Mapping of a Cell-binding Domain in the Cell Adhesion Molecule gp80 of *Dictyostelium discoideum*

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Abstract. At the aggregation stage of *Dictyostelium discoideum* development, a cell surface glycoprotein of M_r 80,000 (gp80) has been found to mediate the EDTA-resistant type of cell–cell adhesion via homophilic interaction (Siu, C.-H., A. Cho, and A. H. C. Choi. 1987. *J. Cell Biol.* 105:2523–2533). To investigate the structure–function relationships of gp80, we have isolated full length cDNA clones for gp80 and determined the DNA sequence. The deduced structure of gp80 showed three major domains. An amino-terminal globular domain composed of the bulk of the protein is supported by a short stalk region, which is followed by a membrane anchor at the carboxy terminus. Structural analysis suggested that the cell-binding domain of gp80 resides within the globular domain near the amino terminus. To investigate the relationship of the cell-binding activity to this region of the polypeptide, three protein A/gp80 (PA80) gene fusions were constructed using the expression vector pRIT2T. These PA80 fusion proteins were assayed for their ability to bind to aggregation stage cells. Binding of ^{125}I-labeled fusion proteins PA80I (containing the Val123 to Ile514 fragment of gp80) and PA80II (Val123 to Ala258) was dosage dependent and could be inhibited by precoating cells with the cell cohesion-blocking mAb 80L5C4. On the other hand, there was no appreciable binding of PA80III (Ile174 to Ile514) to cells. Reassociation of cells was significantly inhibited in the presence of PA80I or PA80II. In addition, ^{125}I-labeled PA80II exhibited homophilic interaction with immobilized PA80I, PA80II, or gp80. The results of these studies lead to the mapping of a cell-binding domain in the region between Val123 and Leu173 of gp80 and provide direct evidence that the cell-binding activity of gp80 resides in the protein moiety.

During development of the cellular slime mold *Dictyostelium discoideum*, solitary cells migrate in response to environmental cAMP to form aggregates that eventually differentiate into fruiting bodies consisting of two major cell types: spores and stalk cells (Loomis, 1975). Multicellularity during development is achieved by the expression of at least two types of cell–cell adhesion sites (Gerisch, 1980). One type is sensitive to low concentrations of EDTA. These EDTA-sensitive binding sites are expressed on the cell surface soon after the initiation of development (Knecht et al., 1987; Siu et al., 1988*b*). Cells begin to acquire the EDTA-resistant cell–cell binding sites ~6 h later at the onset of the aggregation stage (Rosen et al., 1973).

A membrane glycoprotein of M_r 80,000 (gp80) has been implicated in mediating the EDTA-resistant binding sites by Muller and Gerisch (1978). They found that the adhesion-blocking activity of a univalent polyclonal antibody preparation can be neutralized by a highly enriched gp80 preparation. Subsequently, several polyclonal and monoclonal antibodies directed against gp80 have been reported (Murray et al., 1981; Lam and Siu, 1982; Ochiai et al., 1982*a*; Murray et al., 1983; Springer and Barondes, 1985). Since they showed cross-reactivity with other glycoproteins, it has been difficult to use them to evaluate the role of gp80 in cell–cell adhesion. Recently, we succeeded in raising an mAb (80L5C4) which is monospecific for gp80 (Siu et al., 1985). 80L5C4 IgG is also capable of inhibiting the EDTA-resistant type of cell–cell adhesion at the aggregation stage of development, thus confirming the role of gp80 in cell adhesion (Siu et al., 1985). Purified gp80, when conjugated to Covaspheres, can mediate the binding of Covaspheres to cells in a stage-specific manner (Siu et al., 1987). Also, gp80-conjugated Covaspheres associate preferentially with the two polar ends of streaming cells and with filopodia. Filopodia are transient surface structures characteristic of aggregation stage cells during development (De Chastellier and Ryter, 1980). Many can be observed making direct contacts with adjacent cells, and they have been implicated in mediating the initial stages of cell–cell adhesion (Choi and Siu, 1987). These observations clearly establish that gp80 is a cell adhesion molecule. Moreover, the binding of labeled gp80 or gp80-conjugated Covaspheres to cells can be inhibited by precoating cells with 80L5C4 Fab, suggesting that gp80 mediates cell–cell binding via homophilic interaction

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(Siu et al., 1987). However, the domain of gp80 involved in homophilic binding and the mechanism of homophilic interaction remain to be determined.

Qualitative and quantitative binding studies indicate that 80L5C4 IgG binds to a single epitope on gp80 (Siu et al., 1985, 1988*a*). The 80L5C4 epitope is sensitive to proteolytic digestion, indicating that the antibody recognizes a region on the polypeptide (Siu et al., 1985, 1988*a*). Recently, we have mapped the 80L5C4 epitope to a protein region near the amino terminus of gp80 (Kamboj and Siu, 1988). Since 80L5C4 IgG is a potent inhibitor of cell-cell adhesion, it is likely that the cell binding site of gp80 is also located in a protein domain close to the 80L5C4 epitope.

To investigate the structure-function relationships of gp80, we have constructed a λgt11 expression library and cloned cDNAs for gp80 (Wong and Siu, 1986). In this report, we determined the nucleotide sequence of a full length gp80 cDNA. Several different segments of the gp80 cDNA were cloned into an expression vector and fusion proteins were purified and assayed for their ability to bind to aggregation stage cells and gp80 immobilized on nitrocellulose. The results of these studies confirm our prediction that the cell-binding site of gp80 resides in the close vicinity of the 80L5C4 epitope and led to the mapping of the cell-binding domain for the region near the amino terminus.

Materials and Methods

Cell Strain and Culture Conditions

The wild-type strain NC4 of *D. discoideum* was used in all experiments. NC4 cells were cultured in association with *Klebsiella aerogenes* and developed as described (Sussman, 1966). Growth phase cells were collected from the partially cleared bacterial lawn, washed free of bacteria, and plated at 2×10^8 cells/ml on filter paper (No. 50; Whatman Inc., Clifton, NJ) for development. Under these conditions, cells began to aggregate between 6 and 8 h and aggregation was essentially complete by 12 h with the formation of round mounds. Culmination began at ~18 h. Alternatively, cells were resuspended at 10^7 cells/ml in 17 mM Na_2/K phosphate buffer, pH 6.4, and shaken at 180 rpm for development.

Purification of gp80

gp80 was purified from the axenic strain AX2. AX2 cells were cultured in liquid medium as described by Cocucci and Sussman (1970) and then developed in 17 mM phosphate buffer, pH 6.4, for 12 h, with cAMP pulsing (2×10^{-8} M final concentration) at 7-min intervals. Cells were then collected and homogenized for gp80 isolation as described previously (Siu et al., 1987). Purified gp80 was stored either in lyophilized form or in solution at −70°C. Samples were usually dialyzed against 0.1% octyl glucoside in PBS before use.

cDNA Cloning

A λgt11 expression library (Wong and Siu, 1986), constructed from cDNA of aggregation stage cells using the method of Young and Davis (1983), was used to screen for full length gp80 cDNA. We have previously characterized a cDNA clone λDdgp80c-19, which contains a 1.0-kb insert and encodes the amino-terminal portion of gp80. A Hae III to Eco RI fragment of 770 bp derived from the λDdgp80c-19 insert was used to screen the library. Positive recombinant phages were subjected to five cycles of plaque purification. Three different recombinant phages, with two containing full length cDNA inserts, were isolated. The gp80 cDNA inserts were subcloned into the Eco RI site of pEMBL18 (Dente et al., 1983). DNA sequencing was performed for both strands using the dideoxy method of Sanger et al. (1977).

Construction of Expression Vectors Containing gp80 cDNA Inserts

Standard recombinant DNA methods were followed according to Maniatis et al. (1982). gp80 cDNAs of different sizes were derived from the insert in the recombinant phage λDdgp80-12. This insert was sequenced in both directions and the DNA sequence shows that it begins with the codon for Val123 at the 5′ end and has a 3′ untranslated sequence of 101 bases. The first gene fusion was constructed by ligating the complete insert of λDdgp80-12 to the unique Eco RI site of the expression vector pRIT2T (Pharmacia Fine Chemicals Inc., Piscataway, NJ) (see Fig. 2). The second one was constructed by fusing the Eco RI–Hind III fragment to the pRIT2T vector. In this case, the Hind III end was blunt-ended by either filling in the ends with the Klenow fragment of DNA polymerase I or treatment with mungbean nuclease, followed by ligation to the Eco RI/Sma I site of the vector. Both constructs yielded identical results. To construct the third gene fusion, an Xmn I–Eco RI fragment was released from λDdgp80-12 and ligated with an appropriate Eco RI linker at the 5′ end before insertion into pRIT2T. The expression vector pRIT2T contains part of the coding sequence for protein A and it is designed for temperature-inducible expression of fusion proteins in a suitable host cell, *Escherichia coli* N4830-1, which contains the temperature-sensitive λ-cI_{857} repressor. When the temperature was shifted from 30 to 42°C, the repressor was inactivated and the λP_R promoter was turned on, thus initiating the expression of fusion protein. Plasmids containing gp80 cDNA inserts in the proper orientation were selected and expressed. Lysates containing the fusion protein were analyzed by SDS-PAGE and immunoblotting using the mAb 80L5C4 or an anti-gp80 polyclonal antibody.

Expression and Purification of Protein A–gp80 Fusion Proteins

Plasmid DNA was used to transform the *E. coli* strain N4830-1. Cells were grown at 30°C in the presence of ampicillin (50 μg/ml) until $OD_{600} = 1.0$ and the culture was shifted to 42°C by mixing the growth medium with an equal volume of medium preheated to 54°C. The culture was incubated at 42°C for 90 min before harvest. Cells were chilled immediately before collection. The cell pellet was resuspending in cold PBS containing 0.05% Tween-20, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. Cells were lysed by sonication and the sample was centrifuged at 25,000 *g* for 20 min. The supernatant was diluted fivefold with the same buffer minus EDTA before applying to an affinity column. Fusion proteins were usually purified by two passages through an IgG–Sepharose 6FF affinity column (Pharmacia Fine Chemicals Inc.). Fusion proteins were eluted with 0.5 M acetic acid, pH 3.4. Fractions containing fusion protein were neutralized with 0.5 M Na_2HPO_4, pooled, and dialyzed against PBS.

To raise polyclonal antibodies against fusion proteins, 100 μg of purified fusion protein was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into New Zealand white rabbits. Animals were boosted four times with 50 μg of fusion protein emulsified in Freund's incomplete adjuvant at 2-wk intervals. Serum samples were collected after the second boost.

PAGE

SDS-polyacrylamide slab gels were prepared according to Laemmli (1970). Protein samples were solubilized and reduced in 2% SDS, 8 M urea, and 2% β-mercaptoethanol by boiling for 5 min. Electrophoresis was carried out using the minigel apparatus (Bio-Rad Laboratories, Richmond, CA) at a constant current of 20 mA for 40 min. The following proteins were used as molecular mass markers: myosin (200 kD), phosphorylase B (97 kD), BSA (68 kD), ovalbumin (42 kD), and α-chymotrypsinogen (25 kD). Gels were subjected to silver staining using the method of Morrissey (1981).

Binding of Fusion Proteins to Intact Cells

Affinity-purified protein A/gp80 fusion proteins and gp80 were dialyzed against 0.1% octyl glucoside in PBS. Samples were radioiodinated using the chloramine T method, as previously described (Siu et al., 1977). Different concentrations of the ^{125}I-labeled protein were prepared in phosphate buffer immediately before addition to cell suspension, giving a final detergent concentration ≤0.005%. Cells were developed on filter pads for 10 h and then collected for the binding assay as previously described (Siu et al., 1987). Cells were washed and resuspended in 17 mM phosphate buffer, pH 6.4, and resuspended at 5×10^6 cells/ml in the same buffer plus 5 mM

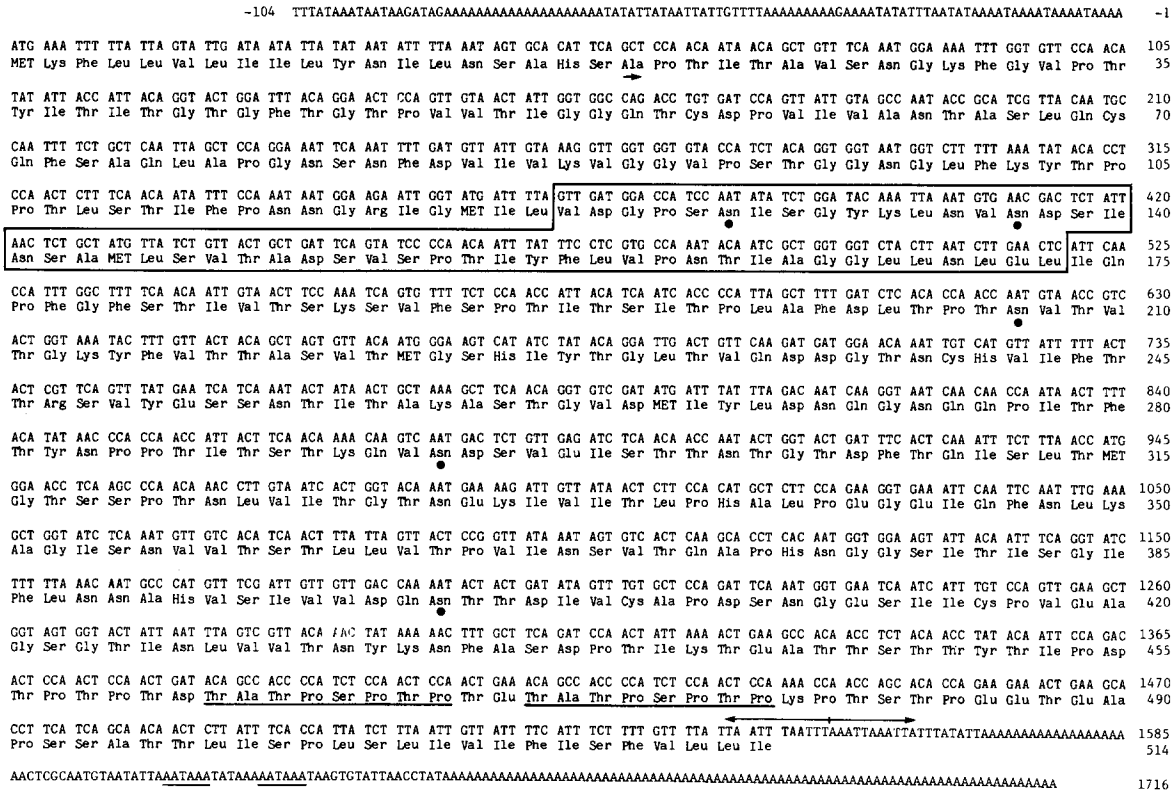

Figure 1. Nucleotide sequence of the full length cDNA and the deduced amino acid sequence of gp80. Nucleotides are numbered at the right and labeled positively in the coding and 3′ untranslated regions and negatively in the 5′ untranslated regions. Amino acids are also numbered at the right. The arrow under Ala at amino acid position 20 marks the beginning of the mature protein. The five potential N-glycosylation sites are marked by a black dot. The two octapeptide repeats and sequences that may serve as polyadenylation signals are underlined. The domain containing cell-binding activity is boxed.

EDTA. Samples of 0.1 ml were incubated with different concentrations of ^{125}I-labeled protein for 45 min at 4°C on a platform shaker. Unbound material was removed by washing twice with cold phosphate buffer. As a control, cells were incubated with similar concentrations of ^{125}I-labeled protein A and the amount of bound radioactivity was used to estimate the nonspecific background binding of fusion proteins. Background binding generally represented <5% of the total amount of radioactive counts bound.

Competition of Fusion Protein Binding to Cells by mAbs

10-h cells were washed and resuspended at 2 × 10^7 cells/ml in 17 mM phosphate buffer, pH 6.4, containing 5 mM EDTA and then precoated with different concentrations of 80L5C4 IgG for 15 min at 4°C. Cells were washed once and resuspended at 5 × 10^6 cells/ml in EDTA/phosphate buffer containing 0.25 mg/ml goat anti-mouse IgG Fab (Cappel Laboratories, Inc., Cochranville, PA) for 15 min at 4°C. Samples were briefly vortexed before the addition of ^{125}I-fusion protein, followed by a 45-min incubation period at 4°C.

Filter Binding Assay

Fusion protein or gp80 samples were immobilized on nitrocellulose disks, which were blocked with 5% skim milk and 0.05% Tween-20 in PBS. The disks were then incubated with different amounts of ^{125}I-labeled gp80 or fusion protein for 30 min at room temperature. Background binding was determined by the inclusion of a blank disk in the incubation mixture. At the end of the incubation period, disks were washed with at least three changes of 5% skim milk followed by two changes of 0.05% Tween-20 in PBS. Disks were air dried and counted in a gamma counter. Background radioactivity was subtracted from the counts bound on the corresponding sample disk. Generally, it represented 25–35% of the total amount of radioactivity bound on the sample disk.

Competition experiments using fusion proteins were carried out by immobilizing fixed amounts of gp80 (0.1 μg) on nitrocellulose disks, which were first blocked with 5% skim milk in PBS and 0.05% Tween-20. Then the disks were incubated with a mixture of ^{125}I-labeled gp80 and unlabeled fusion protein at different molar ratios, with the concentration of the labeled gp80 kept constant at 200 ng/ml. Subsequent steps were identical to the assay protocol.

Cell Cohesion Assay

Cell–cell adhesion was measured using a method modified from Gerisch's (1961) roller tube assay (Lam et al., 1981). Cells were dissociated and suspended at 2–3 × 10^6 cells/ml in 17 mM phosphate buffer, pH 6.4, containing 10 mM EDTA. 200-μl samples were rotated vertically on a platform shaker at 180 rpm. Cells were counted at different time intervals using a hemacytometer. Both singlets and doublets were scored as unaggregated cells. The percentage of aggregated cells was calculated by dividing the difference of the total number of cells and the number of single cells by the total number of cells. Cells were also reassociated in the presence of different concentrations of fusion protein to assess the effect of fusion proteins on cell–cell binding.

To block cell reassociation with antibodies directed against fusion protein, the method described by Springer and Barondes (1980) was used. Cells were suspended at 2–3 × 10^7 cells/ml in phosphate buffer containing 10 mM EDTA and rabbit anti-fusion protein antiserum. The sample was incubated at 4°C for 20 min. Cells were vortexed briefly and diluted 1:10 in phosphate buffer containing 10 mM EDTA and goat anti-rabbit IgG Fab (0.5 mg/ml) (Cappel Laboratories). Cells were incubated for another 10 min on ice and then vortexed briefly to dissociate the cells. The sample was then rotated on a platform shaker at 180 rpm at room temperature and the reassociation of cells was monitored microscopically at regular intervals. All experiments were repeated one to three times and almost identical results were obtained in all cases.

Protein Determination

Protein determination was carried out with the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), using crystalline BSA as the standard.

Results

cDNA Sequence and Deduced Structure of gp80

Three recombinant phages carrying nearly full length cDNA inserts were isolated and characterized. The longest cDNA insert was sequenced in both directions and results were confirmed by partial sequences obtained from the other clones. A total of 1,820 bp were sequenced, containing a single open translational reading frame (Fig. 1). The 5′ and 3′ flanking regions are extremely AT rich and the initiation methionine codon is preceded by four A nucleotides, characteristic of *D. discoideum* translational starts (Kimmel and Firtel, 1983). The coding region spans 1,542 nucleotides, terminating at a TAA codon. The 3′ flanking region has two poly-A tracks. Two consensus polyadenylation signals (AATAAA) are situated 17 and 29 nucleotides upstream from the second poly-A track, suggesting that the second polyadenylate tail was added posttranscriptionally whereas the first oligo-A track was transcribed from the gene.

The open reading frame codes for a protein of 514 amino acids with M_r 53,703 (Fig. 1). An analysis of the deduced primary structure of gp80 shows that the amino terminus has a stretch of hydrophobic amino acids, serving as the leader sequence. The first 19 amino acids are cleaved off in the mature glycoprotein, which starts with Ala at the amino terminus (Stadler et al., 1982). The deduced protein shows three major structural domains. The carboxy-terminal region is composed of 18 hydrophobic amino acids, which is a potential membrane-spanning domain. Adjacent to the carboxy-terminal domain is a hydrophilic region, located between amino acid positions 431 and 496. Within this domain is a unique Pro-rich segment, which contains two 8-amino acid-repeats. Since many of the amino acids in this domain have high probabilities for turns, it may take the form of a relatively rigid stalk structure. The third domain consists of the amino-terminal 434 amino acids. This domain is characterized by many short stretches of hydrophobic segments alternated by hydrophilic residues and it may have a highly folded globular structure. There are five potential N-glycosylation sites within this domain.

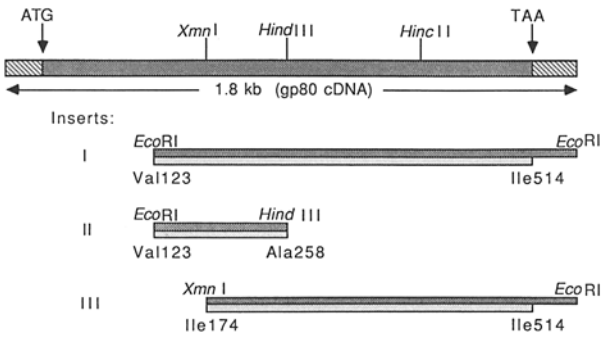

Figure 2. gp80 cDNA inserts cloned into the pRIT2T expression vector. Three different gp80 cDNA inserts were fused in frame to the carboxyl-terminal coding region of the protein A gene as described in Materials and Methods. The protein A gene codes for a 31-kD polypeptide.

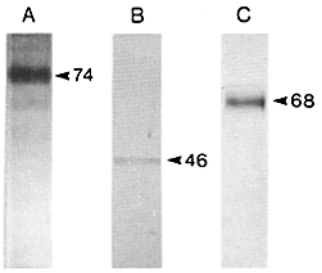

Figure 3. Gel profiles of protein A/gp80 fusion proteins. Transformants carrying the different gp80 inserts were induced to produce the protein A/gp80 fusion protein. Fusion proteins were purified by two passages through an IgG-Sepharose affinity column and samples were analyzed by SDS-PAGE. The slab gel was subjected to silver staining. Lanes *A*, *B*, and *C* are PA80I, PA80II, and PA80III, respectively. Values given to the right of the gel lanes represent M_r in kD.

Expression and Purification of Fusion Proteins

We have recently found that the cell adhesion-blocking mAb 80L5C4 recognizes an epitope in the amino-terminal domain of gp80 between amino acid residues 123 and 173 (Kamboj and Siu, 1988). It is, therefore, likely that the cell binding site of gp80 resides in the close vicinity of this epitope. To test this hypothesis, gene fusions containing three different cDNA fragments were constructed using the expression vector pRIT2T (Fig. 2). These gp80 cDNA fragments were cloned into the unique Eco RI site of pRIT2T. Transformants carrying these recombinant plasmids produced soluble fusion proteins which contained the amino-terminal 31-kD fragment of Staphylococcal protein A and a portion of the gp80 polypeptide. The plasmid carrying insert I (Eco RI to Eco RI fragment) coded for a protein A/gp80 fusion protein (PA80I) of 74 kD, which contained a gp80 fragment between amino acid residues 123 and 514 (Fig. 3). Transformants carrying insert II produced a fusion protein (PA80II) of 46 kD, which contained a gp80 fragment between residues 123 and 258. Those carrying insert III produced a fusion protein (PA80III) of 68 kD, which contained a gp80 fragment between residues 174 and 514. When subjected to immunoblot analysis, both PA80I and PA80II reacted with the mAb 80L5C4, while PA80III reacted with a polyclonal antibody but not 80L5C4 (data not shown).

To purify these three fusion proteins for further characterization, cell lysates were passed twice through an IgG-Sepharose affinity column. The purified protein preparations were subjected to SDS-PAGE analysis and the silver-stained gel profiles were shown in Fig. 3. Only slight degradation was observed in these preparations and all three samples showed major bands corresponding closely to the expected molecular sizes of the three protein A/gp80 fusion proteins.

Binding of Fusion Proteins to Intact Cells

To test whether the fusion proteins have cell-binding activity, they were radioiodinated and assayed for their ability to bind to aggregation stage cells. Fusion protein samples were solubilized in 0.1% octyl glucoside before use. Cells were incubated with the ^{125}I-labeled fusion protein at 4°C for 45

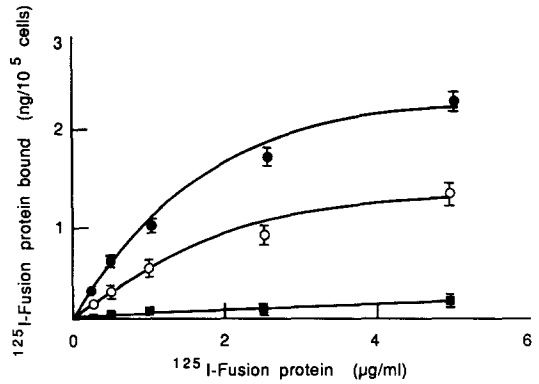

Figure 4. Binding of fusion proteins to aggregation stage cells. Purified fusion proteins were dialyzed against 0.1% octyl glucoside and labeled with ^{125}I using chloramine T. NC4 cells were developed in liquid medium for 10 h and then collected for the binding assay. Cells were washed and resuspended in 5 mM EDTA and phosphate buffer at 5 × 10^6 cells/ml. Cells samples (0.1 ml) were incubated with different amounts of ^{125}I-labeled protein for 45 min at 4°C. Nonspecific background binding was estimated by the binding of different concentrations of ^{125}I-labeled protein A (sp act, 5.1 × 10^4 cpm/ng) to similar cell samples and the amount bound was substracted from the corresponding samples. (●) PA80I (sp act, 2.4 × 10^4 cpm/ng); (○) PA80II (sp act, 5.0 × 10^4 cpm/ng); and (■) PA80III (sp act, 2.6 × 10^4 cpm/ng). Assays were performed in triplicate and the means ± SD were plotted.

min in the presence of 5 mM EDTA to slow down the kinetics of cell reassociation and thus minimize the extent of nonspecific trapping. Background binding due to protein A was also estimated by incubating cell samples with different concentrations of ^{125}I-labeled protein A. After subtracting the background binding from each corresponding sample, the amount of fusion protein bound per cell was estimated

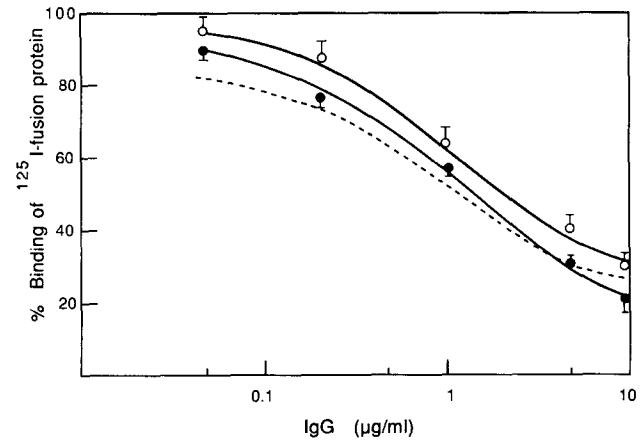

Figure 5. Inhibition of fusion protein binding to intact cells by 80L5C4 IgG. Aggregation stage cells (10 h) were resuspended at 2 × 10^7 cells/ml in phosphate buffer containing 5 mM EDTA and then precoated with different concentrations of 80L5C4 IgG at 10 μg/ml for 15 min at 4°C. Cells were resuspended at 5 × 10^6 cells/ml in the same buffer containing 0.25 mg/ml goat anti-mouse IgG Fab before the addition of ^{125}I-labeled fusion protein (2 μg/ml). The rest of the binding assay was carried out as described in Fig. 4. (●) PA80I; (○) PA80II; and (---) gp80. Values represent means ± SD with n = 3.

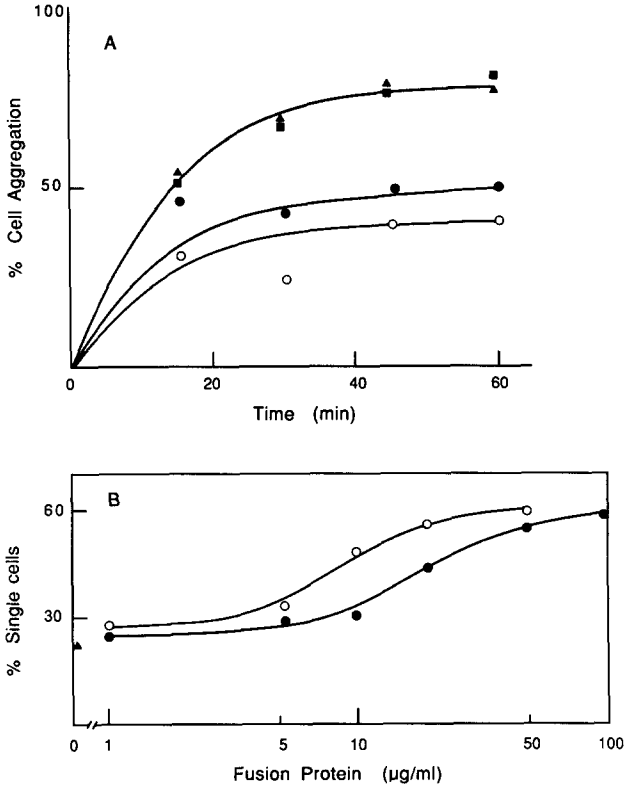

Figure 6. Inhibition of cell-cell adhesion by fusion proteins. Cells were developed on filter pads for 10 h and then collected for the cell cohesion assay. Cells were suspended at 2.5 × 10^6 cells/ml in 10 mM EDTA/phosphate buffer. At 0 min, either octyl glucoside or fusion protein was added and the sample was vortexed briefly to disperse all aggregates. Samples (0.2 ml) were rotated vertically at 180 rpm and reassociation of cells was monitored microscopically at regular intervals. (*A*) Cells were reassociated in the presence of 50 μg/ml PA80I (●), 50 μg/ml PA80II (○), or 50 μg/ml PA80III (■). (▲) Control sample reassociated in the presence of 0.005% octyl glucoside. (*B*) Cell samples were reassociated in the presence of different concentrations of PA80I (●) or PA80II (○). The data were taken from the 45-min point.

(Fig. 4). The binding of fusion proteins PA80I and PA80II to cells were both dosage dependent and saturable. The number of molecules bound per cell was estimated to be 1.7 × 10^5 and 1.6 × 10^5 for PA80I and PA80II, respectively. On the other hand, the binding of PA80III was essentially at the background level. The data thus indicate that the cell-binding domain is retained in PA80I and PA80II but not in PA80III and probably resides within the segment between Val123 and Leu173.

To further characterize the cell-binding activity of PA80I and PA80II, we tested whether they bound specifically to gp80 molecules on the cell surface. Cells were precoated with different amounts of the anti-gp80 mAb 80L5C4 before carrying out the binding assay with fusion proteins. The binding of ^{125}I-labeled PA80I or PA80II was inhibited in a dosage-dependent manner (Fig. 5). In both cases, the amount of IgG required to achieve 50% inhibition was within a twofold difference in comparison with the control using ^{125}I-labeled gp80 for binding. These results indicate that the binding of PA80I and PA80II to cells is similar to that of solu-

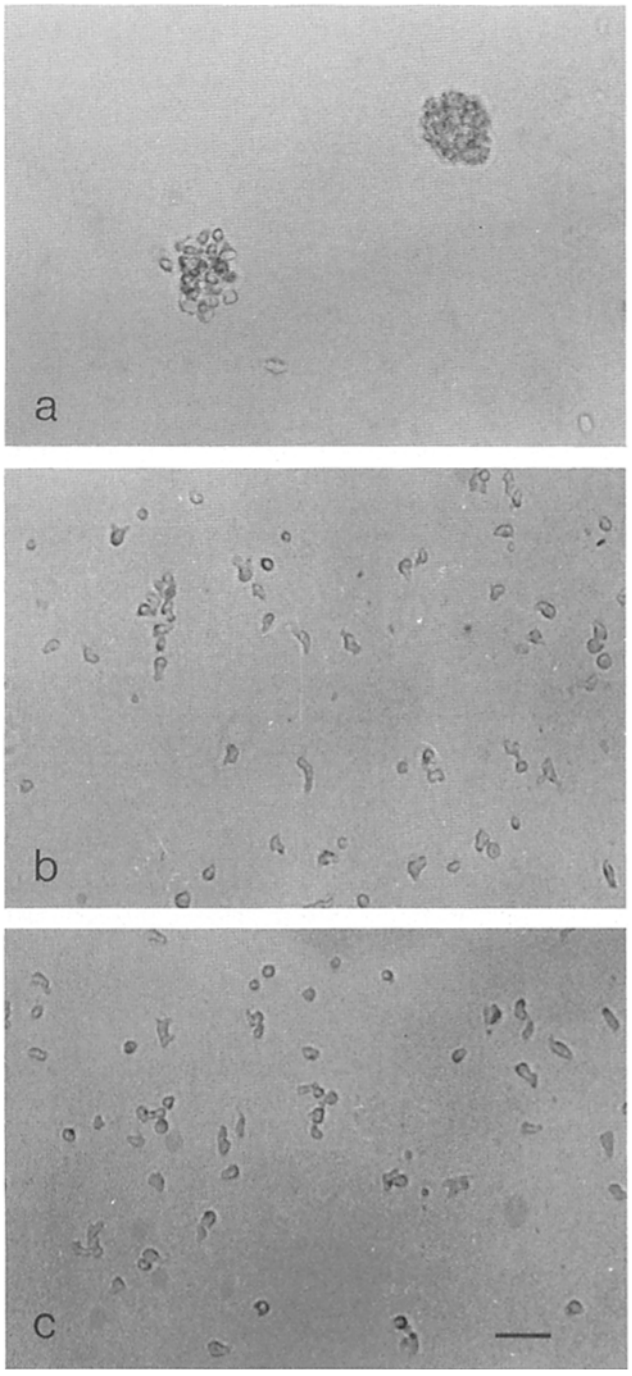

Figure 7. Phase micrographs of cells reassociated in the presence or absence of fusion proteins. 10-h cells were reassociated in EDTA phosphate buffer as described in Fig. 6 and phase micrographs were taken at 30 min of reassociation. (*a*) Cells reassociated in 0.005% octyl glucoside, (*b*) cells reassociated in 100 μg/ml PA80I, and (*c*) cells reassociated in 50 μg/ml PA80II. Bar, 20 μm.

ble gp80 to surface-associated gp80 on aggregation stage cells.

Effect of Fusion Proteins on Cell–Cell Adhesion

If both fusion proteins PA80I and PA80II exist in monomeric form in solution and each monomer consists of only one

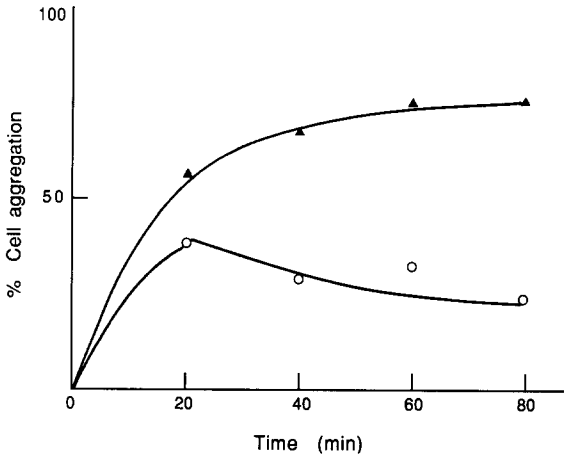

Figure 8. Inhibition of cell reassociation by anti-PA80II antibody. Aggregation stage cells were incubated with the anti-PA80II antiserum (1:20 dilution) and then resuspended in goat anti-rabbit IgG Fab at 2.5 × 10^6 cells/ml. Samples were rotated at 180 rpm on a platform shaker for cell reassociation. (○) Sample precoated with the anti-PA80II antibody; (▲) control sample precoated with preimmune rabbit serum.

gp80 binding site, binding of either fusion protein to cells should block cell–cell adhesion mediated by the homophilic interaction between membrane-associated gp80 molecules. This was tested by incubating aggregation stage cells with different concentrations of detergent solubilized PA80I or PA80II before performing the cell cohesion assay. Cell reassociation was significantly inhibited in the presence of either fusion protein at 50 μg/ml (Fig. 6A). However, the inclusion of PA80III at the same concentration had no appreciable effect on cell reassociation. The control in which cells were incubated with an equivalent amount of octyl glucoside showed the normal kinetics of reassociation. The inhibitory effect of both fusion proteins PA80I and PA80II was dosage dependent (Fig. 6B). At 100 μg/ml of PA80I or 50 μg/ml of PA80II, ~60% of the cells failed to reform aggregates and their effect was halved at 20 μg/ml and 10 μg/ml, respectively. Since the molecular mass of PA80I was ~60% higher than that of PA80II, the molar concentrations required to achieve 50% inhibition were quite comparable for these two fusion proteins. The effect of both fusion proteins became negligible at a concentration of 1 μg/ml.

The morphology of cell aggregates formed in the presence of PA80I and PA80II was quite different from that of the control (Fig. 7). Aggregates formed in the control sample were indistinguishable from those formed in the absence of detergent, while most of the aggregates were significantly smaller in samples incubated with the fusion protein.

Inhibition of Cell–Cell Adhesion by Antifusion Protein Antibody

The observation that the fusion protein PA80II was able to inhibit cell reassociation suggests that it contains the cell-binding site of gp80. It is, therefore, of interest to test whether antibodies directed against PA80II would have any effect on cell aggregation. Antibodies against PA80II were raised in rabbit and added to aggregation stage cells before the cohesion assay. Cell reassociation was inhibited by

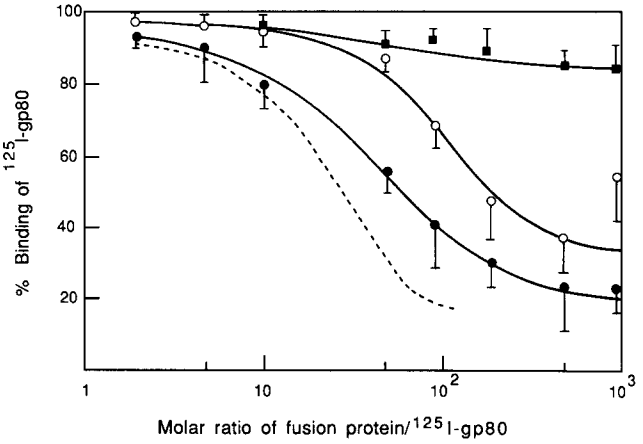

Figure 9. Competition of ^{125}I-gp80 binding to immobilized gp80 by fusion proteins. Samples of 0.1 μg native gp80 were immobilized on nitrocellulose disks. After blocking with 5% skin milk in PBS, the disks were incubated with different molar ratios of ^{125}I-labeled gp80 (sp act, 1.2×10^4 cpm/ng) and unlabeled fusion protein, with the labeled gp80 kept constant at 200 ng/ml. Background binding was estimated by the inclusion of a blank disk in the incubation mixture and subtracted from the bound radioactivity on the corresponding sample disk. Competitive inhibition was carried out with PA80I (●), PA80II (○), protein A (■), and gp80 (---). Samples were done in triplicate and the means ± SD were plotted. In the control where no competitor was added, the amount of radioactivity bound was 1.1×10^5 cpm.

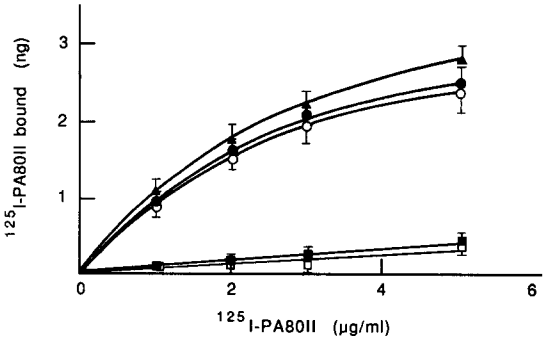

Figure 10. Homophilic interaction of PAII. Samples of 0.1 μg PA80II (○), 0.2 μg PA80I (●), 0.2 μg gp80 (▲), 0.2 μg PA80III (■), or 0.1 μg protein A (□) were immobilized on nitrocellulose disks. Filter binding assays were carried out in triplicate with different concentrations of ^{125}I-labeled PA80II (sp act, 5×10^4 cpm/ng) as described in Materials and Methods. Values represent means ± SD.

~70% in the presence of the antibody (Fig. 8). The result is consistent with the notion that the gp80 fragment in PA80II has cell-binding activity.

Competition of ^{125}I-labeled gp80 Binding to Immobilized gp80 by Fusion Protein

To provide direct evidence that fusion proteins PA80I and PA80II can indeed bind to gp80, an in vitro filter binding assay was used to test their ability to compete for gp80 binding. We have previously demonstrated that ^{125}I-labeled gp80 binds quantitatively to gp80 immobilized on nitrocellulose filter and the binding can be competed off by a 50- to 100-fold excess of cold gp80 (Siu et al., 1987). Similar studies were carried out using either PA80I or PA80II as the competitor and the results are shown in Fig. 9. A 70-fold excess of PA80I or a 200-fold excess of PA80II was required to achieve 50% competition. In comparison, native gp80 achieved the same extent of competition at 25-fold excess, while protein A did not have significant inhibitory effect on gp80–gp80 interaction.

Homophilic Binding of Fusion Protein PA80II

It is, therefore, evident that the cell-binding site of gp80 is located within the gp80 fragment of PA80II. It remained to be determined whether PA80II was interacting with a receptor element in the same region or in a different part of gp80. To resolve this, the ability of ^{125}I-labeled PA80II to bind to PA80II immobilized on nitrocellulose disks was assayed. For comparison, approximately equal molar amounts of protein A, PA80I, PA80III, and gp80 were immobilized separately on nitrocellulose disks for binding with ^{125}I-labeled PA80II.

Fig. 10 shows that PA80II was able to undergo homophilic binding with immobilized PA80II in a dosage-dependent manner and the binding was saturable. Almost identical binding curves were obtained for both PA80I and gp80 (Fig. 10) indicating that essentially the same number of PA80II molecules were bound in each case. However, the binding of labeled PA80II to immobilized PA80III was essentially at the background level, similar to results with bound protein A.

Discussion

Full length cDNAs for the cell–cell adhesion molecule gp80 were isolated and sequenced. An analysis of the gp80 cDNA sequence shows many characteristics common to other *D. discoideum* genes (Kimmel and Firtel, 1983). The coding region makes use of mainly codons with either an A or T in the third base. The 5′ and 3′ flanking regions are characterized by homopolymers of A and T. Since we have obtained several clones with recombined fragments at either 5′ or 3′ ends of the insert, recombination between different cDNA fragments probably occurs frequently at these poly-A tracks.

Our cDNA sequence for gp80 is similar but not identical to that reported by Noegel et al. (1986). In the coding region, differences are located at nucleotide positions 480 (G), 647 (T), 773 (C), 774 (T), 779 (C), and 780 (T), which they reported T, G, T, C, T, and C, respectively. These differences would result in three amino acid changes, involving Val, Ala, and Gly. In addition, our sequence upstream from nucleotide position −39 is completely different from the one they reported. We have obtained identical sequences from two independently isolated inserts of different sizes. Our cDNA sequence has also been confirmed by sequencing the genomic DNA for gp80 (Lam, T. Y., and C.-H. Siu, unpublished observations). While our sequence is extremely AT rich (95%), theirs is relatively GC rich (40%), which is typical of a coding sequence and thus might have resulted from recombining with another cDNA fragment at the poly-A track beginning at nucleotide position −39.

We have made use of the expression vector pRIT2T which allowed us to obtain protein A/gp80 fusion proteins in soluble form. Previous attempts using other expression vectors have often resulted in the precipitation of the fusion protein,

which was difficult to solubilize for subsequent binding studies. Another advantage of this vector is that protein A/gp80 fusion proteins can be easily purified by passage over an IgG-affinity column. Although some degradation was observed in these preparations, the cell-binding activity of the fusion protein appeared to be relatively stable.

Several in vitro binding assays have been performed with three protein A/gp80 fusion proteins. Since all three fusion proteins harbor either the cell-binding site or the hydrophobic carboxy-terminal region of gp80, they have a tendency to aggregate in the absence of detergent. Prior treatment with a mild detergent is necessary for all the assays. Two of the fusion proteins, PA80I and PA80II, can bind to aggregation stage cells. In both cases, 1.6–1.7 $\times$ 10^6 molecules are bound per cell. This value agrees closely with the number of gp80 molecules expressed on cells at this stage (Siu et al., 1988*a*). The binding of PA80I and PA80II to cells is inhibited by precoating cells with the mAb 80L5C4, supporting the idea that both fusion proteins are interacting with gp80 molecules on the cell surface. The binding of either one of these two fusion proteins to aggregation stage cells has an inhibitory effect on cell reassociation. This is consistent with the notion that PA80I and PA80II are competing for the cell-binding site on gp80.

Both PA80I and PA80II are capable of competing for gp80 binding in the filter binding assay. Despite the fact that PA80I contains only part of the gp80 protein moiety, native gp80 is only two to three times more effective than PA80I. Even in the case of PA80II, a molar ratio that is only eightfold higher than native gp80 is required to achieve 50% competition. Since PA80II contains a much shorter fragment of gp80 than PA80I, it is conceivable that the conformation of its cell-binding domain is less stable, resulting in a lower concentration of molecules with the proper gp80-binding site and thus a lower apparent affinity for native gp80. The protein A portion of these fusion proteins might also contribute a certain amount of steric hindrance to the cell-binding domain.

Results of the binding studies lead to the mapping of a cell-binding domain in gp80 to a 51 amino acid region between residues 123 and 173. It is of interest to note that it corresponds exactly to the region which harbors the 80L5C4 epitope (Kamboj and Siu, 1988). Since the anti-gp80 mAb 80L5C4 is a potent inhibitor of cell–cell adhesion, it is likely that 80L5C4 IgG recognizes this cell-binding site or an epitope close to it. The region between Val123 and Leu173 consists of a hydrophilic stretch of amino acids followed by a relatively hydrophobic segment. There are two potential N-glycosylation sites within this region. Therefore, this part of the gp80 molecule is most likely exposed to the surface, allowing it to participate directly in cell–cell binding. Although the precise mechanism of homophilic binding between two gp80 molecules is not known, our data suggest that the major binding force is contributed by protein–protein interaction. Future studies focusing on smaller segments of this region should lead to a better understanding of the binding mechanism.

Since PA80II contains the smallest gp80 fragment, it was used to determine whether its binding site recognizes one or more receptor elements on gp80. PA80II binds equally well with immobilized PA80I, PA80II, and gp80, but not with PA80III. It is, therefore, evident that PA80II binds to a single site on gp80 and that the homphilic binding site of gp80 is also located between Val123 and Leu173. Since labeled PA80III does not bind gp80 (Kamboj, R., and C.-H. Siu, unpublished results), it precludes the presence of another binding site between Ile174 and Ile514.

We have previously proposed three different models for gp80–gp80 interaction (Siu et al., 1987). One of them requires direct protein–protein interaction, while the other two involve the participation of oligosaccharide(s) in the binding activity. The fact that soluble PA80II is capable of undergoing homophilic binding with immobilized PA80II (Fig. 10) clearly demonstrates that the cell-binding site mapped between Val123 and Leu173 is one that involves only protein–protein interaction. Our results suggest that there are no other protein–protein interacting sites on gp80, but they do not rule out the possibility of a second binding site involving protein–carbohydrate interaction.

The role of carbohydrate in gp80 function in vivo is still an unsettled issue. gp80 has two types of N-linked oligosaccharides. Type 1 is heavily sulfated while type two is not (Hohmann et al., 1985). Type 2 carbohydrate is immunodominant and many of the reported anti-gp80 antibodies are directed against it. Several mAb that belong to this class exhibit inhibitory effect on cell reassociation (Ochiai et al., 1982*a*; Siu et al., 1985; Springer and Barondes, 1985). Therefore, the type 2 carbohydrate of gp80 has been implicated in mediating cell–cell adhesion. The role of type 2 carbohydrate in gp80 has been reevaluated recently using strains with mutations in the *mod*B locus, which affects a posttranslational modification of certain membrane glycoproteins. Mutations in this locus result in the loss of the type 2 carbohydrate in gp80 (Murray et al., 1984) and mutant cells accumulate a mutant gp80 of lower molecular size (Gerisch et al., 1985; Siu et al., 1985). The amount of gp80 expressed in these mutant cells is considerably lower than that of the parental strain (Siu and Lam, 1988). However, they are capable of forming EDTA-resistant binding sites and undergo normal morphogenesis. Cell–cell adhesion among *mod*B mutants can be blocked by the mAb 80L5C4 (Siu and Lam, 1988). These observations are consistent with the idea that the cell-binding activity of gp80 resides in its protein moiety and that the type 2 carbohydrate does not directly participate in cell–cell adhesion. Since the cell-binding domain potentially has two oligosaccharide side chains, it is conceivable that binding of immunoglobulin molecules to carbohydrate epitopes in this region would sterically hinder the cell-binding activity.

Although it is evident that the cell-binding activity of gp80 resides in the protein moiety and not the carbohydrate moiety, treatment of cells with tunicamycin, which inhibits N-glycosylation, has been found to block cell–cell adhesion (Lam and Siu, 1982; Ochiai et al., 1982*b*; Yamada et al., 1982). Hirano et al. (1983) found that EDTA-resistant cell–cell binding sites in tunicamycin-treated cells can be restored when tunicamycin is added in the presence of the protease inhibitor leupeptin. It is, therefore, likely that the involvement of this carbohydrate moiety in cell adhesion is indirect in that it acts to protect the protein moiety of gp80 from proteolytic degradation (Hirano et al., 1983; Hohmann et al., 1987). Since the type 1 carbohydrate of gp80 is heavily sulfated (Hohmann et al., 1985), it is conceivable that they may play a modulatory role in cell–cell adhesion similar to the model proposed for the neural cell adhesion molecule (Edel-

man, 1983). The role of type 1 carbohydrate should be further evaluated when specific antibodies directed against it become available.

In addition to gp80, another glycoprotein (gp69/73) has been implicated in mediating the EDTA-resistant type of cell–cell adhesion (Brodie et al., 1983). It is possible that several separate but interdependent adhesion systems are operating at the aggregation stage of development. In this regard, it is of interest to note that endoglycosidase H–resistant glycopeptides derived from 8-h cells are able to inhibit the reassociation of aggregation stage cells (Ziska and Henderson, 1988). Since these glycopeptides were derived from a mixture of plasma membrane glycoproteins, their relationship to gp80 or gp69/73 remains to be clarified. It is also possible that these glycopeptides may belong to a cell adhesion molecule yet to be identified. The purification and characterization of these molecules will be important to our future endeavor to dissect the complex phenomenon of cell–cell interaction.

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