COMMENTARY

How do cells sense and respond to adhesive contacts? Diffusion-trapping of laterally mobile membrane proteins at maturing adhesions may initiate signals leading to local cytoskeletal assembly response and lamella formation

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Introduction

A great deal is known about the ways in which diffusible molecules bind to receptors and trigger transductive mechanisms located in the plasma membrane. Only recently has there been a wider appreciation of the fact that contacts between cells and surfaces on which they can spread may also initiate cytoplasmic signals. This embraces cell-to-cell contact as well as adhesion to non-cellular surfaces. In the former category, signal transduction through the T-cell receptor complex (Littman, 1989) can activate adhesion based on LFA-1/ICAM-1 by a servo-motor effect (Dustin and Springer, 1989; van Seventer et al. 1990; review, Springer, 1990). Very recently, Lo et al. (1991) have demonstrated that adhesion of neutrophils to endothelium by means of E selectin (ELAM-1) generates signals responsible for increased adhesion by the LFA-1/ICAM-1 mechanism. One of the first clearly defined examples of a signal initiated by the adhesion of cells to a substratum was provided by the important experiment of Wright et al. (1983). They showed that when macrophages spread on a surface bearing fibronectin, engagement of a β-integrin resulted in a signal that spread to the upper membrane where it enabled the two distinctly different complement receptors CR-1 (immunoglobulin superfamily) and CR-3 (one of the leucocyte integrins, now called Mac-1 or CD11b/CD18) to internalize particles bearing the corresponding ligands C3b or iC3b. No change in the number of surface receptors is involved. The effect was reversed on re-spreading the cells on a surface lacking fibronectin. Similar results with a laminin-coated surface were reported by Bohnsack et al. (1985). Blood platelet activation by contact with the substratum also involves signalling (reviews by Parise, 1989; Andrews and Fox, 1990). Almost any surface except vascular endothelium and certain polymers (Gingell et al. unpublished) can activate platelets. The response includes rapid spreading, exocytosis and cytoskeletal reorganization associated with changes in the levels of second messengers and the activation of protein kinases, apparently regulated by glycoprotein IIb, IIIa (Ferrell and Martin, 1989; Golden et al. 1990). Localized signalling processes have also been implicated in the recognition of extracellular matrix molecules and other surfaces by fibroblasts (reviewed by Zetter and Brightman, 1990). This involves integrin-mediated adhesion and the assembly of linkages between membrane receptors and the cytoskeleton, resulting in the formation of focal contacts. Massia and Hubbell (1991) have recently studied the relationship between the density of RGD peptides bound to a substratum and the clustering of integrin receptors, leading to focal contact formation. Curtis (1987) has argued that adhesion, particularly to fibronectin, may trigger cell activation, and an innovative study by Curtis et al. (1992) has strongly implicated integrin-mediated signalling in the activation of a non-integrin-based adhesion mechanism in cultured BHK cells.

A number of other contact-mediated responses may fall within this broad heading. These include the stimulation of mitosis, whose probability depends on the degree of cell spreading on a substratum (Folkman and Moscona, 1978); the contact inhibition of growth, studied with membrane glycoproteins immobilized on silica beads (Wieser and Oesch, 1986); the adhesion-dependent targeting of synthesized proteins, which is a feature of polarized cells such as epithelia (Kabat et al. 1985) and the dependence of intracellular pH and control of the cell cycle on adhesion to a solid substratum (Margolis et al. 1988, 1989).

Contact-triggered cell response in Dictyostelium

We have described a cytoplasmic contractile response of Dictyostelium amoebae that follows contact with a range of chemically distinct surfaces (Gingell and Vince, 1982; Gingell and Owens, 1991). This is an

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essentially non-locomotory shape transformation from a hemisphere to a “fried-egg” with very thin peripheral cytoplasm. After spreading, the cells begin to form a uniform lamellar sheet (around 100 nm), starting at the perimeter and sweeping smoothly inwards. The resulting lamellae often occupy a large proportion of the total cell area, before breaking up into dendritic forms prior to cell locomotion. The “permissive” surfaces that cause this response include glass with adsorbed polylysine, which is strongly cationic, and amino-derivatized glass over a range of ionic conditions where electrostatic repulsion is minimized, as well as the hydrophobic surfaces of pure unmodified polystyrene and glass derivatized with paraffinic octadecyl groups (Owens et al. 1988a). Under normal conditions hydrophilic glass and glass bearing a deposited monolayer of docosanol (Owens et al. 1988b) or lipid-linked monosaccharides - all rich in OH headgroups - fail to elicit the response. Cell locomotion does not occur on the monolayers, despite cell spreading. The permissive surfaces share no common features of wettability, electrostatics, smoothness or chemical composition and it seems that their effectiveness is due to a range of different properties that result in strong adhesiveness for the cells (Gingell and Owens, 1991). Our results can be compared with those of Hafeman et al. (1982), who found that neutrophils adhered and spread both on glass and silanated glass, but spread poorly on a monolayer of dipalmitoylphosphatidylcholine. The cytoplasmic response of the amoebae shows that there are changes transmitted to the cytoplasmic face of the membrane that follow adhesion at the external face. This implies the existence of an adhesion-sensitive signalling mechanism.

Mechanisms of adhesion-dependent signal generation

Many years ago, one of us suggested that the membrane might act as a contact transducer (Gingell, 1967; Wolpert and Gingell, 1969). Non-specific electrostatic stimuli due to the proximity of surfaces were predicted to alter the membrane field and modify permeability or activate membrane enzymes; such field changes have since been shown to affect membrane permeability (Glaser, 1990). It was later argued that external events that reduce the repulsive potential between laterally diffusible cell surface glycoproteins would cause them to aggregate, and that this might trigger contraction of actin filaments linked to the glycoproteins (Gingell, 1976). The model that we shall develop here is related, as it hinges on the role of transmembrane protein aggregation in cell signalling, but it is distinct in identifying diffusion and adhesive trapping as the cause of the aggregation.

In the case of Dictyostelium, how can we envisage signals being generated by adhesion to the substratum, regardless of the mechanism of adhesion? The question arose from thinking about the lamellar response, and much of our discussion relates to this organism, but we shall argue that our model may well have wider applicability. Even in the restricted sense of a mechanism for initiating lamella formation, it may also apply to leucocytes and tissue cells that can form similar cytoplasmic sheets, suggesting that the amoeboid reaction may be one particular instance of a wider class of contact-triggered processes, leading to a cytoskeletal response.

Consider a cell whose surface membrane includes transmembrane glycoproteins that are free to diffuse laterally in the bilayer, and whose headgroups can stick to a substratum if they are sufficiently close to it. It follows that random diffusion will carry these molecules from the dorsal surface of the cell into the contact zone, where they will be immobilized. This process of “diffusive trapping” will tend to concentrate the adhesive molecules in the contact zone, where the substratum acts like molecular fly paper. Such diffusive trapping is responsible for the disappearance of Fe and complement receptors from the dorsal surface of monocytes and macrophages that have spread on a surface bearing the corresponding bound ligands (Michl et al. 1979, 1983; Griffin and Mullinax, 1981). The only remaining ingredient needed for a contact-signalling system is that the two-dimensional clustering of adhesive transmembrane molecules, which concentrates their cytoplasmic segments, thereby activates a cytoplasmic response. Although the Dictyostelium response (in common with platelet activation by surfaces) is non-specific, occurring on any sufficiently adhesive surface, signalling by diffusive trapping might in other cases exhibit selectivity. Aggregation of molecules of type (A) by substratum molecules A′ might lead to a given response, aggregation of (B) by B′ might lead to either the same response or a different one, while aggregation of (C), however caused, gives no response. In the last category, it would be surprising if the aggregation of Thy-1, which is a phospholipid-linked protein lacking transmembrane and cytoplasmic domains, could itself initiate a signal (see Ishihara et al. 1987).

An apparent example of non-specific trapping has been reported by Ursitti et al. (1991), who allowed intact red cells to adhere to polylysine-coated surfaces and then subjected them to viscous shearing forces to disrupt the cells and remove elements of the membrane cytoskeleton. They show that intramembranous particles, consisting mostly of dimers of band 3 protein, aggregate when freed from cytoskeletal restraints. These authors do not however discuss their striking results in terms of diffusion and trapping.

There is ample evidence that bringing certain transmembrane proteins into close proximity by crosslinking them can initiate a signal (Shinitzky, 1984). One of the best known is the antigen-mediated linking of IgE on the surface of Mast cells, which results in massive degranulation, releasing histamine (review, Metzger et al. 1986; Menon et al. 1986a,b). Adhesive interactions involving the binding of the CD11a/CD18 complex (LFA-1) on mature T-lymphocytes with ICAM-1 or ICAM-2 on target cells is implicated in signal generation (review, O’Rourke and Mescher, 1990). The interaction of immature lymphocytes with the major
histocompatibility complex (MHC) of thymic epithelial cells, has long been recognized as central to the selection of T-cell clones. It is now known that MHC-I presents bound antigen to the T-cell receptor and simultaneously keys into CD8 and the CD3 complex on the T-cell. Contiguity of CD8 and CD3 (held together by the complex "hand" of the MHC molecule) triggers kinase production in the T-cell. This has been demonstrated by linking them directly with an antibody, causing kinase production in the absence of MHC (review, Littman, 1989).

Curtis et al. (1992) have provided evidence that localized adhesion can cause signalling events whose actions are manifested far beyond the initial site of contact. BHK cells in suspension were exposed to 2.8 μm beads covalently derivatized with fibronectin. Attachment of even a single bead significantly increased subsequent cell adhesion and spreading on a solid surface bearing adsorbed haemoglobin, to which bead-free cells were poorly adherent. Significantly, the beads were found to be restricted to the dorsal surfaces of cells that had spread on the substratum, so that their action was remote from the site of contact with the substratum. In contrast, prior attachment of beads derivatized with albumin failed to increase adhesion to haemoglobin. It therefore appears that the attachment of fibronectin-coated beads triggers a systemic increase in adhesiveness, probably employing a non-integrin mechanism. This sequence of events is similar to the adhesion servo-motor effect, first recognized in cytotoxic T-lymphocytes by Dustin and Springer (1989). They found that engagement of the T-cell receptor complex somehow potentiates LFA-1-mediated adhesion to ICAM-1 on a target cell without any increase in the number of LFA-1 molecules at the lymphocyte surface. It therefore seems virtually certain that adhesion can lead to the generation of signals. Such signals may lead to pleiotropic responses that include secretion, spreading and the recruitment of further adhesion mechanisms. In certain cases the evidence strongly suggests that receptor clustering is sufficient to trigger off the signal cascade. Although we favour such a mechanism, particularly as an explanation for signalling caused by non-specific adhesion (as in *Dictyostelium* lamella response), this is not the only possibility. The obvious alternative is that receptor engagement with ligand is sufficient, without cross-linking or aggregation. Although this cannot at present be ruled out in all cases, evidence relating to responses to diffusible activators is strongly in favour of the receptor clustering concept (Shinitzky, 1984). A well-documented example of this concerns the IgE receptors (FcεRI) of Mast cells, which must be cross-linked for triggering and degranulation to occur (Menon et al. 1986a,b; review, Metzger et al. 1986). FRAP measurements show that the receptor is normally laterally mobile (D~10^{-10} cm^2 s^{-1}). Small oligomers of IgE or the application of anti-IgE reduce the diffusion coefficient D by 2 orders of magnitude, strongly implicating reversible attachment of the receptors to the cytoskeleton. The diffusion-restriction is closely correlated with the triggering of degranulation. Most significantly, despite the fact that binding is strong, receptors labelled with monomeric IgE have a high lateral mobility and do not initiate degranulation, showing that receptor clustering is an essential pre-requisite for signalling. Direct evidence of receptor clustering in response to anti-IgE labelled with gold particles has been provided by Stump et al. (1989).

**Diffusion restriction**

Not all membrane proteins are free to diffuse laterally. The various constraints to which they may be subjected are discussed by Zhang et al. (1991) and briefly reviewed by Gumbiner (1991). It is also evident that some are under metabolic control and can show phases of mobility or stasis according to prevailing conditions. The first example of this was provided by the remarkably imaginative study of Michl et al. (1979). It was found that resident macrophages did not show redistribution of complement receptors CR-1 (binding C3b) or CR-3 (=Mac-1, binding iC3b) when spread on a substratum coated with the appropriate ligand. In contrast, thioglycollate-elicited cells possessed complement receptors that were free to diffuse in the plane of the membrane and these become trapped on a ligand-coated substratum. Fc receptors for IgG were able to diffuse in both stimulated and unstimulated macrophages. A similar result was published soon after by Griffin and Mullinax (1981), who showed that treatment with a lymphokine enabled macrophage C3b receptors to diffuse freely and become trapped by ligands immobilized on the substratum. CR-1 is a member of the immunoglobulin superfamily, whereas Mac-1 is a leucocyte integrin (Law, 1988), but they are both transmembrane receptors with cytoplasmic segments, which can presumably link to the cytoskeletal actin, since they mediate endocytosis in a cytochalasin B-dependent manner (Axline and Rea, 1974). It is therefore clear that not all receptors that are potentially capable of mediating adhesion can suffer diffusive trapping in a given situation, and that their behaviour can be restrained by delicate and complex controls.

**Events following receptor clustering**

The events occurring immediately after transmembrane molecules have been brought into proximity may fall into one of two categories. The first is transduction involving second messengers, in which case effects on the cytoskeleton are mediated by small molecules such as cAMP, cGMP, inositol triphosphate (IP_3) or diacylglycerol. These could influence cytoskeletal assembly in the cortex by protein phosphorylation and calcium-dependent processes, which are discussed below. The second involves direct influence on cytoskeletal organization at the cytoplasmic face of the adherent membrane. There is evidence that clustering of certain transmembrane proteins can promote actin polymerization (see below). It may also influence the binding of myosin I (the single-headed form) to the membrane,
perihaps to the cytoplasmic domains of the clustered molecules themselves. Although conceptually distinct, these two classes of events are both likely to be involved.

Having outlined a model of adhesion-dependent cellular responses, we shall review recent work on the molecular components of the motile system in Dictyostelium and then discuss the mechanisms of signal reception. This will enable us to make some educated guesses about the events that take place in the cortical cytoplasm during the lamellar response to adhesion.

**Cytomechanics of motility in Dictyostelium**

**Actin organization**

In amoebae F-actin is concentrated in active pseudopodia. This was demonstrated by Roos et al. (1986) by means of rhodamine-phalloidin/phalloin staining, and by Condeelis et al. (1988). Polymerization of G-actin to filamentous F-actin, shown by incorporation of actin into the Triton-insoluble cytoskeleton, is an early event both in Dictyostelium chemotaxis (review, Newell et al. 1988; Dharmawardhane et al. 1989) and in neutrophil chemotaxis (review, Omm et al. 1987). A kinetic analysis of F-actin assembly on isolated Dictyostelium membranes, by means of photoactivated cross-linking, was interpreted to show that actin assembly is initiated at sites where two adjacent transmembrane proteins bind three actin monomers (Schwartz and Luna, 1988). The F-actin filaments are thought to grow parallel to the membrane in the early stages, with each additional monomer interacting with one more transmembrane molecule recruited to the cluster. This results in multiple anchorage for each actin filament. Such linkage might stiffen the cortex, an effect associated with receptor clustering induced by lectin binding (Pasternak and Elson, 1985). Schwartz and Luna suggest that “extracellular factors that cluster membrane proteins may create a site for the formation of actin nuclei and thus trigger actin polymerization in the cell”, and speculate that this could explain how external signals trigger the large increases in actin polymerization that have been found. This suggestion is of particular interest in relation to our diffusion-trapping model. It has recently been shown by Chia et al. (1991) that the actin-binding membrane protein investigated by Schwartz and Luna is the same as that described earlier by Wuestehube and Luna (1987), who found that F-actin binds to a 17 kDa transmembrane glycoprotein named ponticulin. It was shown that F-actin linked to a Sepharose column binds ponticulin and that anti-ponticulin Fab prevents ponticulin binding. Pre-adsorption of the Fab at the outer surface of the cell does not reduce the effectiveness of the antibody, showing that specific binding occurs at the cytoplasmic face, presumably to the cytoplasmic tail of ponticulin.

Polymerization of actin occurs during the initial phase of the chemotactic response to cAMP - termed the cringe response - which occupies about 50 s (Dharmawardhane et al. 1989). During this period myosin II (the double-headed form found in vertebrate striated muscle) is dephosphorylated on the heavy chain and phosphorylated on the light chain, with the result that it can assemble into thick bipolar filaments with Mg^{2+}-ATPase activity (Fukui and Yumura, 1986). During the cringe response the cell retracts, due to cytoplasmic contraction, after which energetic pseudopodial extension occurs in the direction of the chemotactic gradient. In the latter phase there is evidence of extensive actin cross-linking by a 120 kDa protein (Condeelis et al. 1988; Dharmawardhane et al. 1989).

**Myosin organization**

The part played by myosin in amoeboid locomotion is most intriguing (Adams and Pollard, 1989; review, Korn and Hammer, 1990; Pollard et al. 1991). Until recently, the contractile analogy with vertebrate striated muscle and the demonstrated co-localization of actin with the normal double-headed myosin II in Dictyostelium (as in other motile cells), together with ATP-induced contraction of isolated cytoskeletons, made it almost axiomatic that actomyosin contraction powers the molecular motor (Clarke and Baron, 1987). Then came the astonishing discovery from two laboratories that Dictyostelium lacking myosin II can locomote without major impediment. Removal of myosin II by antisense RNA or by homologous gene recombination was simultaneously reported by De Lozanne and Spudich (1987) and Knecht and Loomis (1987); similar results soon followed from Wessels et al. (1988) and Manstein et al. (1989). Furthermore, Dictyostelium amoebae lacking myosin II produce a normal chemotactic response to cAMP and show the normal rise in F-actin association with the cytoskeleton (Peters et al. 1988). This traumatic dislocation for the amoeboid establishment was swiftly followed by the major discovery of Fukui et al. (1988, 1989) that myosin II and myosin I play distinctly different roles in Dictyostelium movement. Myosin I differs from myosin II in having a single head consisting of one heavy chain and at least one light chain. It cannot polymerize into thick filaments, but the head has two binding sites for actin, one of which is a Mg^{2+}-ATPase with mechanochemical activity; it can therefore cross-link actin filaments and cause traction between them. Myosin I, which can bind to membrane lipids and perhaps to membrane proteins as well, is characteristically found in association with membranes (Baines and Korn, 1990). First discovered by Pollard and Korn in Acanthamoeba, it has subsequently been found in a variety of cells (reviewed by Pollard et al. 1991) and it is known that several genetic isoforms can be formed in a given cell. Each isoform is characterized by a distinct separately coded heavy chain. Fukui et al. (1988, 1989) found that myosin II is responsible for contraction of the cleavage furrow and is also found at the rear end of the locomoting amoebae. In marked contrast, myosin I is virtually absent from these regions but is concentrated in advancing pseudopodia and phagocytic cups. Myosin I may be responsible for cytoplasmic protrusion and taking this in association with the continuation of cell
locomotion in the absence of myosin II it seems that myosin I is the more important molecule for amoeboid movement. However, even this comforting conclusion has been challenged. Genetic ablation of myosin I results in amoebae that are reputed to locomote and suffer only partial impairment of phagocytosis (abstract, Jung and Hammer, 1989). But this conclusion may be unjustified; since there are several loci coding for myosin I, inactivation of one locus or its RNA transcript would not necessarily cause complete cellular depletion of myosin I.

Confirmation that myosin I, but not myosin II, is closely associated with isolated membranes of Acanthamoeba (Miyata et al. 1989) lends further support to the notion of a functional linkage between the membrane and myosin I. They argue that attachment is independent of actin, via a binding site near the Mg$^{2+}$-ATPase site but distinct from it, and may be direct to a transmembrane protein.

Dictyostelium had further surprises in store. It has recently become evident that several major actin binding proteins of are also inessential for locomotion. Complete genetic removal of a-actinin or severin or gelation factor (the latter is similar to a-actinin; Noegel et al. 1989) leaves amoebae with little motile impairment (Schleicher et al. 1988; Andre et al. 1989; Titus et al. 1990; review, Gerisch et al. 1991). It is not yet known whether the Dictyostelium analogue of brain spectrin (fodrin) is equally dispensable (Bennett and Condeelis, 1988; also review, Bennett, 1990).

**Mechanism of response to signals**

Having discussed the motile machinery of Dictyostelium, what can be said about the way in which signals originating outside the cell activate motility? The chemotactic response to cAMP, which leads to aggregation, has been extensively studied (review, Newell et al. 1988). The two major signal transduction systems based on G-proteins are represented in aggregation competent amoebae. The adenylate cyclase system provides for cAMP reception and signal amplification by making more cAMP. The phosphatidylinositol diphosphate (PIP$_2$) system also responds to cAMP, making inositol triphosphate (IP$_3$) and diacylglycerol. The former releases Ca$^{2+}$, from non-mitochondrial intracellular stores, which somehow stimulates cGMP synthesis (Gang Liu and Newell, 1987) and promotes rapid actin polymerization. One of the actions of cGMP is to recruit myosin to the actin cytoskeleton. Presumably this involves myosin activation by phosphorylation (Fukui and Yumura, 1986) and it should be noted that myosin I is activated by phosphorylation of the heavy chain (Pollard et al. 1991). Newell and his colleagues have studied the streamer mutant which exhibits a prolonged motile response to cAMP. These cells have defective cGMP-specific phosphodiesterase, which results in a tenfold prolongation of the peak of cGMP activity in response to cAMP. This correlates with a tenfold prolongation of the phase of myosin incorporation into the cytoskeleton, which seems sufficient to explain the persistence of locomotion.

The results of extensive studies that have been published on neutrophil chemotaxis are also relevant to the discussion. Chemotaxis occurs in response to a range of factors, including the complement cleavage product C5a, leukotriene B$_4$, platelet activating factor and the chemotactic peptide fMLP, all of which bind to different specific receptors and stimulate phospholipase C via the ubiquitous G-proteins. The fMLP receptor has been identified by photoactivated cross-linking, and is probably a transmembrane glycoprotein. Activation of the PIP$_2$ pathway and the formation of diacylglycerol and IP$_3$ leads to rapid actin polymerization (paralleling that in Dictyostelium) as well as myosin phosphorylation and association of cytoskeletal proteins with the membrane (review, Omann et al. 1987). A report by Cano et al. (1991) based on the kinetics of actin filament depolymerization shows that the number of actin filaments doubles after fMLP stimulation but their mean length is unchanged. Most of the new filaments are associated with the lamella. However, the molecular details of cytoskeletal reorganization in response to fMLP binding are still opaque. Indeed, even the role of PIP$_2$ is questionable. Bengtsson et al. (1988) reported that fMLP stimulation causes a rise in F-actin concentration, but that no IP$_3$ is generated. Since Pertussus toxin suppresses the fMLP-induced polymerization of actin, the authors speculate that G-protein activation is the crucial event that influences actin polymerization in an unknown way (for a new mechanism see Yang et al. 1990). A recent report by Gundersen and Devreotes (1990) shows that the G-protein a-2 subunit, a key element of the transductive concatenation, is phosphorylated within 40 s of cAMP binding. Stephens et al. (1991) have investigated the recently described PIP$_2$ signalling pathway and have argued that phosphatidylinositol (4,5)bisphosphate-3-kinase action seems to be a controlling factor in the fMLP-induced generation of 3-phosphorylated lipids.

In the case of IgE-mediated signalling, earlier confusion over the question of consequential phosphorylation has been resolved by the recent elegant work of Paolini et al. (1991). They have shown that cross-linking the FceRI receptor complex with antigen results in immediate phosphorylation of tyrosine and serine on the beta subunit and of tyrosine and threonine residues on the gamma subunit by at least two different kinases, which are probably of the src family (Brickell, 1991; review, Brickell, 1992). Importantly, these authors conclude that phosphorylation is the first identifiable step of the signalling process. Interestingly, phosphorylation is rapidly reversible upon receptor disengagement, due to the action of an undefined phosphatase. How phosphorylation leads to interaction with G-proteins and whether cytoskeletal molecules become involved is not known.

These results suggest comparison with the rapid reversibility of the action of phorbol ester in the protein kinase C-mediated potentiation of endocytosis in monocytes and macrophages reported by Wright and
Fearon (1986) to result in phosphorylation of the 260 contrast to neutrophils, in which Wright and Meyer complement receptor CR-1 but not Mac-1 (CR-3), in myelomonocytic cells was reported by Changelian and particles bound to them. Phorbol ester treatment of complement receptors (Michl et al. 1979; see Diffusion p. 257, above) but cannot endocytose restriction, of Mac-1. It seems likely that the potentiation of complement-mediated endocytosis by signals derived from engagement of the fibronectin receptor (Wright et al. 1983) involves protein kinase activation, but this appears not to have been examined.

A question that arises from the endocytosis studies concerns the relation between receptor phosphorylation and the assembly of the actin cytoskeleton that is implicated, since cytochalasin B inhibits internalization (Axline and Reaven, 1974). Is phosphorylation necessary for the attachment of actin filaments? Does it mediate direct linkages between receptors, as postulated by Wright and Detmers (1988)? Alternatively, if receptor clustering is alone sufficient to initiate assembly of cytoskeletal molecules (as ponticulin can) phosphorylation may play some other role in facilitating endocytosis.

**Events In lamella formation**

We shall now return to our model for adhesion-dependent signalling and try to relate it to the facts that have emerged from the discussion of motility, in order to construct a plausible series of events in lamella formation. We argued that the earliest component of the cellular response to surfaces that stimulate the lamella response is the diffusion and trapping of laterally mobile membrane receptors, which move from the dorsal surface of the cell and consequently cluster at the adhesive surface. Such trapping of mobile receptors was first demonstrated in the innovative experiments from Silverstein's laboratory mentioned earlier (Michl et al. 1979, 1983) as well as by Griffin and Mullinax (1981). Macrophages were allowed to spread on surfaces coated with oriented IgG molecules (such that their Fc tails pointed away from the substratum) or with complement components C3b or C3b' (now referred to as iC3b). The fixed ligands specifically engaged and trapped the corresponding receptors, which correspondingly disappeared from the dorsal surface of the cell. Similar experiments showed that suitably activated macrophages can redistribute the integrins LFA-1 and p150,95 (Wright and Jong, 1986; Bullock and Wright, 1987). Since we have proved that the Dicyostelium lamella response is chemically non-specific, the trapping of membrane molecules would differ from Silverstein's example only insofar as it would not need to involve specific interactions with the substratum. Results on the Con A-Rh staining of amoebae (Gingell and Owens, 1991) lend some support for such a process. Pre-stained cells form lamellae on permissive surfaces. Subsequent locomotion leaves a dendritic trail of labelled lamellar fragments, but the cell bodies show only weak staining. The trails are most intensely stained at their origins, where the cells first adhered and formed lamellae. This indicates that membrane glycoproteins binding Con A become concentrated near adhesion sites on the substratum where the lamella response was initiated. It does not enable us to decide whether the membrane glycoproteins become concentrated at the edges or beneath the lamellae, but total internal reflection fluorescence (TIRF) should resolve the question. When cells were allowed to settle and move on a lamella-permissive surface and then stained with Con A-Rh, the absence of lamella staining is consistent with the idea that membrane glycoproteins are depleted on the more-accessible dorsal surface. If this is correct, it should be possible to show that the diffusion of labelled lectin beneath the cells is restricted, since studies using TIRF have shown that the diffusion of macromolecules can be severely restricted beneath locomoting tissue cells (Gingell et al. 1985). These labelling experiments are consistent with a membrane glycoprotein diffusion-trapping model.

Our results can be compared with those of Hafeman et al. (1982). They examined the distribution of C3b receptors on neutrophils that had spread on glass or siliconized glass, using fluoresceinated anti-C3b Fab', which does not cause patching. C3b receptors were seen to cluster at the substratum. Most interestingly, clusters were also seen at the upper surface, showing that the events occurring in the membrane adjacent to the substratum had triggered a systemic membrane response. Redistribution was selective, as shown by the failure of HLA molecules to redistribute when cells spread on a surface coated with C3b. No clustering was seen on a phospholipid monolayer on which the cells hardly spread and were poorly adherent. This was confirmed by FRAP measurements.

Where the cell membrane is sufficiently close to the substratum for trapping to begin, the pattern of trapping will depend on the pattern of cell contacts. For example, if a cell makes discrete adhesions but is arched away from the substratum between them, adhesive trapping would first occur around the contacts and may even cause them to spread. On the other hand, if the initial contact is uniform, diffusive trapping should predominate around the edges of the contact zone. Dicyostelium amoebae exhibiting the lamella response are known to make a uniform contact with the substratum, as shown by analysis of total internal reflection fluorescence images (Todd et al. 1988). This study proved that the black images of the lamellae seen so strikingly under interference reflection (IRM) optics (Gingell and Vince, 1982) are not caused by especially intimate contact beneath the lamellae (contrary to the conventional interpretation of such images). The IRM appearance is due to the very thin lamella itself, as predicted by the finite aperture theory of microscope interferometry (Gingell and Todd, 1979; Gingell, 1981). Since the amoebae do make uniform contact with the
substratum, the diffusion-trapping model provides an explanation for one of the main features of lamella formation. If the accumulation of diffusible glycoprotein occurs principally at the edges of the spread cell, and there triggers cortical contraction, we have a neat explanation for the fact that cytoplasmic thinning invariably begins at the extreme edges. Another example of activation at the edge comes from the intriguing observation of Svitkina et al. (1986) that when spread fibroblasts are released from azide inhibition the microfilament network is re-established at the extreme periphery and extends centripetally to re-form the cortical microfilament sheath. The time course of centripetal initiation after inhibition (~1 min) is of the order found after low temperature inhibition of lamella formation in Dictyostelium (Gingell and Vince, 1982). Svitkina et al. (1986) suggest that there might be an increased density of nucleation sites for actin assembly at the cell edge.

The distribution of cell surface glycoproteins on locomoting epidermal cells was reported by Kucik et al. (1991) using succinylated Con A, which does not cross-link membrane glycoproteins. There was no evidence of a general increase in glycoprotein concentration at the leading edge, but the authors emphasize that this does not imply that individual adhesion receptors are evenly distributed over the cell surface. Pytowski et al. (1990) similarly report that receptors for Fc, C3bi and a differentiation antigen are uniformly distributed when quantitative fluorescence measurements are normalized with respect to a lipid probe. They elegantly demonstrated that the apparent high anterior concentration of membrane receptors of motile neutrophils merely reflects the local accumulation of surface membrane, presumably as folds, though no direct evidence is given. In contrast, Letourneau and Shattuck (1989) found high concentrations of an integrin (but not of several other adhesion molecules) at the tips of nerve growth cone filopodia. In this site, interpretational errors due to membrane folding are unlikely. It should be borne in mind in all these experiments that the degree to which selective adhesion receptors may be clustered bears a distinct resemblance to the lamellipodia of tissue cells, both being around 100 nm thick (340 measurements on thin-section electron micrographs of 18 monocytes gave 110±19nm: unpublished results). Both regions exclude cytoplasmic organelles (Forscher and Smith, 1988; Rinnerther et al. 1991) and have similar electron microscopic appearances. Like the pseudopodia of amoebae, lamellipodia have myosin I but lack myosin II. However, the extent to which the centrifugal protrusion of polarized lamellar sheets is mechanically related to their centripetal formation in Dictyostelium by withdrawal of cytoplasm is uncertain. Membrane and cytoplasmic flow in lamellipodia has attracted a lot of attention over the years, and has been the source of much disagreement (review, Heath and Holifield, 1991). While Bretscher's (1984) hypothesis of rearward lipid flow seems to have been disproved by elegant fluorescent tagging experiments, principally from Jacobson's laboratory (Holifield et al. 1990; and particularly, Lee et al. 1990), there is no concensus of opinion regarding the behaviour of membrane proteins and cytoplasmic actin. The first direct evidence for the rearward motion of actin by was obtained from photobleaching measurements by Wang (1985), who reported a rate of 0.8 μm/min with respect to the edge of the cell. From the published results it appears that there was little or no advance of the edge of the lamella during the period of measurement. Holifield et al. (1990) interpret their complex immunofluorescence labelling experiments on fibroblasts as indicating that certain intercalated proteins can attach to actin filaments, which drag them to the rear. This action rakes back other membrane proteins that are not themselves attached to the cytoskeleton. However, it is hard to discern whether such motion is referred to the leading edge or the substratum. On the basis of observations on the lamellae of 3T3 cells, Fisher et al. (1988) envisage a single process involving active contraction based on myosin I or II, which is responsible for the centripetal motion (i.e. away from the edge of the cell) of actin and cell surface components (including capped proteins) in lamellipodia at about 12 μm/min relative to the substratum, accompanied by the assembly of actin filaments at the tip. However, their measurements (as opposed to some visual observations) were confined to cells with non-advancing lamellae. Burmeister et al. (1991) briefly refer to the movement of particles attached to growth cones of Aplysia neurons. Beads are reported to move centripetally at 1-2 μm/min with respect to the substratum under conditions where the lamellar edge advances at 7 μm/h, or only 0.12 μm/min. In this case therefore, particles move centripetally on the surface of slowly advancing lamellae. In a meticulous study of stationary nerve growth cones treated with cytochalasin B (CB), Forscher and Smith (1988) showed that loss of lamellipodial actin began at the cell margin and proceeded centripetally at about 5 μm/min, indicating that CB caps the barbed end of each filament, causing detachment from the membrane insertion site. They conjecture that a myosin motor that is stationary with respect to the substratum is respon-

Relationship between tissue cell and Dictyostelium lamellae

The lamellae formed by Dictyostelium bear a distinct...
sible for drawing the actin filaments centripetally. Their argument is supported by the finding that after CB is washed out, regeneration of actin begins at the margin and proceeds centripetally at about 5 \( \mu m/\text{min} \). The centripetal flow of actin in this growth cone, and its rate of translation, suggest comparison with the centripetal development (around 2 \( \mu m/\text{min} \)) of the *Dictyostelium* lamella.

In contrast to the results showing a centripetal actin flow in lamellae under conditions where they are stated to be non-advancing (or appear to be thus), fluorescence photobleaching of a novel actin label injected to be non-advancing (or appear to be thus), fluorescent photobleaching of a novel actin label injected into fish keratocytes with actively extending lamellipodia by Theriot and Mitchison (1991) shows without doubt that in these cells net polymerization of actin takes place at the margin and F-actin subsequently remains stationary with respect to the substratum. These authors argue from kinetic data that F-actin is likely to be present as short cross-linked filaments, which may not be strongly directionally polarized. A similar conclusion was reached in a recent report by Kucik et al. (1991), who followed Con A-labelled latex microspheres placed on the lamellae of actively advancing epidermal keratocytes by means of laser “optical tweezers”. Beads attached preferentially at the extreme edge and remained virtually stationary with respect to the substratum as the lamella advanced. From this it was deduced that transmembrane glycoproteins that bind the microspheres established links with the cytoskeleton within 0.5 \( \mu m \) of the edge of the lamellae and that their subsequent stasis with respect to the substratum reflects the dynamics of the actin core. We shall return to the apparent distinction between the dynamics of advancing and stationary lamellae below.

A further question attaches to the cyto mechanics of marginal lamellae, which may form as cells settle and then spread on a substratum. A recent paper by Heiple et al. (1990) describes the spreading of macrophages on a carpet consisting of the Fc ends of Ig molecules bound to glass using the method of Michl et al. (1979). Some spread cells show a dark peripheral ring when seen by IRM. The authors argue that this represents a zone of particularly tight contact with the substratum, rather than being generated by the thin cytoplasmic edges of the cells. Their conclusion rests principally on an unconvincing analysis of the way in which the IRM image is affected by changing the refractive index of the medium (see Gingell and Vince, 1982, for the application of this method to the thin lamellae of *Dictyostelium*). Heiple et al. (1990) argue that the dark IRM zones are contact seals that prevent the diffusion of antibodies under the cells. This conclusion is weakened by their own results, which show that diffusion is also restricted beneath cells that form incomplete (horse-shoe-shaped) “seals” as well as the large proportion that do not exhibit dark IRM zones at all. Our own results (unpublished), using a combination of IRM, TIRAF and transmission EM of vertical sections, show that human monocytes can form 100nm lamellae that generate dark IRM zones. TIRAF using volume markers of low molecular weight shows unambiguously that the cell to glass aqueous “gap” into which 10 kDa probes can diffuse is the same in the dark and paler IRM zones. Lack of diffusion of macromolecules from the medium into the dark IRM areas may be a consequence of the diffusive trapping of Fc receptors, which (as we have argued) results in a region of tightly packed proteins under the lamellae. The exclusion of large probes and the admission of smaller ones into the contact zones of migrating fibroblasts has been demonstrated by Gingell et al. (1985) by means of TIRAF.

**Mechanism of *Dictyostelium* lamella formation**

Taking into account the major features of the current body of knowledge that we have discussed relating to the molecular basis of motility and the mechanisms by which cells respond to external signals, what conclusions can be reached about the cytoplasmic mechanics of the *Dictyostelium* lamellar response? We think that diffusive trapping of adhesion molecules is the first step. What happens next? Although we are conscious of the pitfalls of pasting together a patchwork of facts drawn from several different systems, it may be useful to present an outline that can at least stimulate discussion.

Lamellar thinning could be caused by one of two things. First, active withdrawal of fluid cytoplasm could result in the collapse of those regions destined to become lamellae, leaving a 100nm sandwich composed of two apposed layers of cortical cytoplasm, bounded by dorsal and ventral surface membranes. Second, a zone of mechanical stricture incorporating links between the dorsal and ventral membranes could squeeze out cytoplasm as it sweeps across the cell, like rolling up a toothpaste tube. The fact that the cytoplasm does not collapse en masse but thins in a highly regulated way, along a line or front, as if an active zone is moving smoothly and uniformly inwards, is far easier to reconcile with the second mechanism. One way that this could come about is that the dorsal and ventral membranes are pulled together by contractile links consisting of actin and myosin. Linkage with the membrane might involve either myosin I or actin interacting with intercalated membrane proteins. A less-obvious scheme has a certain attraction, as it incorporates findings on tissue cell lamellae and nerve growth cones (Fig. 1). Suppose that adhesive ponticulin-like molecules suffer diffusive trapping at the substratum and thereby become aggregated. They may nucleate short F-actin filaments that in turn bind the non-mechanochemical site on myosin I. The adhesive molecules thus act as anchorage points against which cytoplasmic contractile events can obtain the necessary mechanical reaction (Bray and White, 1988). The second binding site on myosin I interacts with a group of cross-linked actin filaments running away from the cell margin parallel to the substratum. Activated by kinases, resulting from a signal cascade triggered by the clustered membrane proteins, myosin interacts with the central actin assembly, drawing it rearwards. If the actin...
Cell response to adhesive contacts

Rearward travel of (H + I) causes necking zone which also moves rearwards, "rolling out" the lamella.

**Fig. 1.** Scheme for contractile formation of *Dictyostelium* lamella. Diffusible membrane proteins (E) become trapped and aggregate (A) at the substratum, nucleating actin filaments (B), which bind myosin I molecules (C) that are activated by myosin kinase (D). Longitudinal cross-linked actin filaments (F) polymerized from G-actin subunits (G) at the cell periphery are clawed centripetally en-masse by interaction with the mechanochemical site (black spot) on the myosins (C). This drags centripetally myosin I molecules (I) at the dorsal surface, which are bound to membrane molecules (H) (or directly linked to the lipid bilayer) as well as to the actin core by their non-mechanochemical sites. This results in a force on the upper membrane (filled arrows) with a component directed towards the substratum. This component thins the cell periphery. Unengaged myosins are brought into the thinning zone by diffusion (J). We show the orientation of the longitudinal actin filaments with their "barbed" ends, where net polymerization takes place, associated with the membrane at the edge of the cell. This accords with the results of Mooseker and Tilney (1985) and Mooseker et al. (1982).

core assembly is attached to the dorsal membrane, perhaps by myosin I acting as a passive linkage via the non-mechanochemical site, the rearward motion of the transmembrane molecules by which it is attached will exert a force on the membrane that will have a component directed towards the substratum. The edge of the cell will therefore thin, to a constant value, in a rearward (centripetal) direction with respect to the substratum. Addition of G-actin at the tip of the lamella, at the growing end of the actin filaments, would ensure continuity of the thinning process.

This proposal embodies the major features of the observations on pre-formed non-advancing lamellae that we have outlined above. The basic plan can also provide a mechanism that is in accord with the most recently demonstrated facts relating to advancing lamellae. Suppose that the lower myosin I links act passively rather than mechanochemically; lamella protrusion is then accompanied (perhaps driven) by polymerization of actin at the leading edge. This would be accompanied by a forward flow of fluid cytoplasm, perhaps driven by hydrostatic pressure, carrying actin monomers to the edge. A forward flow of lipid (Lee et al. 1990) would accommodate the anteriorly displaced volume. In this case, the actin and all transmembrane molecules linked to it remain stationary with respect to the substratum as the lamella advances. Protrusion, versus stasis or retreat, might be controlled by switching the lower myosins between the passive-link and mechanochemical states, perhaps by reversible phosphorylation or the availability of ATP. In the absence of ATP, myosin I heads bind to actin, giving a passive link. If ATP is available, phosphorylation of the heavy chain in the head region increases the rate of ATP hydrolysis 20-fold without influencing the energy of actin binding at the mechanochemical site on the myosin head (Pollard et al. 1991). In addition, some forms of myosin I include a calmodulin binding site near the C terminus of the head, which might provide a further regulatory mechanism. If myosin I molecules can be functionally switched in this way, their role would resemble that of motor plus gear box.

One particular feature of this mechanical model requires comment. We have illustrated the actin and the lower (fixed) myosins as a polarized assembly. A recent report by Rinnerthaler et al. (1991) based on a combined video and EM study has enabled them to image negatively stained whole mounts of neutrophil...
lamellae that were known to have been fixed during active protrusion. These show an apparently randomly criss-crossed array of filaments. If this does represent the state of F-actin in vivo, it presents no conceptual problem for lamellar advance. Actin polymerized at the leading edge during protrusion will remain static with respect to the substratum if it is attached, directly or indirectly, to adhesion sites. This is quite in accord with our model. However, it is less easy to reconcile the results of Forscher and Smith (1988) on centripetal actin displacement in cytochalasin-treated non-advancing growth cones with the existence of apparently unpolarized actin. There is a similar difficulty for the centripetal formation of Dityostelium lamellae if actin is not arranged perpendicular to the local margin of the cell. In these cases, actin of the lamella could be imagined to move centripetally by virtue of an “intrinsinc” mechano-chemical interaction with myosin I associated with the lower membrane, or it could be pulled “extrinsically” by a tension-generating motor located in a more central part of the cell. This would be unlikely if centripetally moving actin is in fact depolymerized near the inner limit of the lamella (Wang, 1985). The results of Forscher and Smith (1988) rule out any possibility of the actin being pushed forwards from the cell edge.

Centripetal translocation of actin defines a local polarity or axis pointing away from the adjacent margin of the cell. If this motion is caused by an intrinsic myosin I-based motor within the lamella, three possible situations arise. (1) Myosins associated with adhesion sites are polarized so that their heads swing along the local axis. Those filaments of the adjacent actin network that happen to be aligned with the axis will be engaged, with the result that the whole crosslinked network will be dragged along the axis. (2) Myosins associated with adhesion sites are not polarized, but the adjacent actin filaments are. (3) Neither myosins nor the adjacent actins are polarized. In this case net progression of actin along the axis is not possible. One variant of (2), is that centripetal actin motion depends on the strongly polarized actin of growth cone filopodia (microspikes) interacting with fixed myosins, and that this pulls back the intervening unoriented actin network. It should be noted that Dityostelium lamellae develop branched structures somewhat similar to filopodia. Another variant of (2) is that while the bulk of the lamellar actin filaments do not point along the axis, those nearest to the membrane-associated myosins do. Linkage to those above would result in mass centripetal translocation. These suggestions should be experimentally testable.

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References


