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Cell adhesion in the life cycle of *Dictyostelium*

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Abstract. Three forms of cell adhesion determine the life cycle of *Dictyostelium: i)* adhesion of bacteria to the surface of the growing amoebae, as the prerequisite for phagocytosis; *ii)* cell-substrate adhesion, necessary for both locomotion of the amoebae and migration of the slug; *iii)* cell-cell adhesion, essential for transition from the unicellular to the multicellular stage. Intercellular adhesion has received the most attention, and fruitful approaches have been developed over the past 25 years to identify, purify and characterize cell adhesion molecules. The csA glycoprotein, in particular, which mediates adhesion during the aggregation stage, is one of the best defined cell adhesion molecules. The molecular components involved in phagocytosis and cell-substratum adhesion are less well understood, but the basis has been laid for a systematic investigation of both topics in the near future.
Key words. *Dictyostelium*; *Polysphondylium*; slime mold; cell adhesion; phagocytosis; cell differentiation; morphogenesis; immobilized ligands; membrane glycoproteins; cytoskeleton.

Introduction

A footnote in the history of biology: the momentous role of *Dictyostelium* in the study of cell adhesion

The ability of cells to adhere to each other is a prerequisite for metazoan organisms to exist. Since the pioneering experiments of Wilson[126], and Holtfreter[64], developmental biologists have realized the key role of cell adhesion in the shaping of embryos and in the maintenance of adult tissue organization, making adhesion one of the most intensively studied subjects in experimental biology[29,116]. However, major breakthroughs in the understanding of the molecular mechanisms underlying cell adhesion were not realized till the late seventies, when the first 'cell adhesion molecules' were identified and purified. This was the result of a radical change in the way of dealing with cell adhesion which was strongly influenced by pioneering studies on *Dictyostelium* cell adhesion.

Models involving specific cell surface molecules in adhesion had been already proposed in the late forties[117,123] and in modified form later[94]. However, the notion that 'cell adhesion molecules' exist was by no means unquestioned in the biology of that time. As a matter of fact, the experimental tools available made it very difficult to identify and purify cell surface molecules in sufficient amount to perform biological assays, and unequivocally prove the presumed role of a given molecule in cell adhesion. In addition, an alternative, influential view maintained that cell adhesion could be explained as resulting from the cohesive properties of the cell surface as a whole and not of a few specific 'cell adhesion molecules'[25,30,92]. The cell membrane, it was argued, is made up of a variety of lipids, proteins and carbohydrates, each of them inevitably contributing to building up a constellation of attractive and repulsive forces, the net value of which has to be different in different cell types; this could explain why cells are differentially cohesive.

The attraction of this theory was that by treating cells just as 'sticky particles' immersed in a hydrophilic milieu, it became possible to design quantitative bioassays of cell adhesion, and to assign to each cell population a cohesiveness value that enabled its adhesive behavior when mixed with different cell populations to be predicted. Steinberg's very elegant sorting out studies are the best example of this approach[106,107]. On the other hand, searching for specific 'cell adhesion molecules' within this theoretical framework was, to say the least, a sterile exercise, because it was obviously implicit in this approach that *any* change in protein or lipid composition would affect to a certain extent the adhesive properties of the cell surface.

In contrast, the major tenet of what may be called a 'naive biological view' of cell adhesion was that cell adhesion depended on the mutual interaction of only a minor subclass of membrane proteins and/or glycolipids expressed on the cell surface specifically to perform that function.

In the absence of selective tools, such as monoclonal antibodies, that allowed targeting of minor components of the cell surface, the burden of identifying and purifying 'cell adhesion molecules' was a cumbersome and very often unsuccessful task. Biochemical purification and quantitative adhesion assays required, in fact, large and homogenous cell populations from embryonic or adult tissues to start with; furthermore, the same tissues had to be first disaggregated either mechanically or

enzymatically, in both cases altering the properties of the cell surface, and thus introducing variables from experiment to experiment as well as among labs. Purifying membrane proteins such that they maintained their biological activity was not an easy task, and almost always required detergents which could affect the biological assay. The biological activity to be measured, and the bioassays for measuring it, were themselves ambiguous: what should one expect to be the 'activity' of a presumptive cell adhesion molecule in a cell adhesion assay, an inhibitory or a stimulatory one? How to detect unequivocally the one or the other or both with a given assay?

In this context, *Dictyostelium* offered many advantages as an experimental model: cells could be easily grown, and let develop, in large amounts in suspension cultures, and homogenous populations collected in a few days; moreover, cells were very little cohesive at the beginning of development, and started to adhere tightly a few hours after starvation[47]; this allowed for both a biochemical purification of developmentally-regulated 'adhesion molecules', without any need to enzymatically disaggregate cell tissues, and a genetic analysis of aggregateless, adhesion-defective mutants.

The potential of *Dictyostelium* as an experimental system was fully exploited by Gerisch and his coworkers, who devised an immunological strategy to the study of cell adhesion (for a still very instructive review of this early work see ref. 48). They first showed that the weak adhesion of growth-phase cells could be abolished by treating cells with EDTA, whereas this was not possible with cells at the aggregation stage, suggesting that a new EDTA-resistant adhesion system appears during development[47]. They argued that if aggregating *Dictyostelium* cells adhered through two different adhesion systems, one of which was developmentally regulated, it should be possible to identify the second one by raising a polyspecific serum against membrane particles of aggregating cells and making it specific for developmentally-regulated cell surface adhesion molecules by extensive absorption with growth-phase cells. The specific serum could then be applied in form of monovalent antibodies (Fab) in an adhesion-blocking assay to detect operationally-defined 'cell adhesion molecules' or 'contact sites', as they were called, that should neutralize the adhesion-blocking activity of the Fab[4]. The immunological strategy, systematically applied, was successful and led first to the definition of two classes of contact sites, the developmentally-regulated, EDTA-resistant A sites, and the non-developmentally regulated, EDTA-sensitive B sites[5]. A major breakthrough was showing that adhesion-blocking Fab reacted with a small subset of membrane antigens, amounting to maximally 10^5 molecules per cell. In contrast, covering the cell surface with Fab from an antiserum raised against heat-denatured membranes, that reacted with at least ten fold more binding sites, was not sufficient to block adhesion[6]. This clearly indicated that *i)* specific cell surface molecules, and not global membrane properties, were responsible for cell adhesion; and *ii)* such molecules could be detected, in carefully designed experiments, by an unambiguous quantitative bioassay, namely neutralization of adhesion-blocking Fab. A few years later, an 80 kDa glycoprotein that neutralized the contact sites A-blocking Fab was purified by K. Muller in Gerisch's laboratory[85].

These results were very influential in favoring the view that adhesion depends on specific cell adhesion molecules and establishing the immunological strategy as the most effective approach in the study of cell adhesion. Its application to higher eucaryotes led to the discovery of CAM's[17,29] and cadherins[46,66,118], and to the contemporary molecular studies of cell adhesion.

Neglected opportunities in *Dictyostelium* research on cell adhesion

Although *Dictyostelium* has been successfully used for studying the molecular basis of intercellular adhesion, its potential as an experimental model for adhesion studies has not been fully exploited. It is worth remembering that *Dictyostelium* cells grow as unicellular amoebae during the growth-phase of their life cycle, by ingesting bacteria. Phagocytosis involves, as the first step, bacterial binding to the cell surface of the amoebae and, very probably, self-nonself recognition. Little is known of the molecular mechanisms underlying this process, particularly whether or not specific receptors exist, and whether bacterial binding elicits signals leading to reorganization of the cytoskeleton, as it has been suggested occurs in other systems[7].

As mentioned above, the molecular basis of intercellular adhesion just before and during the aggregation phase has been intensively studied. Much less is known about the cell surface components mediating cell adhesion during the multicellular stage, and on the potential role of cell adhesion in the establishment of pattern formation and morphogenesis. This is partly due to the multicellular aggregate and the slug not being as easily amenable to biochemical analysis as aggregating cells. In addition, the prevailing notion is that pattern formation and morphogenetic movements in *Dictyostelium* depend mostly on diffusible signals, such as cAMP, DIF and ammonia, with cell-cell adhesion playing just the passive role of keeping the cells together (for recent reviews see references 53, 97). Thus, cell adhesion at later stages of development has not received much attention by *Dictyostelium* researchers.

Similarly, cell adhesion to the substratum and the interplay between adhesion and motility have only recently started to be studied systematically. Obviously, cell locomotion requires adhesion to the substratum: cells must adhere strongly enough to gain the traction neces-

sary for movement, but not too strongly, otherwise they would become stuck, or too weakly, else they would slip. Many tissue culture cells behave so, and an inverse correlation exists between the number of focal adhesions and locomotion[75]. *Dictyostelium* cells are highly motile, ameboid cells; in addition, several genes for both cytoskeletal and adhesion proteins have been identified and are easily amenable to disruption[26,98]. Therefore, *Dictyostelium* could be the best candidate for studying the mutual relationship of adhesion and contractility, and there is some indication that this subject will be developed in the next few years[66a,97a,99,121a].

In the next sections, we will first review the larger literature on cell adhesion in *Dictyostelium* and then discuss the relatively few data concerning phagocytosis and cell-substratum adhesion.

Intercellular adhesion during development

Developmental changes in cell adhesiveness

Development of *D. discoideum* is accompanied by discrete changes in the adhesiveness of cells. Growth-phase cells are barely cohesive: on agar or glass they adhere to each other only transiently, whereas in shaken suspension they form loose aggregates, which are easily dissociated with 1–2 mM EDTA or EGTA.

During aggregation, cells adhere through two different classes of adhesion molecules, which can be discriminated by their sensitivity to EDTA as well as serologically[4,5]. In addition to the EDTA-sensitive form of adhesion, which persists throughout development, a new EDTA-resistant adhesion appears shortly before cells enter the aggregation stage[5]. Aggregating cells assume an elongated shape and form streams; in streaming cells, EDTA-stable cohesiveness is preferentially restricted to polar cell-cell contacts, whereas the lateral cell-cell contacts are EDTA-sensitive[5].

At the end of aggregation, the aggregates undergo compaction and develop a tip. Compact and tipped aggregates are more difficult to dissociate with EDTA than aggregation-stage cells; both serological and genetic evidence suggest that the molecular basis of EDTA-stable cohesiveness changes between aggregation and the tipped mound stage[108,124].

When slugs are formed, a new change becomes manifest: the previously homogenous cell population now shows a biomodal distribution in the EDTA-resistant cohesiveness, which reflects differentiation into prespore and prestalk cells. Prespore cells are much more resistant to EDTA dissociation than prestalk cells[70], though other lines of evidence indicate that prestalk cells are more cohesive than prespore cells[114,127].

In conclusion, during development of *Dictyostelium* we can distinguish at least four different adhesion systems, three of which are EDTA-resistant (fig. 1). Cell adhesion molecules mediating one or the other adhesion system have been detected, basically with the immunological approach pioneered by Gerisch and coworkers.

Membrane glycoproteins involved in EDTA-sensitive cell adhesion

Two glycoproteins of 126 and 24 kDa have been suggested to mediate EDTA-sensitive adhesion. Gp126 was found to neutralize the adhesion-blocking activity of a rabbit antiserum raised against growth-phase cells[21]. Rabbit antibodies raised against the purified glycoprotein inhibited cell adhesion and phagocytosis, suggesting that the glycoprotein could be involved both in EDTA-sensitive cell-cell contacts and in bacterial attachment to *D. discoideum* cell surface[20,21]. Unfortunately, the glycoprotein has not been further characterized.

The second glycoprotein, gp24, was the major component of a membrane fraction which neutralized adhesion-blocking Fab raised against a *modB*-mutant defective in the expression of csA (see next section). The antibody completely blocked adhesion of a wild-type strain at preaggregative stage, but only partially at aggregation stage[69].

Synthesis of gp24 correlates well with the appearance of contact site B expression[69], although it has recently been suggested that EDTA-sensitive adhesion is mediated by two types of adhesion sites, which are differentially affected by EDTA or EGTA[38]. The first type, equally sensitive to both chelators, is already present in cells developed for two hours, whereas the second type appears between two and ten hours of development, is sensitive to EDTA only, and can be rescued by Mg^{2+} in the presence of EDTA. Gp24 appears to be identical with the first class, as suggested by the fact that a dodecapeptide similar to a carboxyterminal region of gp24 inhibits EGTA-sensitive, but not EDTA-sensitive, adhesion[38].

EDTA-resistant adhesion during the aggregation stage and the csA glycoprotein

As mentioned in the Introduction, EDTA-resistant adhesion during aggregation is mediated by an 80 kDa glycoprotein, called csA (contact site A). Since its discovery[85], csA has been one of the most intensively studied cell adhesion molecules. The glycoprotein was identified and purified based on its ability to neutralize Fab from a polyspecific antiserum that blocked the EDTA-resistant form of adhesion[85]. A rabbit antiserum as well as some monoclonal antibodies raised against the purified glycoprotein also blocked this form of adhesion[86,101].

Despite the overwhelming acceptance nowadays of the immunological strategy as a tool for the molecular analysis of cell adhesion, it is worth remembering that the definition of contact sites as the target site of adhe-

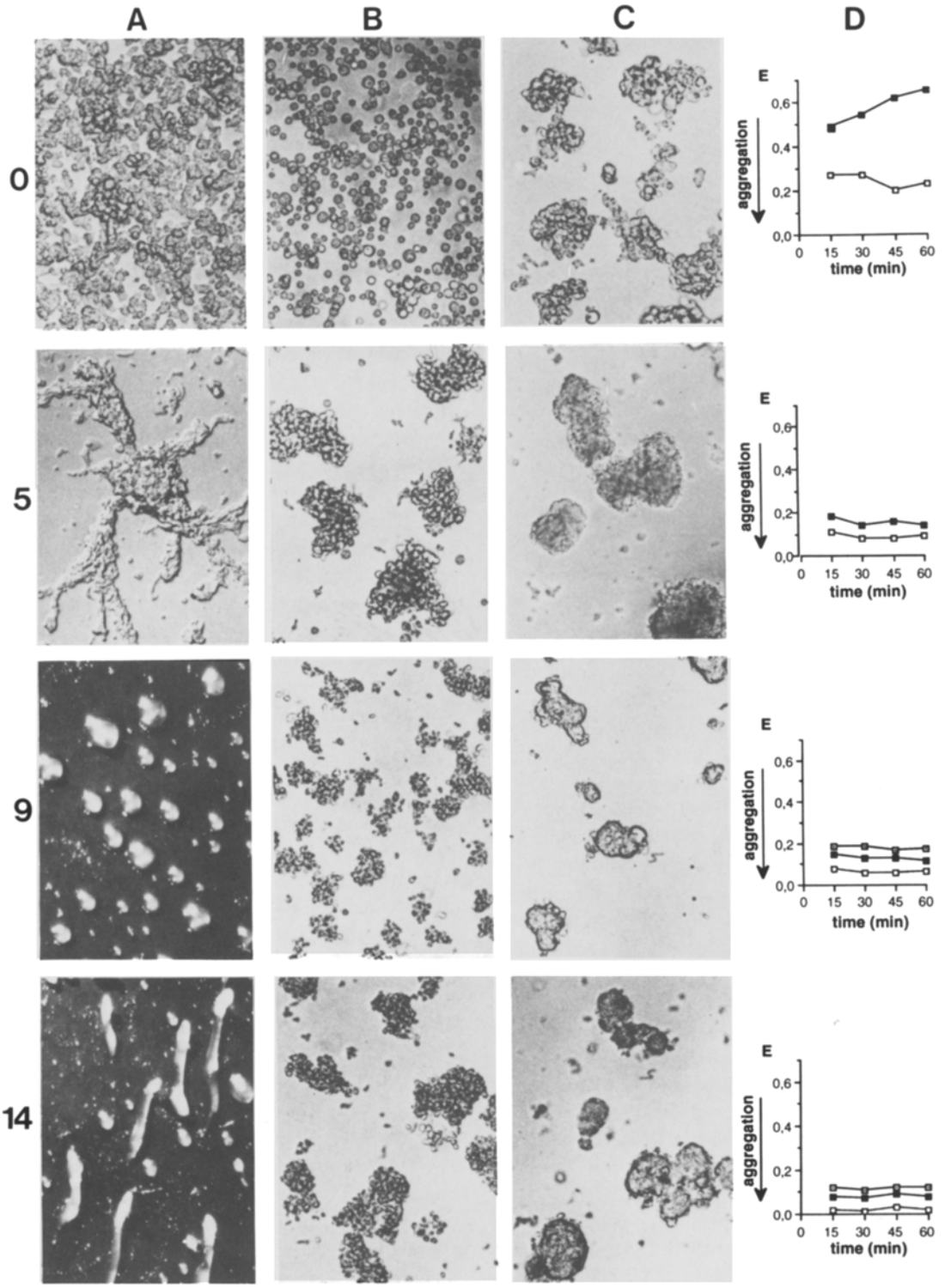

Figure 1. EDTA-sensitive and EDTA-resistant adhesion during development of *D. discoideum*.
A) Different developmental stages of *D. discoideum:* amoebae at the beginning of development (0 h) and during aggregation (5 h); mounds (9 h); migrating slugs (14 h).
B, C, D) At the indicated time of development *(*left*)*, cells were tested for adhesion either in the absence (□) or presence of (■) 10 or (⊡) 20 mM EDTA, and the formation of aggregates measured in the agglutinometer of Beug and Gerisch[5]. The quantitative data are shown in *D;* the morphology of cells or aggregates at the end of the incubation in the agglutinometer are shown in *B* (10 mM EDTA) and *C* (no EDTA). EDTA-resistant adhesion appears before cells enter the aggregation stage, and persists thereafter. Note the compact morphology of the aggregates at mound and slug stage in the absence of EDTA.

sion-blocking antibodies is merely operational[48]. The activity of neutralizing adhesion-blocking antibodies is suggestive of a potential role in adhesion, but is no proof that a given antigen is a 'cell adhesion molecule', i.e. that it is the actual link between cells. For this, additional lines of evidence are required, the most convincing being a genetic one.

In the case of csA, it has been shown that *i)* disruption of the gene by homologous recombination results in aggregating cells with drastically reduced EDTA-resistant adhesion[55]; *ii)* constitutive expression of csA results in cells forming EDTA-stable aggregates while still growing in nutrient medium[32]. Thus, csA is one of very few proteins for which there is compelling evidence for a direct role as a 'cell adhesion molecule' (fig. 2).

The csA glycoprotein appears on the cell surface shortly before aggregation and disappears slowly after tight aggregate formation. Its activity, therefore, seems to be restricted to the aggregation stage only. As will be discussed later, csA mediates cell adhesion in a homophilic manner, thus disruption of the gene completely abolishes any csA contribution to EDTA-resistant adhesion. In csA-null mutants, however, EDTA-stable contacts are drastically reduced, but not totally absent[55]. This raises the question of whether 'adhesion molecules' other than csA are involved in EDTA-resistant adhesion during the aggregation stage and what could be the functional relationship between these putative molecules and csA. The residual EDTA-resistant cohesiveness detected in null mutants could be due to postaggregative adhesion molecules, assuming they start to be expressed at low level during aggregation. Alternatively, aggregation-competent cells could possess other molecules, in addition to csA, responsible to a lower degree for EDTA-stable adhesion. The availability of csA-null mutants should allow us to investigate this question.

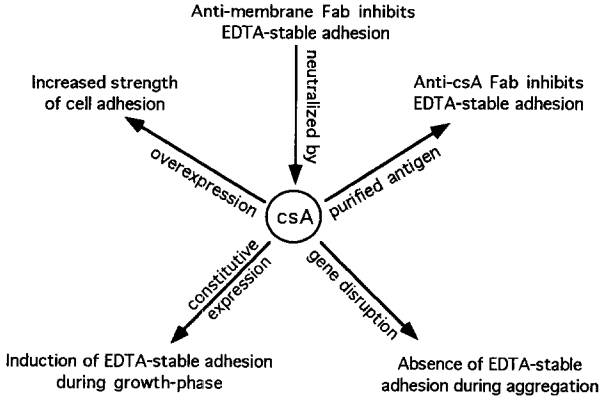

Figure 2. A summary of the methodological approaches and different lines of evidence suggesting that the csA glycoprotein is a cell adhesion molecule. For explanation and references see text.

EDTA-resistant adhesion systems at postaggregative stage: gp95 and gp150

After formation of tight aggregates, EDTA-stable adhesion persists, but is related to new adhesion sites specific of postaggregative stage cells[108,124]. Two different glycoproteins, gp95 and gp150, have been suggested to take over the adhesive function of csA.

Gp95 shares with csA antigenic determinants that react with wheat germ agglutinin (WGA)[96]; the glycoprotein was reported to neutralize an Fab preparation that specifically blocked adhesion of tight aggregate cells[108]. The Fab was derived from an antiserum raised against slug-stage plasma membranes and preabsorbed with aggregation-stage cells to remove any activity against csA and other antigens common to both stages. A membrane fraction enriched in gp95 restored cohesiveness in a temperature-sensitive mutant[19,96]. Gp95 has not been purified to homogeneity and the encoding gene has not been cloned.

The other glycoprotein, gp150, is a Concanavalin A (Con A)-binding protein present at low level in vegetative cells and accumulating during development, with maximal expression at ten hours of development[45]. Fab against the glycoprotein inhibited adhesion of tip and slug stage cells, and to a lesser degree of aggregation stage cells[45,71,74]. At slug stage, the Fab blocked more efficiently prespore than prestalk cell adhesion[71]. The involvement of gp150 in adhesion is a matter of debate. The antiserum against gp150 was found to crossreact with csA, and its adhesion-blocking activity could be completely absorbed with csA[74]. The purified glycoprotein, on the other hand, inhibited adhesion when added to preculmination stage cells[44]. Since the protein is purified, it should now be possible to obtain genetic evidence that gp150 acts as adhesion molecule.

Properties of the csA glycoprotein and mechanism of csA-mediated adhesion

Of all the putative adhesion molecules involved in intercellular adhesion, only csA has been investigated in detail. We will therefore discuss the biochemical properties of csA and their relevance for the biological activity of the glycoprotein.

csA mediates homophilic adhesion

It is established that csA mediates adhesion in a homophilic manner. The evidence for this is as follows: *i)* Covaspheres coated with purified csA form small aggregates in suspension, besides binding to cells in a stage-specific manner; in addition, ^{125}I-labeled csA binds specifically to csA immobilized on a nitrocellulose filter[100]; *ii)* glutaraldehyde-crosslinking of cell mixtures expressing different forms of csA does not reveal any protein other than csA molecules bound to each other[34]. In addition, when cells expressing constitutively csA are

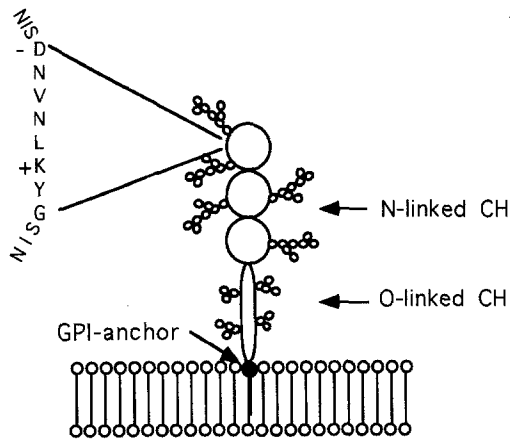

Figure 3. Structural features of the csA glycoprotein.
The amino-terminal region consists of three homologous globular subdomains, containing five N-glycosylation sites. Two of these flank the putative binding site mediating homophilic adhesion, and whose sequence is shown on the left. The hydrophilic, carboxy-terminal domain is rich in Pro-Thr/Ser and is O-glycosylated. The glycoprotein is anchored to the phospholipid bilayer through a ceramide-based GP-anchor. See text for details.

mixed 1:1 with wild-type growth-phase cells in the presence of EDTA, 50% of the cells remain single, and these are the only cells in the mixture that do not bind fluorescently-labeled mAb specific for csA (Bozzaro, unpublished observations). These results indicate that csA also interacts homophilically in vivo, ruling out both non-developmentally- or developmentally-regulated ligands that would allow for heterophilic EDTA-resistant adhesion.

The active site mediating homophilic binding has not been unequivocally identified. After removal of a 19 amino acid-long leader sequence the mature glycoprotein consists of 495 amino acids[89]. N- and O-linked carbohydrate chains contribute about 30% to the apparent M_r of 80 kDa. Three major structural domains have been identified in the protein[102], as shown in figure 3: a short hydrophobic domain in the carboxyl terminus, which is posttranslationally cleaved and substituted by a ceramide-based phospholipid anchor[95,105]; adjacent to it a hydrophilic, Pro/Ser/Thr-rich segment of about 50 amino acids, reminiscent of the 'hinge' region of immunoglobulin and of the cytoplasmic domain of N-CAM[78,89]; this extends out of the membrane and supports the third, large amino-terminal domain, consisting of three globular subdomains with relatively high degree of amino acid sequence homology[102], and, again, some structural similarity to N-CAM and immunoglobulins[78].

Kamboj et al.[68] tested the cell binding activity of fusion proteins containing different segments of the csA polypeptide, and restricted the homophilic interaction site to the segment between Val-123 and Glu-172. By using synthetic oligopeptides in competition experiments, they identified an octapeptide within this region that inhibited EDTA-stable adhesion and csA binding to the cell surface (fig. 3). The two charged residues Lys-133 and Asp-138 were proposed to be directly involved in the binding, since an exchange of the position of Lys and Asp led to a total loss of activity[68]. The latter result has been questioned by Faix[34], who found that substitution of the charged amino acids with uncharged ones did not relevantly affect csA activity.

Oligosaccharide chains and adhesion

The octapeptide region is flanked by two of five N-glycosylation sites present in the polypeptide backbone, which are all concentrated in the globular amino-terminal domain. There is evidence that all five sites are glycosylated[34]. The N-glycosidic chains are added co-translationally in the endoplasmatic reticulum, converting the protein into a 68 kDa precursor, which is further O-glycosylated in the Golgi[62]; the O-linked carbohydrates are attached to the hydroxyamino acid-rich hinge region. In the Golgi, the N-glycosidic chains are also fucosylated and sulphated[63,104]. The N- and O-linked oligosaccharide chains react, respectively, with Concanavalin A or wheat germ agglutinin (it is worth mentioning that WGA also binds to N-linked carbohydrates present in other glycoproteins, but not in csA). The O-linked oligosaccharides are highly antigenic, and they are recognized by a variety of monoclonal antibodies, which do not react with the N-linked oligosaccharides[3,12]. The purified glycoprotein contains D-mannose, N-acetylglucosamine, D-glucose and L-fucose[84], but the exact structures of the glycosidic chains have not been determined. The N-glycosidic chains are probably of the high-mannose type[41,57], whereas the O-linked sugars contain N-acetylglucosamine, mannose and, possibly, glucose[12].

N- or O-glycosylation are necessary for transport of the protein to the cell surface and for its stability to proteases[58,62], but not essential for mediating adhesion. In fact, EDTA-stable contacts are formed by *modB*-mutants defective in O-glycosylation[51,62,86a], as well as transformed cells in which all five N-glycosylation sites of the csA polypeptide have been inactivated by site-directed mutagenesis[34].

The latter result is prima facie in contradiction with earlier experiments, in which N-glycosylation was blocked with doses of tunicamycin low enough that synthesis and O-glycosylation of csA were presumably not blocked[60,90]; the tunicamycin-treated cells failed to form EDTA-stable contacts. However, examination of the published data shows that, under the conditions used, expression on the cell surface of the N-glycosylated form of csA (a 66 kDa glycoprotein) amounted to less than 30% of the mature csA present in control cells[61], both because of impaired transport to the cell

surface[62] and proteolytic degradation of the exposed glycoprotein[58]. This can explain the strong inhibition in EDTA-resistant adhesion observed by several authors in tunicamycin-treated cells[72,79,90,109].

Although the oligosaccharide chains are not essential for mediating adhesion, it cannot be excluded that they play a modulatory role which may be relevant in the actual process of aggregation. Two other reports, in fact, are worth mentioning that implicate glycosidic chains in the process of cell-cell adhesion. Hirano et al.[59] found that mannosidase-treated cells displayed reduced EDTA-resistant adhesion, whereas Ziska and Henderson[129] reported a specific inhibition of EDTA-stable adhesion by some membrane glycopeptides, containing mannose, released by pronase treatment. These results are suggestive of a carbohydrate role in EDTA-resistant adhesion that demands further investigations. It would be interesting to know whether the active glycopeptides bind to csA, or whether they may be responsible for the remaining EDTA-resistant adhesion observed in csA-null mutants.

Short-term modulation of csA activity

Time-lapse movies of aggregating cells reveal that cell adhesion is a dynamic process, with cells in both streams and aggregates undergoing rapid shape changes and movements, and adhering and de-adhering continuously. Streaming cells attached at their polar ends can be induced to change their polarity rapidly and loose their end-to-end contacts by stimulating them with a local gradient of cAMP.

These observations, and the finding that during stream formation csA is evenly distributed all along the cell surface while EDTA-stable adhesion is restricted to the polar ends of the elongated cells, suggest the existence of modulatory controls of adhesiveness. It is conceivable that a local threshold concentration of csA molecules is necessary to trigger or stabilize adhesion, and indeed enrichment of csA in contact regions has been reported[23]. The same contact regions are enriched in actin and a 30 kDa actin-bundling protein[35], suggesting a possible regulation of csA clustering by the cytoskeleton. The absence of a cytoplasmic domain in the csA glycoprotein rules out a direct connection of csA with cytoskeletal elements, but this could be mediated by other transmembrane proteins interacting laterally with csA molecules. The phospholipid anchor, on the other hand, increases the residence time of csA on the cell surface[2] and renders csA highly mobile along the cell surface, thus favoring clustering[36] and, possibly, stabilizing adhesion.

Sulphated carbohydrates attached to the glycoprotein are not essential for the adhesive function of csA, but they could be potential targets of short-term modulation of activity, assuming that reversible sulphation or exposure of sulphated residues occurs[104]. Evidence of this is, however, lacking.

Is the csA glycoprotein redundant? What is the actual role of csA?

Expression of the csA glycoprotein is stringently regulated: the protein is not expressed during growth, and the coding gene starts to be transcribed shortly after starvation[34,77]. With the onset of cAMP signaling, transcription is strongly enhanced[34,77,89]. Several lines of evidence indicate that cAMP pulses stimulate csA accumulation[49]. Mutants defective in adenylate cyclase activation, such as *synag 7* or HSB1, accumulate about 20–30% of the glycoprotein compared to wild type cells, but can be induced to full expression by exogenous cAMP pulses[11,77]. Therefore, csA appears to be subject to a bimodal regulation, the first signal linked to starvation and the second to cAMP binding to membrane receptors[83]. Both signals act at the transcriptional and not at the posttranscriptional level, as shown by run-off transcription experiments with isolated nuclei as well as by measuring the stability of the mRNA coding for csA (Bogliolo, Ceccarelli and Bozzaro, unpublished data).

Formation of tight aggregates results in rapid suppression of csA transcription, though the protein disappears only slowly from the cell surface due to its long half-life[2]. Disaggregation of tight aggregates re-induces high-level transcription of the gene within 30 min (Morandini, Ceccarelli and Bozzaro, unpublished data). Therefore it appears that the csA gene is subject to a stringent regulation which restricts its expression to the aggregation stage.

This favors the idea that csA plays a crucial role during aggregation, which is contradicted, however, by the finding that null mutants for csA aggregate quite normally on agar[55]. The same mutants are unable to form EDTA-stable contacts in shaken cultures, thus suggesting that csA increases the strength of adhesion but is dispensable for aggregation and normal development, at least under laboratory conditions. The adhesion strength induced by csA may be crucial, however, for normal development in the natural habitat.

We propose that csA could be primarily involved in favouring species-specific adhesion during aggregation. Since the early experiments conducted by Raper and Thom[93], data from several laboratories have shown that cells from different species of *Dictyostelium* form species-specific aggregates when mixed together or with related *Polysphondylium* species[10,88,109]. Sorting out depends not only on cells being attracted by different chemoattractants, but also on species-specific differences in cell adhesion[10,50,82,88,109].

We showed several years ago that aggregation stage cells, but not growth-phase cells, of *D. discoideum* and

Table. The csA glycoprotein mediates cell sorting out.

Time of starvation (h)	Combination of strains tested	Total sorting out	Partial sorting out (% of total)	No sorting out
0	D.d. + P.p.	0	10	90
5	D.d. + P.p.	100	0	0
0	D.d. TWT + P.p.	80	20	0
5	D.d. T10 + P.p.	10	90	0
0	D.d. + D.d. TWT	80	20	0
5	D.d. + D.d. T10	80	20	0

Sorting out experiments were performed by mixing cells of the strains indicated at the beginning of development (0 h) or at the aggregation stage (5 h), and shaking the mixtures for 30 min at 100 rpm. At the end of incubation, the aggregates were fixed and fluorescently labeled with monoclonal antibodies specific either for *P. pallidum* (mAb 293) or *D. discoideum* (anti-csA mAb 448). D.d.: *D. discoideum* AX2; P.p.: *P. pallidum* WS320; TWT: AX2 transformed to express constitutively csA[33]; T10: AX2 null-mutant for csA[55].

P. pallidum sorted out when mixed in shaken suspension, and under conditions which ruled out chemotaxis[10,50]. Sorting out could be abolished if cells were pretreated with polyclonal Fab specific for aggregation-stage membrane antigens of both cell types[50]. These experiments were interpreted as evidence that species-specific adhesion was mediated by aggregation-stage specific cell-adhesion molecules.

Recent experiments by using null mutants for csA or transformants constitutively expressing csA have confirmed this hypothesis (table). *D. discoideum* cells, aggregation competent but deficient in csA, show a strongly reduced sorting out when mixed with *P. pallidum* cells. Transformants expressing csA during growth undergo species-specific sorting when mixed with *P. pallidum* growth-phase cells, in contrast to the control (Ponte, Faix, Bozzaro, unpublished observation). These cells sort out even when mixed with growth-phase cells of the same species (table). Clearly, the expression of csA favours selective cell type or species-specific adhesion.

The specificity observed in the sorting out experiments may play a relevant biological role during aggregation in the natural environment, since amoebae of different species can live together and feed on the same bacteria. Species-specific chemotaxis is not sufficient to ensure species-specific aggregation, as cAMP is shared as chemoattractant by different Dictyostelids, and is also secreted by species such as *D. lacteum* or *P. violaceum*, that do not react chemotactically to it[97]. A species-specific form of adhesion would therefore act as a double-insurance mechanism for the formation of species-specific aggregates. If csA mediates species-specific adhesion, then it is plausible for its expression to be restricted to the time of aggregation, as once aggregates are formed there is no need for a specific 'self-recognition' mechanism.

If the hypothesis is correct one should expect to find cell adhesion molecules specific for the aggregation stage in other Dictyostelids. In *P. pallidum*, indeed, two classes of contact sites have been described, one of which, cs2, is specific for aggregation[10,15]. The expression of cs2 could explain why sorting out in mixing experiments of aggregation-stage *P. pallidum* and *D. discoideum* csA-null mutants is reduced, but not totally abolished (table).

Cell adhesion and the control of morphogenesis

Although intercellular adhesion is clearly necessary for aggregate formation and maintenance of the multicellular state, its role as a morphoregulatory factor in differentiation and proportioning of prespore/prestalk cells and in pattern formation within the developing organism remains to be established. It is possible, in fact, that adhesion plays just the passive role of keeping cells in close proximity, and that diffusible signals, which are secreted by the cells in the course of development, act as the only 'morphoregulators' responsible for cell differentiation and sorting out. Cyclic AMP, DIF and ammonia are examples of such molecules, which have been shown to be capable of regulating cell type-specific gene expression and cell differentiation under conditions in which cell adhesion was inhibited[8,54,77,87,97]. Usually, however, high concentrations of these molecules have to be used for being effective, and this raises the question of whether the necessity for cell adhesion is not simply bypassed by such non-physiological concentrations.

Adhesion can elicit signals

Evidence that cell contacts may act as signals during development of *Dictyostelium* is suggested by a few scattered reports. Fontana and Price[39] have provided evidence that cell-cell contact is required for cAMP secretion and relay. Interestingly, they found that cAMP secretion can be induced by any mechanical contact, such as cell-cell or cell-latex beads adhesion; in contrast, cAMP relay was affected only by homologous cell-cell contacts or heterologous interactions, such as bacteria-cell binding.

Mehdy and Firtel[83] reported that cell-cell contacts, which can be replaced by non-specific cell surface interactions, are necessary for prespore gene expression, in addition to cAMP and conditioned factors.

Blocking gp24 with antibodies inhibited postaggregative morphogenesis of the *modB* mutants used to generate the antiserum: cells aggregated by chemotaxis and formed loose aggregates, but further development was blocked. Production of antisense mRNA also delayed development[73].

We have used defined ligands covalently bound to an inert, flat surface to study the effects of cell attachment on development[14]. Cells bound strongly to polyacry-

lamide gel surfaces derivatized with glucose, N-acetylglucosamine and mannose. Cell interaction with the immobilized glucoside, but not with the other two sugars, froze development at the transition point from loose to compact aggregates, though motility and chemotaxis were not impaired; postaggregative gene expression was also blocked[13,14]. Stalk, but not spore, differentiation of isolated, single cells was inhibited on glucoside gels, and the block relieved by nanomolar concentrations of DIF[43]. Interestingly, DIF favoured also compaction of aggregates on glucoside gels[43]. These results and the previous ones raise the possibility that cell contacts may regulate developmental gene expression and morphogenesis by locally altering cAMP and DIF levels, their signalling or the cell response to them.

Overexpression of csA, under a constitutive promoter or its own promoter, allowed for some interesting observations related to the role of cell adhesion molecules in morphogenesis: *i)* in contrast to wild-type cells which in axenic medium do not develop even if they enter the stationary phase, aggregates were formed by csA-overexpressing cells in axenic medium, and the cells expressed some developmentally-regulated genes[32]. The csA effect on gene induction under these conditions is probably indirect, as it is likely due to cells in the core of the aggregates being starved; the results nevertheless show that adhesion modifies the cell environment in a way that is relevant for development; *ii)* overexpression of csA under its own promoter resulted in large, stippled streams, which fragmented into small tight aggregates, that developed eventually into small fruiting bodies[33]. Whether fragmentation is the direct result of the strong cohesion or whether increased adhesion somehow interferes with cAMP signalling and/or chemotaxis remains to be established.

Is adhesion a morphoregulatory signal in *Dictyostelium* morphogenesis?

It is well established that prestalk and prespore cells differentiate randomly in the aggregates, and sort out in response to a combination of environmental stimuli and endogeneous signals (references 9, 110, 113, 125; for a recent review see reference 53). The factors inducing the initially random differentiation are not known, but biases appear to exist from the end of growth[76,81,122]. Apparently, cells starved early in the cell cycle have a tendency to develop earlier than cells starved late, both concerning their sensitivity to cAMP and the appearance of chemotaxis-associated proteins as well as EDTA-stable contacts[80,91,121]. The same cells preferentially enter the prestalk pathway. It is conceivable that quantitative differences in the adhesive properties of the cells introduce local microheterogeneities in the aggregate, which lead to the initially random differentiation of prestalk and prespore cells. However, how these differences are mechanistically linked with cell differentiation is not known.

Once differentiated, prestalk and prespore cells sort out in different areas of the aggregate. Sternfeld and David have provided evidence that sorting out is enhanced by differential chemotaxis to cAMP between prestalk and prespore cells[110]. Their study also shows that the initial asymmetry in the distribution of prestalk vs. prespore cells cannot be explained solely by an endogenous cAMP gradient, but requires additional signals.

A morphoregulatory role for cell adhesion in the sorting out has been proposed by several authors[65,70,91,114], based on observations suggesting differential adhesiveness to exist between prestalk and prespore cells. However, there are conflicting reports as to whether prespore or prestalk cells are more cohesive, possibly due to the fact that cells of different stages (first finger, slug migration or late culmination) were studied. If so, this indicates that quantitative or qualitative changes in cell adhesion during postaggregative development are probably more frequent than we had so far assumed. To confirm this, a systematic study of the molecular basis of cell adhesion during morphogenesis is required, and the availability of sophisticated molecular genetic techniques should facilitate this task. Thus, REMI could be used to search for mutants defective in adhesion during postaggregative morphogenesis, whereas the adhesive properties of specific subpopulations within the slug could be altered by ectopic expression of known cell adhesion molecules, such as csA, or even heterologous proteins, under the control of regulated promoters.

Heterotypic adhesion and phagocytosis

Dictyostelium cells are natural phagocytes, which ingest bacteria as source of food. Phagocytosis involves recognition and binding of a particle by the phagocyte. Binding triggers a transmembrane signal which leads to circumferential attachment of the particle by pseudopodial movement and subsequent internalization by membrane fusion[52,111]; alternatively, particle binding can induce membrane ruffles which direct engulfment[40].

Phagocytosis in *Dictyostelium* has been the subject of few studies, and little is known about the membrane components mediating bacterial binding. By screening for the ability to bind tungsten beads, Vogel et al.[119] isolated phagocytosis mutants which failed to ingest hydrophobic beads, but were still capable of binding and internalizing *E. coli*. Bacterial binding was however inhibited by glucose, leading to the hypothesis that phagocytosis in *Dictyostelium* is mediated by a glucose-binding receptor and by hydrophobic interactions. The latter are probably mediated by more than one membrane component, as the defect in the isolated mutants could be assigned to two different loci[28]. Remarkably,

the mutants were also defective in EDTA-labile adhesion and in cell binding to glass or plastic.

Similar pleiotropic defects have been found in phagocytosis mutants selected for their inability to adhere to immobilized carbohydrates[18], or to ingest fluorescent bacteria[24]. It is likely that the mutations affect either the cytoskeleton or signal transduction elements reponsible for initiating bacterial recognition, but not components involved in bacterial binding.

The possibility that bacterial adhesion is mediated by the same adhesion molecule(s) involved in EDTA-labile adhesion has been raised by Chadwick et al.[20], and would be consistent with the finding that most of the phagocytosis mutants selected so far are also defective in EDTA-sensitive cell contacts. However, the antisera that blocked both adhesion and phagocytosis, raised by Chadwick et al.[20], and independently by Chia et al.[22], were found to react with carbohydrate determinants shared by several membrane glycoproteins[22]. Thus, a specific target cannot be unequivocally identified.

Con A- and WGA-binding sites have been implicated in phagocytosis of both bacteria and yeast, although only for the WGA-binding sites has a suggestive correlation with specific ingestion of yeast particles been found[56].

Further studies are necessary to unravel the molecular basis of phagocytosis in *Dictyostelium*, and to understand the possible relationship of phagocytosis with the initial steps in cell-cell adhesion and in self-versus-non-self recognition. In this context it is worth remembering that slime mold species are usually not predatory or cannibalistic, with the notable exception of *D. caveatum* and its mutants, in which self recognition is, respectively, reduced or totally defective, leading eventually to cells feeding on each other[103].

Cell-substrate adhesion

Dictyostelium amoebae adhere to, and move on, a wide variety of substrata, including agar, glass, cellulose filters, bacteriological and tissue culture plastic. The strength of adhesion increases during development, and is higher for prestalk than for prespore cell[127]. Initial binding to the substratum may involve anionic or cationic sites on the cell surface and hydrophobic interactions, depending on the substratum. Extracellular material accumulates with time as a result of membrane shedding or active secretion by the cells, but it is not clear whether this material acts as an 'extracellular matrix' that mediates cell-substrate focal adhesion and provides attachment for ordered cell motility. The claim that discoidin, a lectin constituting about 1% of intracellular protein in aggregating cells, is secreted and serves as migration trail[103] has not been confirmed. Discoidin is neither released extracellularly nor expressed on the cell surface[1,31]. The finding that RGD-containing synthetic peptides affect cell attachment and spreading on plastic[103] may suggest that *Dictyostelium* cells possess analogs of fibronectin, but they do not belong to the discoidin family[1]. A preliminary report that integrin-like molecules exist in *Dictyostelium*[42] has not been further substantiated (Freeze, personal commun., Faix, personal commun.; Peracino and Bozzaro, unpublished results). The search for such molecules should however continue, especially in view of the presence of cytoskeletal proteins such as talin in *Dictyostelium*[27].

As already mentioned, we have used a chemically-defined surface to study the effect of cell-substrate binding on motility and aggregation, by covalently immobilizing carbohydrates to polyacrylamide gels. Cells bound strongly to glucosides, and to a lesser extent to N-acetylglucosaminides and mannosides[14]. On glucoside gels, larger aggregating streams were formed and aggregation proceeded more slowly in comparison to other immobilized sugars; chemotactic orientation was not blocked, but cell speed slightly reduced[37]. Compaction of aggregates was inhibited on glucoside gels, but because of some specific signalling triggered by cell interaction with the immobilized sugar, and not because of inhibition of cell speed or motility[43].

Overexpression of the csA glycoprotein has been reported to give rise to large streams which break down into small aggregates that develop into small slugs and fruiting bodies[33]. Both orientation to cAMP and cell speed were reduced in csA-overexpressing cells (Faix, personal commun.), but whether this is due to increased cell-substratum adhesion remains to be demonstrated.

Changes in cell shape and projection of membrane protrusions, such as filopodia or pseudopodia, depend primarily on the organization of the cytoskeleton, and they occur even in cells developing in shaken suspension. Preliminary results, studying attachment to a substratum and movement of defined null-mutants for cytoskeletal components with reflection interference contrast microscopy, confirm these observations. During locomotion cell attachment to a substratum is restricted to small focal points of the cell surface, but the size of contact area is subject to variation within minutes, suggesting that the control of adhesion is intrinsic to the cell[97a]; contact to the substratum is not necessary for protrusion of the front region of the amoeba in response to chemoattractant, but formation of a new contact point is required for cell detachment and further movement[121a]. Interestingly, defects in cytoskeletal components affect in different ways formation of focal contact points: compared to wild-type cells, smaller contact areas are observed in a triple mutant lacking two actin cross-linking, and one actin-fragmenting, proteins[121a], whereas larger contact areas are observed in myosin II-null mutants, probably because of reduced cytoskeletal tension[99]. The net result on cell motility is, however, difficult to predict because of compensatory effects, par-

ticularly on strong adhesive surfaces: motility and changes in cell shape are not affected by strong attachment to a substratum, except for myosin II-null mutant cells which show reduced motility, probably because myosin II is involved in retraction of the cell's rear edge[66a, 99, 121a].

Taken together, these results suggest that *Dictyostelium* amoebae are largely autonomous in regulating adhesiveness, motility and shape changes over a wide range of adhesion strength to a substratum. This conclusion is supported by recent data measuring cell spreading and motility in relation to different degrees of cell-substratum adhesion[66a]. It would be interesting to know whether some form of cytoskeletal organization, similar to the one found in cell-cell contact regions[35], is also present in cell-substrate focal adhesions, as reported for mammalian cells[75], and whether the cytoskeletal components and/or organization at focal contacts differ when cells bind to an inert substratum through unspecific binding sites, or through specific membrane binding sites, as in the binding to immobilized carbohydrates or csA.

Overview and future directions

The different facets of cell adhesion in the life cycle of *Dictyostelium* have been reviewed in some detail, with an emphasis on the experimental opportunities offered by the model, which have only been exploited in part. *Dictyostelium* has played a major role in the establishment of our understanding that intercellular adhesion depends on specific cell surface molecules, and in the development of original techniques for detecting and characterizing these molecules.

The biochemical and genetic dissection of the csA glycoproteins has allowed us to define in part the function of different domains and posttranslational modifications of the protein, though the active site involved in homophilic binding is still unknown. However, structural studies to solve this question are facilitated by the availability of the gene and the possibility of purifying large amounts of the glycoprotein from overexpressing cells. Disruption of the gene, on the other hand, has revealed a redundancy in the components of EDTA-resistant adhesion during the aggregation stage which can now be identified and purified thanks to csA-null mutants.

Candidates for mediating adhesion at earlier and later stages of development are also known, though further studies are needed to establish their function, and to extend to them a structural analysis of adhesion.

There is no question of the importance of adhesion in the establishment of the multicellular stage in *Dictyostelium*, but its role in differentiation and pattern formation, and in particular the interplay of adhesion and motility during morphogenesis, are important tasks for future research. Since the cytoskeleton is one of the most intensively studied subjects in *Dictyostelium*, it is also likely that studies on cell-cell and cell-substrate adhesion will receive new impulses from the identification of other cytoskeletal elements involved in membrane-cytoskeleton interactions[75, 99].

In contrast to intercellular adhesion, the molecular basis of phagocytosis remains to be elucidated: how cells interact with bacteria, whether bacterial binding elicits signals leading to cytoskeletal organization, and the potential relationship between phagocytosis and the early cell-cell adhesion system, are some of the problems that deserve more attention. The application to the study of phagocytosis of recently developed molecular genetic approaches, such as restriction-enzyme-mediated tagging of genes, should overcome some of the problems encountered in the past with phagocytosis mutants obtained by classical mutagenesis.

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