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Antibodies Specific for gp40 Inhibit Cell-Cell Adhesion by Cross-Linking the Protein on the Surface of *Dictyostelium purpureum*

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Abstract We have previously suggested a role for gp40 in cell-cell adhesion in *Dictyostelium purpureum* from the fact that antibodies specific for this protein inhibited adhesion in an in vitro assay [Springer: Dev Biol 133:447–455, 1989]. To further confirm this role mutants lacking the protein were isolated and characterized. To our surprise, the mutants had normal adhesive properties both in vitro and in situ. These results lead us to the conclusion that gp40 is not necessary for the cell-cell adhesions observed and may not be a molecule which directly participates in these adhesions. When studied further, we found that adhesion-inhibitory antibodies were only effective as divalent IgG. Monovalent Fab fragments of the same antibodies could not inhibit adhesion. The inhibitory antibodies also caused the cells to remain rounded and incapable of attaching to plastic surfaces. We conclude that when divalent antibodies specific for gp40 cross-link this protein on the cell surface a cytoskeletal change prevents them from attaching to substratum as well as to other cells, thereby inhibiting cell-cell adhesion. We suggest that an alternative mechanism for inhibition of cell-cell adhesion by divalent antibodies exists and should be considered before proposing a direct role for a protein in adhesion.

Key words: cellular slime mold, cytoskeleton, rounding, filopodia, glycocalyx, flow cytometry, immunogold

An often used means of suggesting a role for a specific protein in adhesion has been to show that antibodies specific for the protein are capable of inhibiting the adhesion of interest. Initially the antibody fragment used was the monovalent Fab which cannot cause artifactual agglutination of cells as could be the case with divalent IgG. Several years ago, however, we developed a method [Springer and Barondes, 1980] to screen large numbers of hybridoma supernatants for adhesion inhibition using secondary Fab against the species in which the primary IgG was raised. This technique eliminated the need to purify and digest the primary antibody before screening. We showed that secondary Fab was necessary to prevent agglutination by the divalent primary antibodies we used in developing the assay. In recent years, however, several investigators [Jones and Gillett, 1975; Adler and Chen, 1992; Drake et al., 1992] have also used divalent antibodies without secondary Fab successfully as inhibitors in other adhesion systems. Thus agglutination may be a property of the antibodies and antigens involved and may not present problems in all systems. In our assay there was an exact correlation between antibodies which inhibited as monovalent Fab fragments and as divalent IgG in the presence of secondary Fab [Springer and Barondes, 1980]. We used this technique to implicate specific antigens in the mechanism of cell-cell adhesion in the cellular slime molds.

Upon starvation the individual amoebas of *Dictyostelium discoideum*, and most other species of cellular slime mold, develop adhesiveness which allows them to aggregate with other amoebas to form mounds of cells which eventually differentiate into environmentally resistant spores held aloft on a stalk of dead cells (sori). A significant early discovery (Raper and Thom, 1941) was that within the mounds, cells from different species were able to sort out from each other to form sori of purely one species. The phenomenon of species-specific adhesion makes the cellular slime molds a useful model for or-

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ganogenesis and other types of cell recognition phenomena.

Using the assay described above [Springer and Barondes, 1980] we isolated a monoclonal antibody that inhibited developmentally regulated cell-cell adhesion in *D. discoideum* [Springer and Barondes, 1983]. It was found that this antibody, d-41, bound to a carbohydrate epitope present on the well-documented cell adhesion molecule gp80 [Springer and Barondes, 1985; Loomis et al., 1985] and several other molecules. Further studies showed that d-41 was not species specific but rather inhibited adhesion in all species of *Dictyostelium* tested [Springer and Ahern, 1990]. In the various species, d-41 bound to a number of molecules on Western blots, not all of which were identical [Springer and Ahern, 1990]. The most prominent d-41 binding protein in *D. purpureum* has an apparent molecular weight of 40 kd, much smaller than gp80. This molecule in *D. purpureum* was designated gp40 and was presumed to act as a cell adhesion molecule in *D. purpureum* by the same mechanism that gp80 acts in *D. discoideum*. One peculiarity of gp40 was that it was not developmentally regulated as gp80 was [Springer, 1989] and as would be expected for a protein which mediates a developmentally regulated adhesion. One explanation for this could be that the interaction is heterophilic and the ligand to which gp40 binds on the other cell is developmentally regulated. Thus it was of interest to study what might be an alternative adhesion mechanism in the cellular slime molds.

Since d-41 was capable of binding to a few other molecules in *D. purpureum*, and was, therefore, not totally specific for gp40, we affinity purified another antibody raised against periodate oxidized gp40. Periodate oxidation removes carbohydrate and preserves the polypeptide antigenicity. After affinity purification this new antibody, anti-gp40, was found to exclusively bind to gp40 in Western blots of whole cell extracts [Springer, 1989] and was also capable of completely inhibiting the cell-cell adhesion of *D. purpureum* in our in vitro assay [Springer, 1989]. Thus by criteria used to implicate other cell adhesion molecules, gp40 could be considered a mediator of cell-cell adhesion.

We present evidence here, however, that gp40 is not necessary for the cell adhesion observed in *D. purpureum* and probably does not directly mediate adhesion. By this we mean that it is not a molecule involved in the attachment of one cell to another either by binding to an identical (homophilic) or different (heterophilic) molecule on the opposing cell surface. As a result of this finding we postulate that there exists an alternative mechanism of adhesion inhibition by divalent antibodies which is dependent upon the cross-linking of a specific antigen, in this case gp40, on the cell surface. We suggest that this cross-linking causes cytoskeletal changes which prevent the cell-cell adhesion mediated by other cell surface proteins. Although reported here in a cellular slime mold, this type of adhesion inhibition may exist for other cell types and may require the reevaluation of the role of some immunologically defined cell adhesion molecules.

METHODS

Cell Culture and Development

D. purpureum cells grown in conjunction with *Klebsiella pneumoniae* were harvested and washed with cold water to remove bacteria. The cells were then starved overnight by shaking in 17 mM sodium, potassium phosphate pH 6.0 (SPS). Starved cells were placed on filter pads all as described previously [Springer and Barondes, 1978] or, for electron microscopy, placed on an Anocell 25 tissue culture insert membrane (Anotec Separations Ltd., Oxon, UK) on top of the filter pad. The cells were allowed to aggregate in a moist atmosphere over a period of 1 to 2 h. Aggregated cells were removed from the filters by vortexing and used as described below or, in the case of the Anocell membrane, fixed on the membrane as below.

Assay of Inhibition of Cell-Cell Adhesion

The assay used here has been described in detail previously [Springer and Barondes, 1980; McDonough et al., 1980; Springer, 1989] using *D. discoideum* and various antibody preparations against specific proteins known to be involved in cell-cell adhesion as well as specific cell surface proteins which do not participate in the measured adhesion. Briefly, in the standard assay, aggregated cells are preincubated on ice for 30 min with specific divalent IgG (primary antibody) followed by dilution of this mixture into assay buffer while shaking at 200 rpm. The assay buffer contains Fab fragments of either goat anti-mouse or goat anti-rabbit IgG (secondary antibody), corresponding to the primary antibody species. The number of single cells at 0

and 10 min is determined using an electronic particle counter (Coulter Electronics ZBI, Hialeah, FL). From these determinations the percent inhibition of adhesion can be calculated [Springer and Barondes, 1985]. To determine whether cross-linking of antigen on the cell surface was needed for inhibition, Fab fragments were used in place of primary IgG and secondary Fab was replaced with IgG. For this paper antibody concentrations are given as that used for preincubation, which is 11 times that after dilution into the assay buffer. The concentration after dilution has been reported in some other papers [Barondes et al., 1981; Springer, 1989], but we now consider the preincubation concentration to be the more relevant parameter.

Antibody Preparation

We have previously shown [Springer, 1989] that an affinity-purified polyclonal antibody prepared against the periodate-oxidized glycoprotein, gp40, is specific for the protein and is capable of inhibiting cell-cell adhesion of *D. purpureum* in our standard assay. Sera from three additional rabbits having undergone an identical challenge with periodate-oxidized gp40 were supplied by Calbiochem (La Jolla, CA). The sera were pooled at each bleed. Reactive antibodies were present in the sera after the first bleed at 5 weeks as determined by Western blots. Additional boosts were performed followed by bleeds at 9, 13, and 17 weeks. At 17 weeks the rabbits were euthanized and approximately 100 ml of pooled sera was obtained. Antibody was affinity purified from the sera using purified gp40 attached to Sepharose as described previously [Springer, 1989; Bols et al., 1986]. The affinity-purified antibody was designated APgp40.

Serum R454 was obtained from a single rabbit immunized against the amino-terminal pentadecapeptide of gp40 [Springer, 1989] coupled to bovine serum albumen (BSA) prepared for us by Immuno-Dynamics, Inc. (La Jolla, CA). The serum was affinity purified on gp40-Sepharose as for APgp40. This antibody was designated AP454.

Mutant Selection

A method similar to that developed by Francis et al. [1985] was used to select for mutant cells which did not react with antibody specific for gp40. Mutagenized vegetative cells (survival frequency of 8×10^{-4}) arising from treatment of a wild-type clone (HS1) with *N'*-methyl *N'*-nitro *N*-nitrosoguanidine [Loomis, 1987] were grown with bacteria for 68 h. The vegetative cells were harvested, washed free of bacteria, resuspended to 1×10^7/ml in 5 ml of 5% nonfat dry milk, 0.2% BSA, 0.05% Tween 20, 0.15 M NaCl in 0.05 M Tris-HCl, pH 7.6 (blocking buffer), and reacted with 5 μg/ml APgp40 for 1 h. Blocking buffer was centrifuged at 5,000*g* prior to use to remove insoluble particles. After washing with blocking buffer the cells were incubated with 1/1,000 dilution of affinity purified fluorescein isothiocyanate conjugated goat anti-rabbit IgG (FITC-GARIgG) (CalBiochem, La Jolla, CA) and sorted using a Facstar Plus cytometer (Becton Dickinson, Mountain View, CA). The cells falling in the lower 10% of the fluorescence histogram were recovered and plated with bacteria. This cycle was repeated two more times, at which time the recovered cells were plated at low density and grown until clonal plaques were observed. Cells from individual plaques were picked with sterile toothpicks, touched to a replicate plate or vortexed in 50 μl of 0.5% sodium dodecyl sulfate (SDS) to dissolve the cells. The dissolved cell suspension was dotted onto a nitrocellulose sheet by applying 1 μl from a mechanical pipet. After drying the nitrocellulose was treated as in Western blotting, reacted with APgp40 and horseradish peroxidase conjugated goat anti-rabbit IgG (HRP-GARIgG), and developed as previously described [Springer, 1989]. Cell clones which were negative in the dot blot were retested by performing a Western blot of a sample prepared by dissolving cells at 10^7/ml in Laemmli solubilization buffer [Laemmli, 1970] and applying 10 μl to a well. After electrophoresis and transfer the nitrocellulose was probed with APgp40 and HRP-GARIgG as above. After development the individual lanes were compared to the parent strain for reactivity with gp40.

Flow Cytometry

To determine surface binding of selected antibodies, washed aggregated cells were fixed in 10% buffered formalin and treated as described above for cytometer sorting. Analysis was done on a Cytofluorograf 50H (Ortho Instruments, Westwood, MA).

Antibody Rounding

Fifty microliters of APgp40 at 100 μg/ml in SPS were added to a well of a 96-well microtiter plate. Five microliters of disbursed aggregated

cells at 10^7/ml were added and the plate lightly tapped to distribute the cells. The cells that settled onto the surface of the plate were observed with an inverted microscope over a period of 2 h. Photographs were taken at various times.

Antibody Capping

Aliquots of 10^6 aggregated *D. purpureum* were resuspended in 100 μl of 0 μg/ml of either APgp40 IgG or Fab. After incubating with shaking for 1 h, the two cell suspensions were each diluted with 2 ml of SPS containing 5% goat serum and centrifuged. After washing in the same buffer, half of the cells from each tube were resuspended in 50 μl of 1/50 FITC-GARIgG or 1/50 FITC-GARFab and incubated for 30 min. After washing as above the cells were observed with an epifluorescence microscope. Cells were considered capped when greater than 90% showed fluorescence over less than 50% of the membrane at the equatorial optical section.

Fixation and Embedding for Electron Microscopy

For post-embedding immunogold staining, aggregated cells on Anocell membranes were removed from the filter pad and transferred to an absorbent pad for fixation overnight at 4°C with freshly prepared 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Pieces of the membrane were then incubated with 100 mM glycine in sodium cacodylate buffer for 10 min followed by dehydration with graded ethanol solutions ending with 2 changes of 70% ethanol. Samples were then post-fixed for 1 h at room temperature with a solution containing 1 ml of saturated aqueous uranyl acetate in 25 ml 70% ethanol. Post-fixation with uranyl acetate greatly improved morphology without substantially affecting antigenicity [Erickson et al., 1987].

To better visualize and stain any extracellular material 2.0 mg/ml ruthenium red was added to the primary fixative described above. After a buffer wash the samples were post-fixed in 1% osmium tetroxide, 2 mg/ml ruthenium red in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h at room temperature [Dykstra and Aldrich, 1978]. After another wash in cacodylate buffer these samples were dehydrated with graded ethanol solutions ending in two changes of 80% ethanol. The uranyl acetate fixation described above was omitted. Since this treatment reduced gp40 antigenicity, samples for comparison were taken from the same membrane as used for immunogold fixation.

After dehydration to 80% ethanol all samples were placed in a 1:1 mixture of 80% ethanol:LR White resin (medium grade: Ted Pella Inc., Redding, California) and left overnight. Samples were then transferred to 100% plastic and two changes were made over the next 6 h. Strips of Anocell membrane were then placed in gelatin capsules and polymerization was carried out at 55°C for 24–48 h.

Post-Embedding Immunostaining

Thin sections were picked up on nickel grids that had been coated with a film of 0.5% parlodion. Samples were sectioned perpendicular to the Anocell membrane surface after the membrane had been carefully teased away from the bottom of the aggregate.

On-grid immunostaining was done at room temperature using a slight modification of the procedure detailed in literature provided by Janssen Laboratories [Janssen Biotech, 1988]. Grids were incubated with sections facing down on drops of reagent on Parafilm. All solutions, with the exception of the colloidal gold suspension, were passed through a 0.22 μm Millex-GV filter (Millipore, Bedford, MA) immediately prior to use. Samples were first incubated for 30 min in 50 mM Tris-HCl, 150 mM NaCl, pH 7.6 (TBS), containing 5% goat serum, 0.8% BSA, 0.1% gelatin (IGSS quality: Janssen Laboratories), and 0.02% sodium azide in order to block any nonspecific sites. Grids were then transferred to TBS, 0.8% BSA, 0.1% gelatin, 0.02% azide (wash buffer) containing 1–5 μg/ml APgp40. Control grids were incubated in wash buffer alone or in wash buffer containing 5–10 μg/ml of affinity-purified rabbit antibody to an endogenous *Xenopus* lectin [Bols et al., 1986] found not to react with extracts of whole *D. purpureum* cells. After incubation for 90 min samples were washed twice for 10 min in wash buffer and then transferred to 1/25 dilution of affinity-purified goat anti-rabbit IgG conjugated to 15 nm colloidal gold particles (E-Y Laboratories, San Mateo, CA) in TBS containing 1% normal goat serum, 0.1% gelatin, 0.02% sodium azide. After 90 min grids were washed with wash buffer for 10 min followed by 10 min in TBS and then fixed with 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min. Grids were then washed with 3 × 5 min changes of glass distilled water. Samples were post-stained with satu-

rated uranyl acetate in 50% ethanol followed by bismuth subnitrate before examination with a Zeiss EM10 electron microscope.

Other Techniques

IgG was prepared by ammonium sulfate precipitation [Good et al., 1980] and Fab fragments as described by Porter [1959].

RESULTS

Antibodies Specific for gp40

For this paper antibody APgp40 was prepared after a series of four boosts with purified periodate-treated gp40 over a period of approximately 4 months. The concentration needed to produce maximal inhibition was somewhat higher for APgp40 (50–100 μg/ml) than that previously published for our other antibody preparation, anti-gp40 (20–30 μg/ml [Springer, 1989]). This is probably due to the fact that the sera used to prepare anti-gp40 resulted from a series of monthly boosts over a period of a year. The affinity purified APgp40 was tested for specificity toward gp40 in whole cell extracts of *D. purpureum* run on Western blots (Fig. 1B) and found to react exclusively with a protein of the correct molecular weight.

After affinity purification, AP454, which was raised against the amino-terminal peptide sequence of gp40, reacted well with gp40 on Western blots and only with a band of identical molecular weight to gp40 in whole cell extracts of *D. purpureum* (Fig. 1C), indicating that the peptide was indeed an epitope of gp40. Both APgp40 and AP454 were used as specific reagents to indicate the presence of gp40 in a preparation.

Mutant Selection

The ability of APgp40 to detect gp40 on the surface of living cells was used to select for mutants missing the protein by a method similar to that developed by Francis et al. [1985] (also see Methods). After three cycles of growth, labeling, and sorting, the cells having the lowest fluorescence intensity were plated at low density onto a bacterial lawn and 200 individual colonies picked after 3 days of growth. These 200 clones were grown individually and tested for APgp40 binding in dot blots of 0.5% SDS extracts of whole cells on nitrocellulose. Finally 27 clones showing low binding in the dot blots were subjected to polyacrylamide gel electrophoresis and Western blotting. Since low binding could result from low numbers of cells picked, variability in the solubilization, or a number of artifacts, Western blots are required to definitively prove loss of the gp40 antigens. Therefore after Western blotting 2 of these 27 possible mutant extracts showed no reactivity with APgp40. These isolates were designated HS23 and HS27. Since this method involves enrichment and amplification, it is possible that the two isolates were derived from the same mutational event. Both mutants have similar if not identical properties. HS27 was selected for use in the characterization of the phenotype.

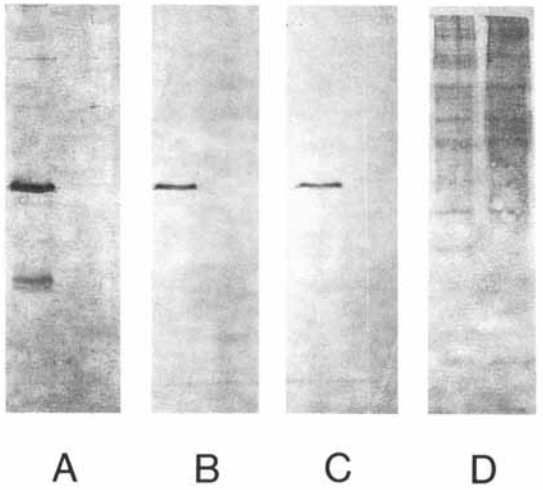

Fig. 1. Western blots of whole cell extracts of *D. purpureum* vs. various antibodies. Whole *D. purpureum* cells of wild type, HS1, or mutant, HS27, were harvested, solubilized, subjected to polyacrylamide gel electrophoresis, and transferred to nitrocellulose as described in Methods. Slices of the nitrocellulose containing extracts from each strain were reacted with (**A**) d-41, (**B**) APgp40, (**C**) AP454, or (**D**) antibody raised against whole *D. purpureum*, washed, reacted with horseradish peroxidase conjugated second antibody, and developed as described in Methods. Left lanes are extracts of HS1 and right lanes of HS27.

Characterization of Mutant HS27

Figure 1 shows that HS27 is missing gp40 as defined by the three antibodies which react with gp40. All three react well with gp40 in the parent strain, HS1, but not HS27. Mouse monoclonal antibody d-41 (Fig. 1A) reacts with a carbohydrate epitope prominent but not exclusively on gp40 [Springer and Barondes, 1985; Springer, 1989]. APgp40 (Fig. 1B) reacts exclusively with the gp40 polypeptide in HS1, as does AP454 (Fig. 1C). The fact that all three antibodies show no reactivity with anything in HS27 suggests that gp40 is indeed missing from the

mutant. The final pair of blots (Fig. 1D) is included to show that an equivalent amount of protein was run and transferred in both cases since a crude antiserum to whole *D. purpureum* reacts equally well with both strains. Lower molecular weight proteins, although not staining with this crude antiserum, did also transfer as determined by amido black staining of the filter after transfer (not shown).

Although these results do not definitively prove that the mutation is in the structural gene for gp40, it can be argued that the protein would have to be greatly modified not to react with all three of these antibodies. Two of these antibodies are protein specific—raised against antigens of the polypeptide backbone of the protein. The fact that the amino-terminal peptide antigen is missing suggests that a peptide fragment of any size also does not blot. Although we do not have data to indicate the number of unique antigens APgp40 reacts with, it is a polyclonal antibody which suggests that there may be more than one. If not in the structural gene, for the purposes of this paper, the mutation results in a protein which must be absent or so grossly modified as to be considered functionally absent from the cell.

The fact that the lower molecular weight bands are also missing when d-41 (Fig. 1A) is used, suggests, as was proposed previously [Springer, 1989], that those bands are the result of proteolytic cleavage of gp40 during differentiation. The fact that neither APgp40 or AP454 reacts with these bands could result from the cleavage eliminating, or altering, the epitopes with which they react. An alternative explanation would be that gp40 and the lower molecular weight bands are co-regulated and the mutation results in a loss of all these molecules. Either interpretation suggests that gp40 must be missing from the cell.

In addition to Western blotting, flow cytometry analysis confirms that surface reactivity of the two specific antibodies is eliminated, or, for d-41, is significantly reduced (Fig. 2). Monoclonal d-41 does, however, retain residual surface binding activity which may be similar to that reported for species of the genus *Polysphondylium*. This genus shows measurable surface binding of d-41 but no reactivity with proteins in Western blots [Springer and Ahern, 1990]. It is presumed that the d-41 carbohydrate epitope can reside on other molecules besides proteins. In addition d-41 does not inhibit adhesion in *Polysphondylium*. Glycolipids and saccharides containing the epitope might be detected by whole cell binding but not Western blots. We will show below, however, that the effects d-41 has on adhesion are dependent upon gp40 on the cell surface and not these other species.

Even though missing gp40, to our surprise, by all other criteria tested HS27 is identical to its parent, HS1. Most importantly, it grows and develops normally, exhibits normal developmentally regulated adhesion, is capable of sorting out from other species in mixed aggregates, and distributes randomly in fruiting bodies and slugs formed by mixing vegetative or aggregated HS1 and HS27 cells (data not shown). To date we have not been able to find any difference in behavior between the mutant and its parent except for those involving antibody inhibition described below.

Gp40 Is Not a Cell Adhesion Molecule, But Antibody Inhibition Is Specific for gp40

If we accept the evidence presented above as indicative of gp40 being missing from HS27, then the fact that this mutant displays normal adhesive properties under all conditions tested leads us to conclude that gp40 cannot be a molecule that participates directly in the adhesion of these cells to each other. Therefore, the only criterion that suggests that gp40 plays a role in adhesion is the fact that antibodies which react specifically with gp40 are capable of inhibiting cell-cell adhesion in the assay we have developed and have shown to work for other adhesion molecules. We must, therefore, postulate an alternative mechanism of how the gp40-specific antibodies can inhibit adhesion. We and others have often shown that adhesion cannot be inhibited simply by binding antibodies to any random surface protein. Indeed we have saturated the surface of *D. discoideum* cells with antibodies to a number of surface proteins and found no effect on adhesion [Barondes et al., 1981]. We assume, therefore, that the ability of antibodies against gp40 to inhibit adhesion results from the properties and associations specific to gp40. The fact that gp40 must be present for inhibition of adhesion by these antibodies can be shown by comparing the ability of our antibody panel to inhibit adhesion in HS1 and HS27. As can be seen from Table I, d-41 and APgp40 are both capable of significantly inhibiting adhesion in HS1 but not the mutant lacking gp40. The fact that d-41 cannot inhibit adhesion

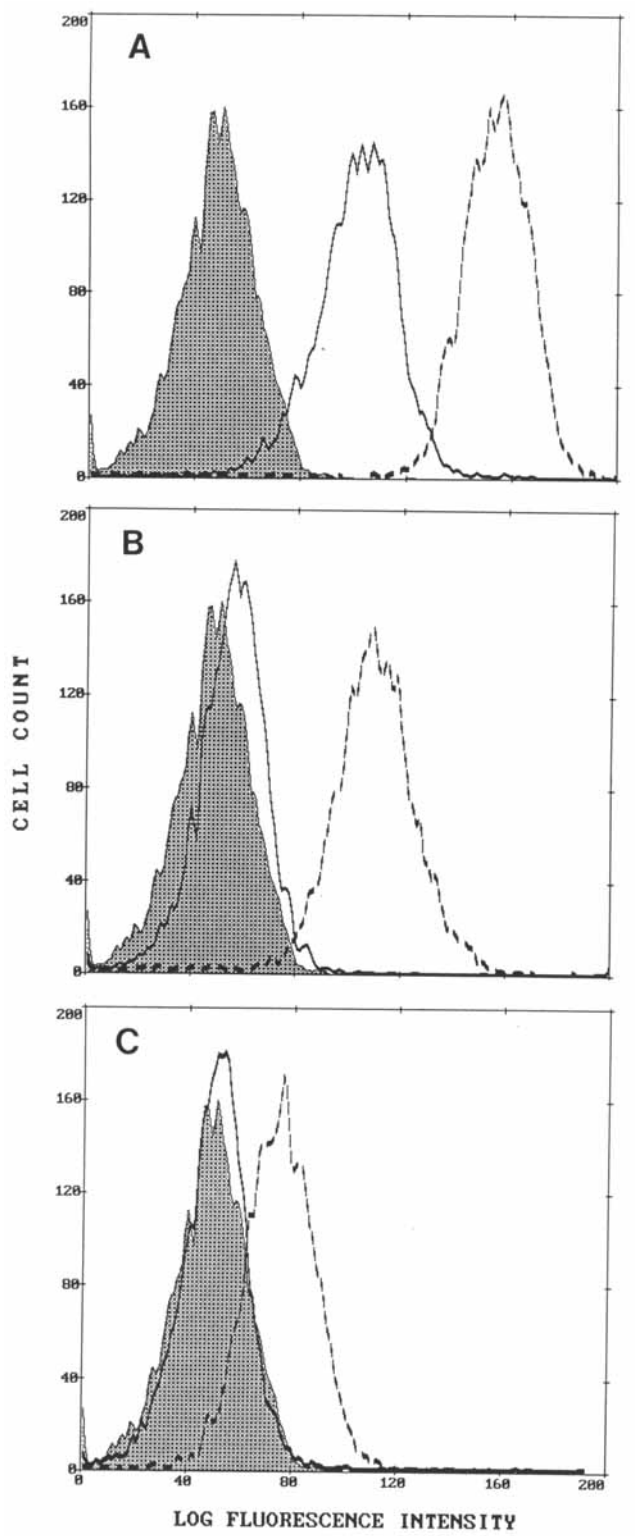

Fig. 2. Fluorescence histograms of HS1 and HS27 cells reacted with various antibodies. Cells were fixed, reacted with the indicated antibodies followed by fluorescein conjugated second antibody, and subjected to flow cytometry as described in Methods. Filled histogram is result of no primary antibody (background) on HS1 cells. The background histogram for HS27 was indistinguishable from that shown for HS1. Solid line is the result using the gp40 mutant, HS27. Broken line is the result using wild type, HS1. **A:** d-41; **B:** APgp40; **C:** AP454.

TABLE I. Inhibition of the Adhesion of HS1 and HS27 by Various Antibodies*

Antibody (conc., μg/ml)	Inhibition of adhesion (%) HS1	HS27
Normal mouse IgG (200)	7	9
Normal rabbit IgG (400)	0	0
d-41 (200)	100	0
APgp40 (350)	87	0
AP454 (400)	5	NT
Anti-purpurin (500)	8	0

*Adhesion assays were performed after incubation in the indicated concentrations of antibody as described in Methods. Percent inhibition was determined. Cells which show no adhesion would be 100% inhibited. NT means not tested at 400 μg/ml (percent inhibition at 100 μg/ml = 3%).

in HS27 even though it exhibits residual binding to the same epitope on other molecules on the cell indicates that antibody must specifically bind to gp40 to affect adhesion. Normal rabbit or mouse IgG or anti-purpurin, which binds to the endogenous lectin on the surface of both cells, have little or no effect at the concentrations used, ruling out the possibility that inhibition results from gp40 being some sort of antibody binding protein or a nonspecific effect of antibody binding to the cell surface. Of significance, however, is the fact that AP454 binds to gp40 and the surface of HS1 (Fig. 2), but does not inhibit adhesion. We attribute this to the fact that steric hindrance of gp40 does not, of itself, inhibit adhesion as would be expected for an antibody which binds to a bona fide cell adhesion molecule.

Since flow cytometry showed that AP454 does not bind to HS27, and, therefore, presumably could not affect the behavior in any specific way, we did not always test it against these cells. Observations of the cells in microtiter wells and specific experiments discussed below always gave identical results with AP454 to those observed with normal rabbit antibodies—indicating that no specific interactions occurred.

Cross-Linking of gp40 Is Required for Adhesion Inhibition

The data in Table I resulted from an assay which uses divalent primary antibody followed by monovalent secondary antibody to prevent artifactual agglutination [Springer and Barondes, 1980; also see Methods]. This method has been shown to reliably detect cell adhesion molecules defined by other criteria, including the use of primary Fab as an inhibitor of adhesion [Springer and Barondes, 1980]. We found previously that Fab prepared from serum raised against periodate treated gp40, but which had not been affinity purified, was capable of inhibiting adhesion in *D. purpureum* [Springer, 1989]. It was striking, however, how much more monovalent antibody was necessary to inhibit adhesion, and to a lesser extent [Springer, 1989]. At the time we attributed this to a lower avidity of the monovalent Fab fragments, but such high concentrations could have small amounts of divalent antibody contaminants or antibodies against other proteins involved in the adhesion. When we tried the affinity-purified APgp40 as monovalent Fab fragments, we found it had little, if any, effect at a concentration at which divalent IgG inhibited quite well (less than 10% inhibition at 150 μg/ml APgp40 Fab).

Figure 3 shows that the reason APgp40 or d-41 IgG is more effective than their Fab fragments is not because of a much higher avidity but because of their divalency. When divalent secondary antibody is added after incubation with monovalent d-41 Fab, the effective d-41 concentration is similar to that for the combina-

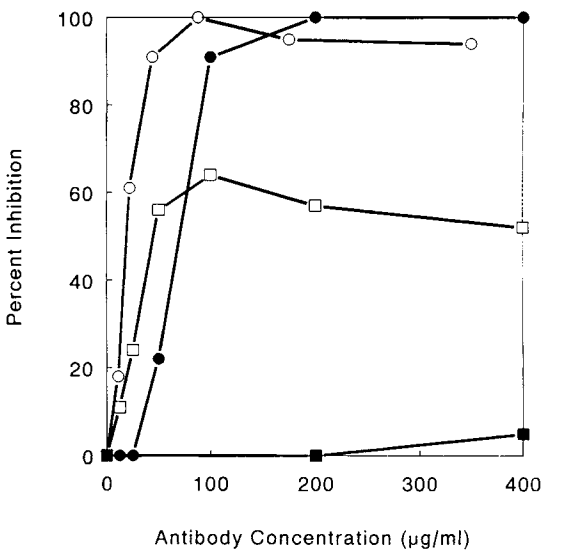

Fig. 3. Inhibition of cell-cell adhesion in *D. purpureum* by monovalent or divalent antibodies. Cells were assayed for adhesion after preincubation in either divalent IgG followed by monovalent secondary antibody or preincubation with monovalent Fab followed by divalent or monovalent secondary antibocies as described in Methods. Percent inhibition of cell adhesion was determined. —○—, divalent APgp40, monovalent secondary; —●—, divalent d-41, monovalent secondary; —□—, monovalent d-41, divalent secondary; —■—, monovalent d-41, monovalent secondary.

tion of d-41 IgG and secondary Fab. As would be predicted, the combination of d-41 Fab and secondary Fab shows no effect over the entire concentration range (Fig. 3). We did not have sufficient APgp40 Fab to do an entire dose/response curve, but at a concentration of 100 μg/ml followed by secondary IgG it inhibited adhesion by 68%. The fact that in both cases Fab plus secondary IgG does not cause 100% inhibition could be the result of agglutination by the divalent second antibody, a condition which the use of monovalent primary or secondary Fab was designed to prevent. It is assumed that adhesion-inhibitory, divalent, primary IgG exerts its effect by cross-linking or perhaps immobilizing gp40 on the cell surface. Cross-linking is also suggested by the fact that antibody combinations which cause inhibition (e.g., APgp40 Fab and secondary IgG) also result in antibody capping on the cell surface (see Methods). Caps were not observed when APgp40 Fab was used in combination with secondary Fab, as would be expected for monovalent fragments. Capping was also not observed when either AP454 Fab or IgG were used in combination with secondary IgG, which would explain its inability to inhibit, if our hypothesis is correct.

In addition to inhibition and capping, d-41 and APgp40, but not AP454 or nonspecific antibodies, also prevent the cells from elongating and attaching to plastic surfaces, a phenomenon called rounding [Springer and Barondes, 1983; Springer et al., 1984; Jones and Gillett, 1975; Adler and Chen, 1992; Drake et al., 1992]. Figure 4 shows what happens when either wild-type or mutant cells are seeded into media containing an inhibitory concentration of APgp40 IgG (or d-41, not shown). Both cells quickly settle to the surface of the plate, but only the mutant HS27 is able to immediately flatten and elongate. The ability of wild-type cells to flatten is delayed by an hour or more when in the presence of antibody. During this time cell-cell adhesion as measured in our assay is also inhibited. Consistent with the other observations is the fact that d-41 IgG does not round the mutant (not shown) even though it shows residual surface binding (Fig. 2). Again this suggests that rounding results from cross-linking of gp40, in this case through the d-41 epitope, and not other surface molecules bearing the epitope. Finally AP454, as either Fab or IgG, had no effect on either wild-type or mutant cells, as might be expected from the results of the lack of adhesion inhibition in the parent or binding to the mutant.

Localization of gp40 on the Cell Surface

APgp40 and AP454 both bound to whole *D. purpureum* cells indicating that gp40 was on the surface of the cell (Fig. 2). Its exact location was determined by electron microscopy and immunogold staining with APgp40 as shown in Figure 5. Although often localized close to the plasma membrane, specific gold label was also found at a considerable distance from the membrane in what has been described as the glycocalyx, a carbohydrate-rich region extending from the surface and staining with ruthenium red [Dykstra and Aldrich, 1978]. No gold particles were observed when identical sections were stained with an unrelated monoclonal antibody as primary antibody and used as a negative control (not shown).

All of the gp40 is strongly associated with a crude membrane fraction resulting from freezing and thawing of cells followed by centrifugation to remove soluble components [Springer, 1989]. Quantitative extraction of gp40 from these membranes requires treatment with either ionic or nonionic detergents [Springer, 1989; unpublished data]. Washing with 1 M NaCl removes no more gp40 from the particulate than washing with SPS (3–6% in each case). A criterion used to define peripheral membrane proteins, extraction with 0.1 M sodium carbonate [Howell and Palade, 1982; Fujiki et al., 1982], resulted in the release of 16 times more gp40 into the medium than treatment with SPS. This treatment, however, was not terribly specific and released a number of proteins from the *D. purpureum* membrane. Therefore, this procedure may not define peripheral membrane proteins in *D. purpureum* as found for other cells. Taken together these results indicate that gp40 associates with the cell surface through what is probably a hydrophobic interaction, but, because of its location beyond the membrane, gp40 may not be an integral membrane protein.

DISCUSSION

We have previously shown [Springer and Ahern, 1990] that the anticarbohydrate monoclonal antibody, d-41, inhibits a form of adhesion in every species of *Dictyostelium* we tested. Since it had been shown [Springer and Barondes, 1983] that the antibody inhibits adhesion in *D. discoideum* by binding to the well-docu-

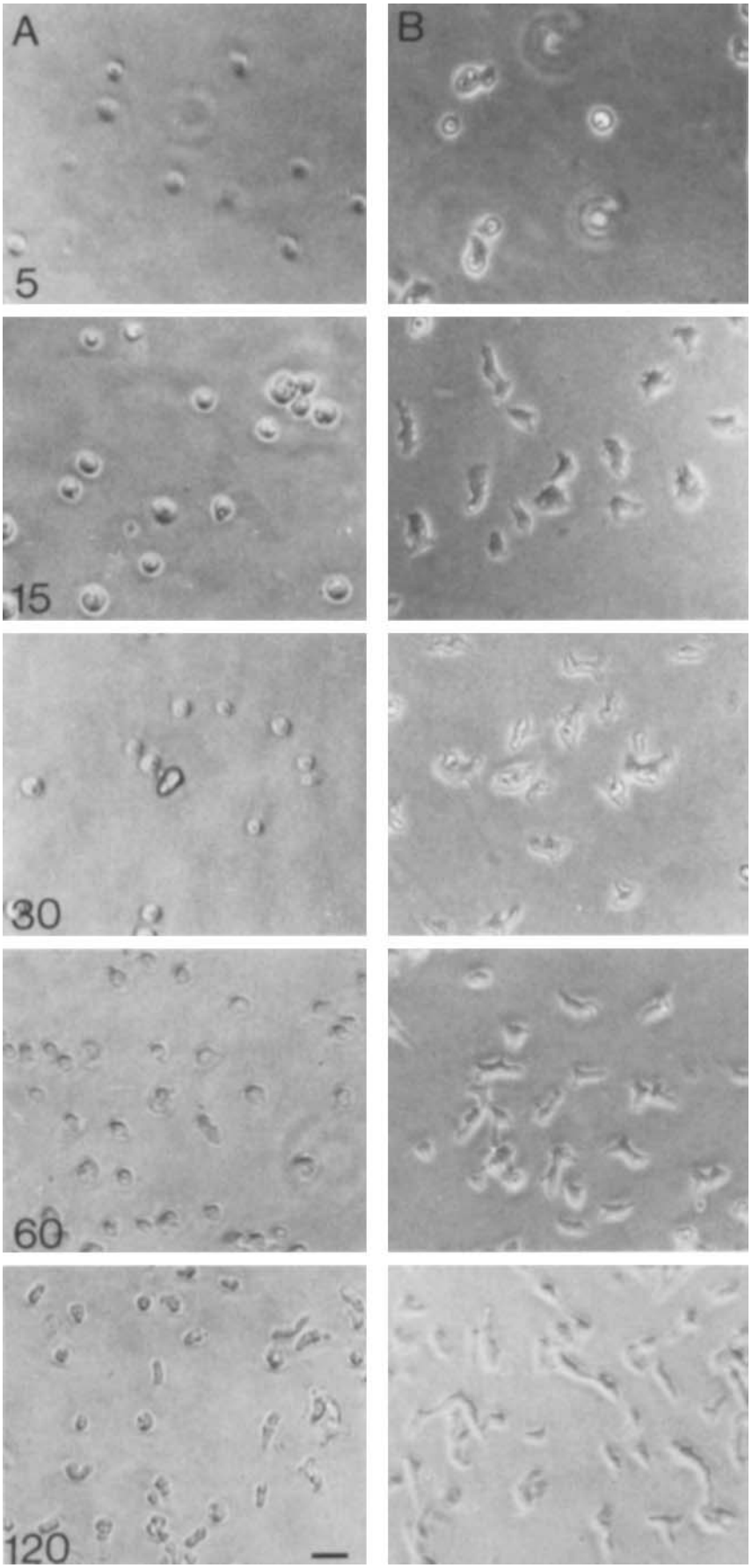

Figure 4

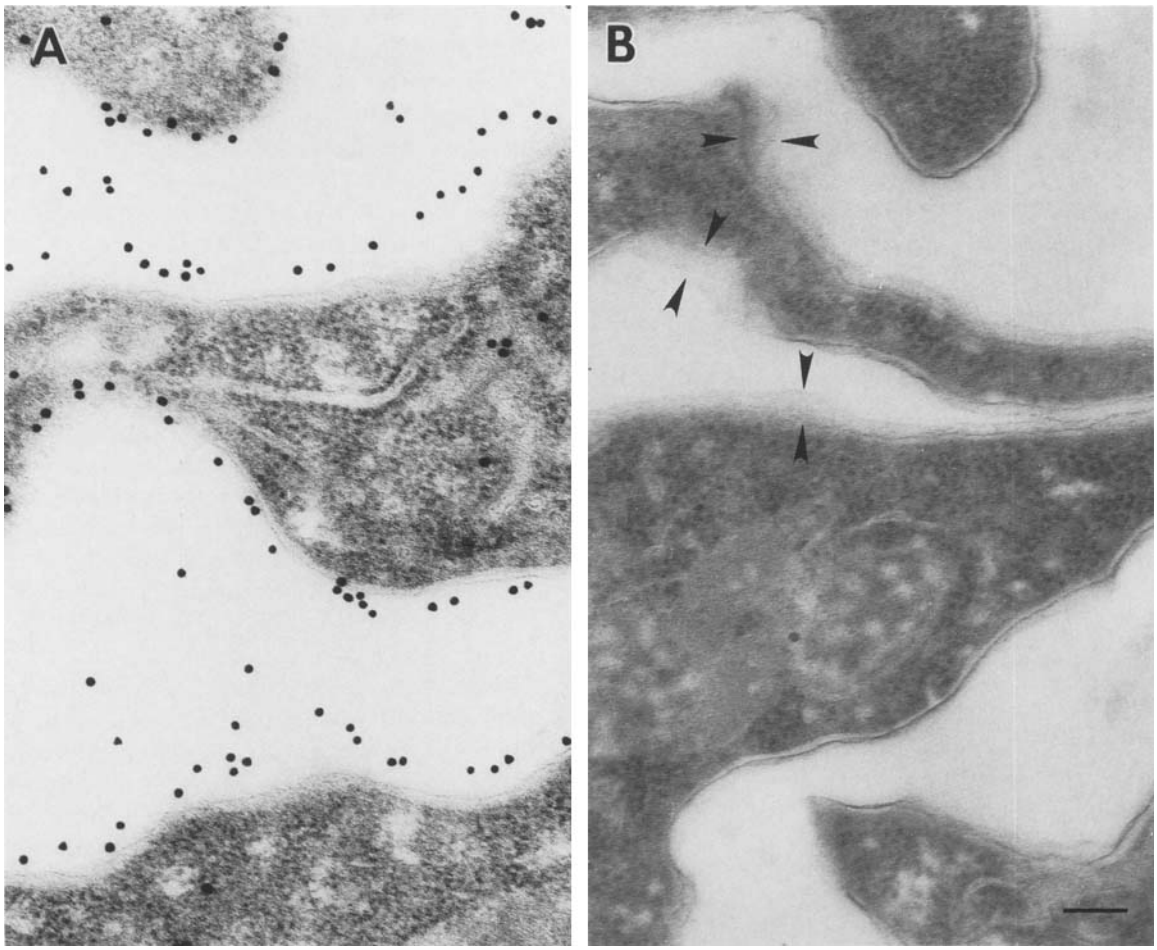

Fig. 5. Immunogold labeling of gp40 and visualization of the glycocalyx. Samples were prepared for electron microscopy as described in Methods. **A:** Immunogold localization of gp40 antigen using APgp40 and colloidal gold conjugated goat anti-rabbit. **B:** A section from the same preparation stained for glycocalyx by ruthenium red as described in Methods. Arrows define extent of ruthenium red staining. Bar equals 100 nanometers.

mented cell adhesion molecule, gp80, and blocking its ability to mediate homophilic adhesion [Siu et al., 1988], we assumed that d-41 was working by a similar mechanism when it bound to the glycoprotein, gp40, in the species *D. purpureum*. A peculiarity of *D. purpureum*, however, was that gp40 is of a different apparent molecular weight than gp80 and is not developmentally regulated, although the adhesion inhibited by d-41 and other antibodies specific for gp40 is. This could be explained by gp40 binding through a heterophilic interaction with a developmentally regulated molecule on the opposing cell, which would also be different from the homophilic binding of gp80.

In an attempt to independently show a direct role for gp40 in cell-cell adhesion in *D. purpureum*, we decided to isolate a mutant which did not have the protein. Since we had several antibodies specific for gp40, we could be reasonably certain that protein was absent from the mutant cell. When such a mutant was characterized it behaved as wild type in all respects including developmentally regulated adhesion. We, therefore, must conclude that gp40 is *not* directly involved in the measured cell-cell adhesion. However, we must also conclude that certain antibodies specific for gp40 are capable of inhibiting this adhesion in *D. purpureum*.

The results presented above indicate that the inhibition of *D. purpureum* adhesion by divalent

Fig. 4. The effect of APgp40 on the morphology of HS1 and HS27. HS1 (**A**) or HS27 (**B**) cells were added to wells containing 100 μg/ml APgp40 and photographed at the indicated times (min). Bar equals 20 mm.

gp40-specific antibodies is due to the cross-linking of gp40 on the cell surface. Concurrent with adhesion inhibition is the inability of the cell to spread on the substratum and elongate (rounding). We show that the adhesion inhibition and rounding are the result of binding of these specific antibodies to gp40 and not some nonspecific effect. The results suggest that when gp40 is cross-linked on the cell surface the cytoskeleton is affected in a way which prevents the cells from spreading on the substratum, as well as preventing the actual cell adhesion molecule from acting. Since electron microscopy indicates that a great deal of the gp40 is not closely associated with the plasma membrane, but is bound in the carbohydrate-rich glycocalyx, it is not clear how the signal is transmitted to the cytoskeleton.

Singer [1992] has postulated that certain associated proteins can cocap when antibody against only one is applied to the cell and this cocapping can prevent adhesion mediated by the second protein. This does not seem to be the case here, however, since the inhibition of adhesion is complete within minutes while capping requires a much longer time (30–60 min). Rounding is indicative of the loss of or inability to produce filopodia [Jones and Gillett, 1975]. Jones et al. [1977] found that adhesion in preaggregative *D. discoideum* was partially dependent upon the existence of filopodia. De Chastellier and Ryter [1980] concluded from their observations that the formation of filopodia was a necessary step in aggregation. Siu et al. [1988] have suggested a four stage model for cell-cell adhesion in *D. discoideum* which involves filopodia in the initial recognition events. Loss of filopodia could result in the improper display of true cell adhesion molecules or the inability to establish initial contacts [Jones and Gillett, 1975].

The adhesion inhibition by d-41 and APgp40 in *D. purpureum* may be similar to that of the monoclonal antibody d-47 [Springer and Barondes, 1983] specific for *D. discoideum* which also causes rounding and inhibition of adhesion in *D. discoideum*. Finally, the mechanism of d-41 inhibition of adhesion in *D. discoideum* is also different, since in this species no rounding is observed yet the antibody inhibits adhesion. It is presumed that in this species d-41 inhibits by sterically hindering the well-documented cell adhesion molecule, gp80. Thus the same antibody can inhibit adhesion by two different mechanisms even in such closely related species as *D. discoideum* and *D. purpureum*.

We do not know why AP454 cannot cross-link, cap, or round, but it may be that the N-terminal peptide is not displayed in a manner which allows cross-linking of gp40 or, more likely, that cross-linking of only two molecules through the single epitope is not sufficient to cause the cytoskeletal effects.

Perhaps the finding of most significance is the fact that antibodies can block adhesion without binding to an actual cell adhesion molecule. The results presented suggest that adhesion inhibition by an antibody, although indicative of the possible direct involvement of a molecule in cell adhesion, is not sufficient to prove that involvement. Additional evidence in the form of direct inhibition by the molecule in question or a correlation of the loss of adhesion with the loss of the molecule is needed to definitely assign the function of cell adhesion molecule.

Finally, we can assume that gp40 performs some yet unknown function in the cell. This function may be in some way supportive of the actual adhesion and undetectable by our assays or something completely unrelated to adhesion. Whether the ability to disrupt the normal cytoskeletal framework when cross-linked is integral to this function is yet to be determined.

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