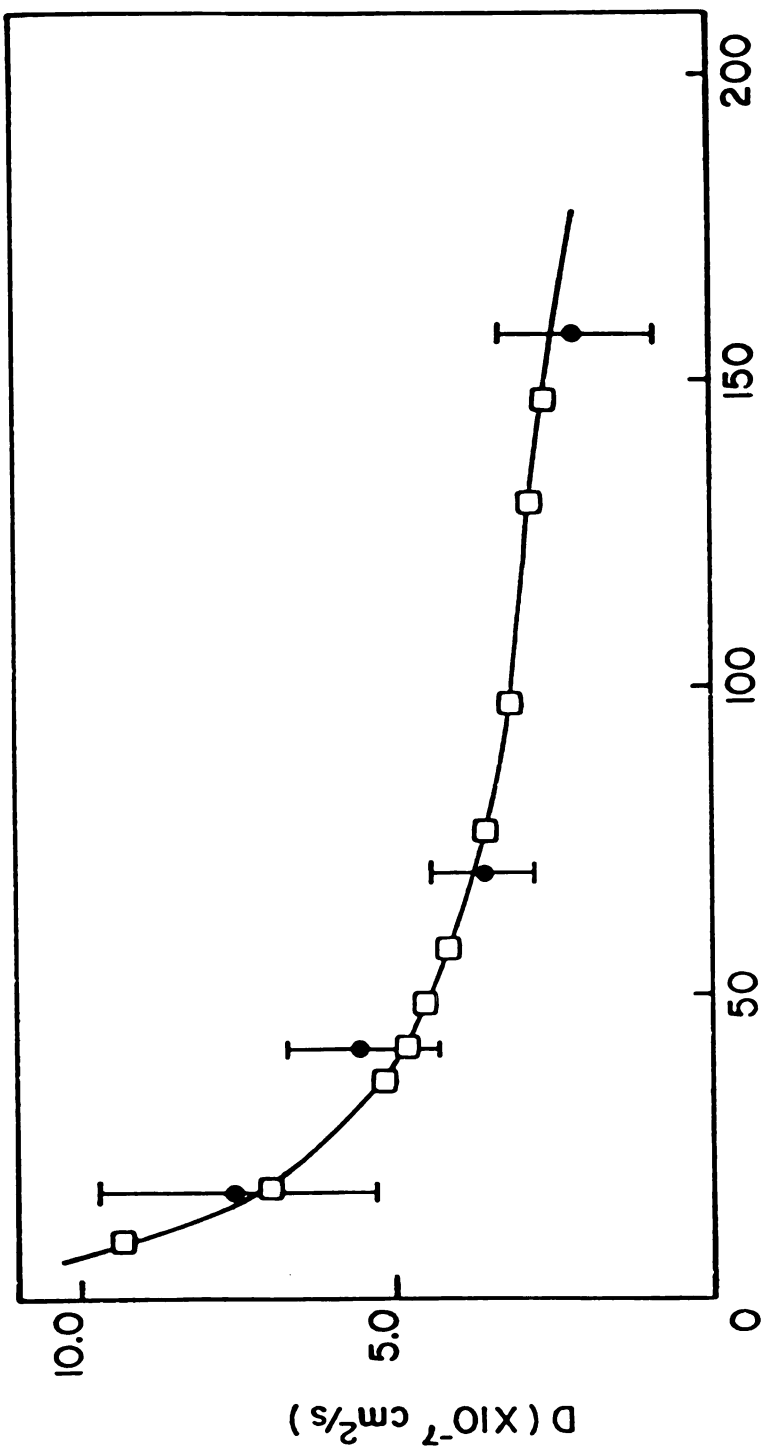

Figure 4. A comparison of the diffusion coefficients of FITC-dextrans measured by the FRAP multipoint analysis (●) and chromatography (□). The line is drawn through the chromatography data. Error bars indicate the standard deviation of the FRAP data.

$$c(x,t) = \sum_{n=0}^{\infty} A_n P_n(x) \exp[-(1/2)n(n+1)\Gamma t] \qquad (7).$$

In this equation the fundamental relaxation rate is Γ, where $\Gamma = 2D/r^2$, x is the cosine of θ shown in Figure 5, the initial concentration distribution determines the coefficients A_n, and P_n is the Legendre polynomial of order n. The normalized "first moment" of the distribution was defined as:

$$\mu_1(t) = \int_{-1}^{1} P_1(x)c(x,t)dx / \int_{-1}^{1} P_0(x)c(x,t)dx \qquad (8),$$

where $P_0(x) = 1$

$P_1(x) = x.$

After the combination of equations (7) and (8) and the application of the orthogonality relation for Legendre polynomials, $\mu_1(t)$ selects the first mormal mode such that

$$\mu_1(t) = (A_1/3A_0)\exp(-\Gamma t). \qquad (9).$$

For a mixed populaton of labeled molecules such as lectin receptors, $\mu_1(t)$ is a weighted sum of exponentials and a time independent background fraction would be immobile on the time scale of the experiment.

In practice the laser beam is scanned along the polar axis of the cell (see Figure 5). The edges of the cell; denoted x=-1 and x=+1, result in the peaks of fluorescence typical of surface labeling. Photobleaching at one of the

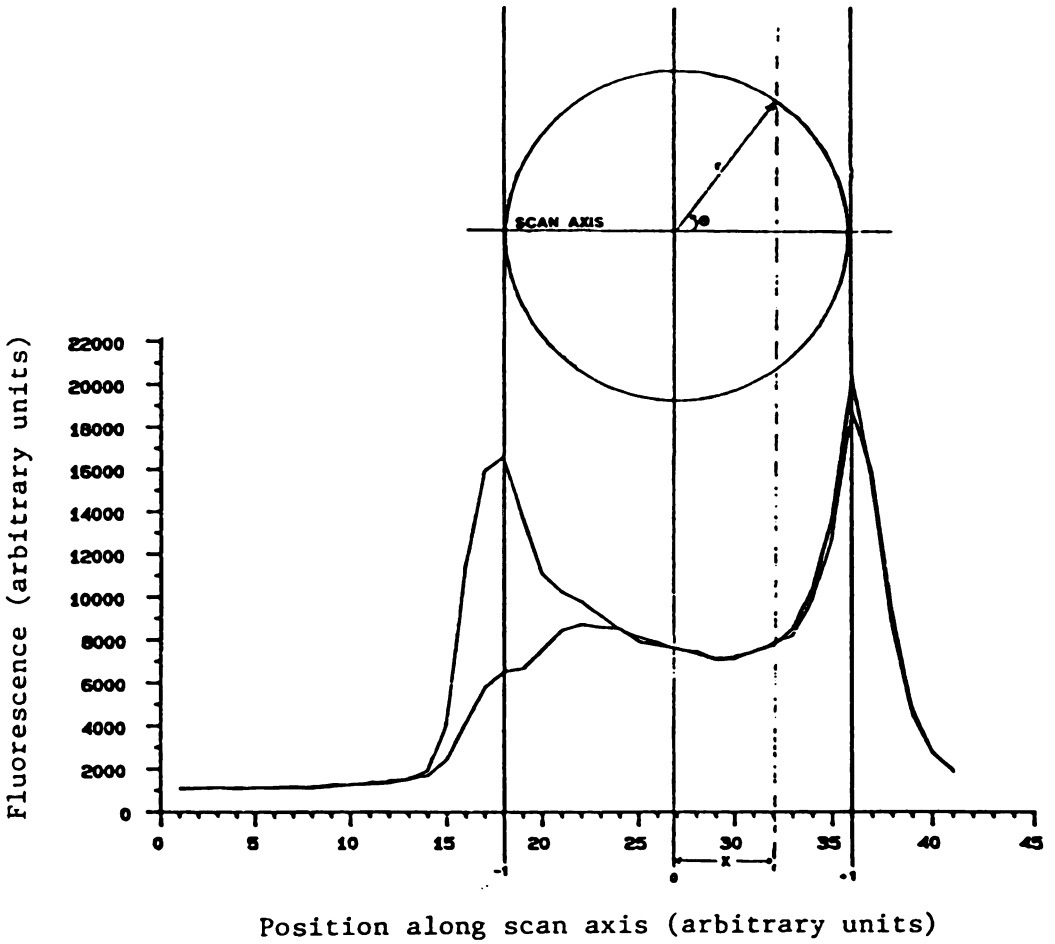

Figure 5. Representative prebleach and postbleach scans of spherical cells labeled with rhodamine WGA. The diagram indicates the relationship of the peaks to the cell edges and the polar scan axis. The diagram also shows the parameters used in the text.

edges results in the decrease in one peak (Figure 5) and produces the required initial nearly azimuthally symmetric distribution. The redistribution of fluorescent molecules results in the recovery of the bleached peak which is monitored by subsequent scanning of the cell with the attenuated laser beam after the photobleaching pulse. Corrections for the effects of cell geometry and possible nonuniform illumination or collection efficiencies can be approximated with point by point normalizations of each postbleach scan by the prebleach scan, F(x,-) so that

$$\hat{\mu}_1(t) = \hat{M}_1(t)/\hat{M}_0(t) \qquad (10),$$

where $$\hat{M}_1(t) = (2/N)\sum_{i=1}^{N} x_i F(x_i,t)/F(x_i,-)$$

$$\hat{M}_0(t) = (2/N)\sum_{i=1}^{N} F(x_i,t)/F(x_i,-).$$

$\hat{\mu}_1(t)$ is a biased estimate of $\mu_1(t)$ since the beam width is finite; therefore, $\hat{\mu}_1(t)$ will deviate slightly from $\mu_1(t)$, but this deviation is generally insignificant. The relaxation time, Γ can be taken from the slope of the line in the plot of ln $\hat{\mu}_1(t)$ versus time (see Figure 6) since $\mu_1(t)$ decays exponentially. Taking the radius (r) of the cell directly from the scan, the diffusion coefficient can be calculated from the equation:

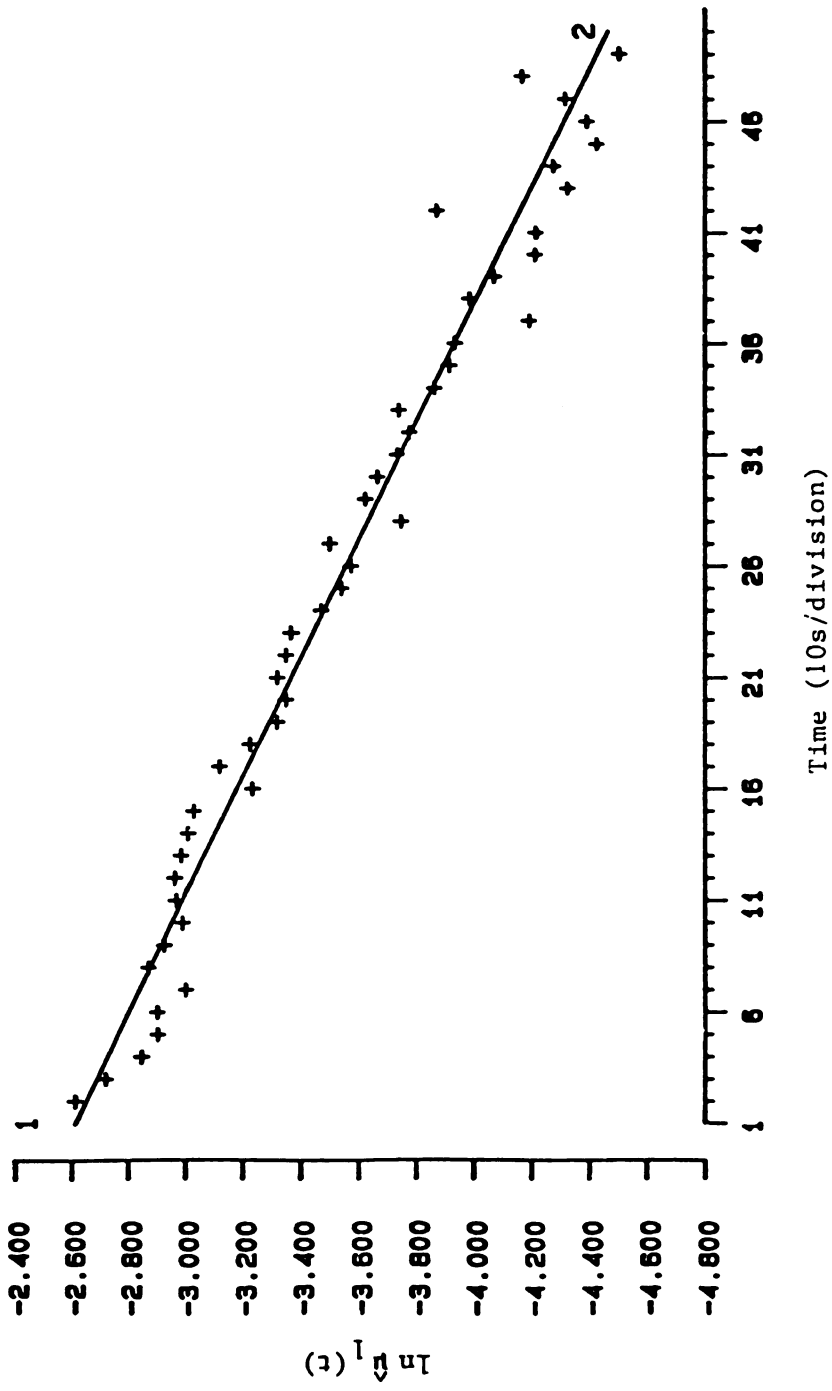

Figure 6. The exponential decay of $\hat{\mu}_1(t)$ as the fluorescence redistributes from the initial azimuthal distribution set up by the photobleach.

$$D = (r^2 \Gamma)/2 \qquad (11).$$

While there has been some doubt of the validity of the equations used and some problems in implementing them, several things indicate that systematic deviations in the calculations are not significant in the type of experiment performed. Analysis of the extent of recovery and diffusion coefficients found no evidence for a correlation between the extent of recovery and the calculated diffusion coefficient which would indicate a systematic error. Finally, the measured diffusion coefficients for lipids in the spherical fibroblast cells were close to those measured in spread fibroblasts using the multipoint analysis and were within the range for lipids reported in the literature (Tank, et al, 1982; Aroeti & Henis, 1986; Cherry, 1979).

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APPENDIX B

Publications

Swaisgood, M. & Schindler, M. "Lateral Diffusion of Proteins in Fibroblast Membranes as a Function of Cell Shape" (to be submitted to Biochemistry)

Swaisgood, M. & Schindler, M. "A Fast Mobility Mutant in Transformed 3T3 Fibroblast - Clonal Selection by Fluorescence Redistribution After Photobleaching (FRAP)" (to be submitted to Biochemistry)

Swaisgood, M. & Schindler, M. "Global Modulation of Proteins in Fibroblast Membranes as a Function of Cell Shape" (to be submitted to Biochemistry)

GLOSSARY

Aggregation- crosslinking of surface receptors to form regions of closely associated receptors (variable in size).

Anchorage dependent- refers to the cell growth requirement for the attachment and spreading (anchorage) of untransformed fibroblasts.

Capping- the coalescence of patches into one large aggregate called a cap.

Contact mediated recruitment- adhesion or contact with the substratum resulting in increased accumulation of actin under the membrane.

Cortical cytoskeleton- a submembranous cytoskeletal assembly (e.g. mammalian erythrocytes) encircling the cytoplasm which is probably actin based and interacts with the medullary cytoskeleton.

E7G1 clone, "fast" mobility population- is a population of cells isolated from K-MSV transformed 3T3 fibroblasts that demonstrate unhindered protein lateral mobility when cell shape is spherical.

F-actin- actin in filamentous form.

Global modulation- a cytoskeleton dependent process reported to restrict protein lateral mobility after crosslinking of surface receptors. The restriction is believed to occur through reorganization of the cytoskeletal systems.

K-MSV 3T3- Kirsten murine sarcoma virus transformed BALB/c 3T3 cell line.

Lateral diffusion, lateral mobility- refers to the random movement of membrane components in the plane of the plasma membrane and the rate is represented by the diffusion coefficient

Medullary cytoskeleton- a cytoskeletal system pervading the cytoplasm composed of microfilaments, stress fibers, microtubules, and intermediate filaments and interacts with the cortical cytoskeleton.

Nucleocytoplasmic transport- the pore mediated movement of particles (dextrans in this instance) between the cytoplasm and the nucleoplasm.

Patching- the aggregation of labeled receptors on the plasma membrane in response to crosslinking agents.

Signal tansduction- the initiation, transmission, and nuclear acquisition of environmental signals or messages.

Tensegrity- a structural support system composed of tension-producing elements and non-interacting compression-resistant elements and are independent of gravity. The model was derived from the highly efficient architectural design for the geodesic dome.

3T3 used alone refers to the untransformed cell line of BALB/c 3T3 fibroblasts.