Involvement of Cell Surface Phosphatidylinositol-anchored Glycoproteins in Cell–Cell Adhesion of Chick Embryo Myoblasts

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Abstract. During myogenesis myoblasts fuse to form multinucleate cells that express muscle-specific proteins. A specific cell–cell adhesion process precedes lipid bilayer union during myoblast fusion (Knudsen, K. A., and A. F. Horwitz. 1977. Dev. Biol. 58:328–338) and is mediated by cell surface glycoproteins (Knudsen, K. A., 1985. J. Cell Biol. 101:891–897). In this paper we show that myoblast adhesion and myotube formation are inhibited by treating fusion-competent myoblasts with phosphatidylinositol-specific phospholipase C (PI-PLC). The effect of PI-PLC on myoblast adhesion is dose dependent and inhibited by D-myo-inositol 1-mc.nophosphate and the effect on myotube formation is reversible, suggesting a specific, nontoxic effect on myogenesis by the enzyme. A soluble form of adhesion-related glycoproteins is released from fusion-competent myoblasts by treatment with PI-PLC as evidenced by (a) the ability of phospholipase C (PLC)-released material to block the adhesion-perturbing activity of a polyclonal antiserum to intact myoblasts; and (b) the ability of PLC-released glycoprotein to stimulate adhesion-perturbing antisera when injected into mice. PI-PLC treatment of fusion-competent myoblasts releases an isoform of N-CAM into the supernate, suggesting that N-CAM may participate in mediating myoblast interaction during myogenesis.

The formation of mature skeletal muscle involves the fusion of single muscle precursor cells (myoblasts) to generate multinucleate syncytia (myotubes) (for reviews see Bischoff, 1978; Wakelam, 1985). In vitro, lipid bilayer union of the myoblasts is preceded by a specific cell–cell adhesion step that is necessary, but not sufficient, for myoblast fusion (Knudsen and Horwitz, 1977, 1978). Myoblast adhesion, like the adhesion of other cells types (Takeichi, 1977; Urushihara et al., 1977; Grunwald et al., 1980; Magnani et al., 1981; Thomas and Steinberg, 1981; Brackenbury et al., 1981; for review see Damsky et al., 1984), appears to include both Ca++-independent and Ca++-dependent molecular mechanisms (Gibralter and Turner, 1985; Pizey et al., 1988). While myoblast adhesion can occur in the absence of Ca++ fusion of myoblasts to form myotubes is dependent upon the presence of Ca++ (Shainberg et al., 1969).

Previously, we showed that myoblast adhesion is mediated by cell surface glycoproteins and that the glycoproteins involved in Ca++-dependent adhesion appear to have a Ca++-dependent conformation (Knudsen, 1985). In this respect myoblast adhesion appears similar to other Ca++-dependent cell–cell adhesion systems (for reviews see Damsky et al., 1984; Takeichi, 1988) mediated by molecules such as uveomorulin (Hyafil et al., 1980, 1981; Vestweber and Kemler, 1985; Ringwald et al., 1987), L-CAM (Bertolotti et al., 1980; Gallin et al., 1983, 1985, 1987), GP 120/80 (Damsky et al., 1983; Wheelock et al., 1987), E-cadherin (Yoshida-Noro et al., 1984), N-cadherin (Hatta and Takeichi, 1986), P-cadherin (Hatta and Takeichi, 1986), N-cal-CAM (Bixby et al., 1987), A-CAM (Volk and Geiger, 1984), and Arc-l (Behrens et al., 1985).

Recently, the modification of proteins by covalently attached lipids has captured the interest of many investigators (for review see Sefton and Buss, 1987). Some proteins have a covalently attached glycosyl-phosphatidylinositol moiety that serves to anchor the polypeptide portion of the molecule to the cell surface lipid bilayer (for reviews see Low, 1987; Low and Saltiel, 1988; Ferguson and Williams, 1988). Included in this group of proteins are two cell-cell adhesion molecules that can be anchored to the cell surface via the phosphatidylinositol-glycan moiety—the Ca++-independent neural cell adhesion molecule, N-CAM (Hemperly et al., 1986; He et al., 1986), and the lymphocyte LFA-3 cell adhesion glycoprotein (Dustin et al., 1987). N-CAM, a member of the immunoglobulin family (Cunningham, 1986; Cunningham et al., 1987), is expressed by muscle cells (Grumet et al., 1982; Rutishauser et al., 1983), has a muscle-specific sequence in the extracellular domain in human muscle cells (Dickson et al., 1987), undergoes changes in its isoforms during myogenesis (Moore et al., 1987), is found at the neuromuscular junction (Reiger et al., 1985), and is thought to mediate neuromuscular interaction (Grumet et al., 1982; Rutishauser et al., 1983; for review see Edelman, 1983). Thy-1 antigen is another glycosyl-phosphatidylinositol–an-
chored protein (Homans et al., 1988; also see Low, 1987; Cross, 1987; Low and Saltiel, 1988; Ferguson and Williams, 1988) expressed by rodent muscle during myogenesis (Lesley and Lennon, 1977), but it does not appear to play a role in myoblast fusion (Schweitzer et al., 1987). The extracellular domain of the above phosphatidylinositol-anchored proteins can be released from cells by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) (for review see Low, 1987).

Some years ago Nameroff et al. (1973) demonstrated that treating muscle cell cultures with phospholipase C (PLC) from Clostridium welchii inhibited the fusion of myoblasts. The authors speculated that PLC treatment altered the surface of myoblasts in a way that prevented the cells from recognizing each other. In a later publication Nameroff and Munar (1976) reported that PLC-treated myoblasts continued to line up on the culture dish, even though they did not fuse. The authors interpreted this to mean that myoblast recognition and fusion were separate events and that PLC inhibited myoblast fusion but not cell–cell recognition.

In the studies reported here we investigated the effect of PI-PLC on cell–cell adhesion of fusion-competent chick embryo myoblasts.

Materials and Methods

Materials

PLC (type III from Bacillus cereus) and d-myo-inositol 1-monophosphate (IP.) were purchased from Sigma Chemical Co. (St. Louis, MO). Purified PI-PLC from Bacillus thuringiensis was a gift of Martin Low (College of Physicians and Surgeons of Columbia University, New York) or was purchased from ICN Biomedicals, Inc. (Cleveland, OH). Neuraminidase (type X from Clostridium perfringens) was purchased from Sigma Chemical Co. Calcium-free DME was made in our laboratory or purchased from Hazleton Research Products (Lenexa, KS) and DME with calcium was purchased from Flow Laboratories, Inc. (McLean, VA). FCS was from HyClone Laboratories (Logan, UT). Embryo extract was made by expressing decapitated, eviscerated 11-d chick embryos through a syringe, adding a twofold volume excess of Ca++-free 10 mM Hepes-buffered Hanks' balanced salt solution (HHBSS), and extracting the tissue material on ice for 30 min followed by centrifugation and recovery of the supernate. Affi-Gel 10 was from Bio-Rad Laboratories (Richmond, CA), lectins were from E. Y. Laboratories (Logan, UT). Embryo extract was made by expressing decapitated, eviscerated 11-d chick embryos through a syringe, adding a twofold volume excess of Ca++-free 10 mM Hepes-buffered Hanks' balanced salt solution (HHBSS), and extracting the tissue material on ice for 30 min followed by centrifugation and recovery of the supernate. Affi-Gel 10 was from Bio-Rad Laboratories (Richmond, CA), lectins were from E. Y. Laboratories, Inc. (San Mateo, CA), alkaline phosphatase-coupled antibodies were from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD), and other reagents were from Sigma Chemical Co.

Antibodies

The antiserum to fusion-competent myoblasts (anti-my) was generated by injecting a rabbit with fusion-blocked, 52-h, CSAT-harvested myoblasts as described (Knudsen, 1985). Anti-my inhibits both myoblast adhesion and myotube formation (Knudsen, 1985). Antisera to PI-PLC–released glycoproteins were generated by injecting mice intraperitoneally three times at 2-wk intervals with 100 µl of Affi-Gel 10–immobilized Lens culinaris lectin (Knudsen et al., 1981) that had been exposed to material released by PI-PLC (R. cereus) from 5–10 × 10^6 cells and then washed free of nonsorbed material. Cell-derived protein was estimated to be ∼1–5 µg/injection. Control mice were injected with similar volumes of inactivated Affi-Gel 10, lectin conjugated to Affi-Gel 10, and PLC (Sigma Chemical Co.) (5–10 µg/injection). CSAT is a monoclonal antibody that binds to avian integrins (Neff et al., 1982). Specific, polyclonal rabbit antiserum to chicken N-CAM was a gift from Drs. M. Watanabe and U. Rathschauser (Case Western Reserve University School of Medicine, Cleveland, OH).

1. Abbreviations used in this paper: anti-my, antiserum to fusion-competent myoblasts; HHBSS, 10 mM Heps-buffered Hanks' balanced salt solution; IP., d-myo-inositol 1-monophosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; PLC, phospholipase C.

Cell Culture

Primary cultures of pectoral skeletal muscle cells and chick fibroblasts were prepared from 11-d chick embryos as described (Knudsen and Horwitz, 1977). To enrich cultures for myoblasts, the cells were routinely preplated on gelatin-coated culture dishes for 2–4 h. When indicated, a second 24-h preplate on gelatin-coated dishes was done to yield cultures highly enriched for myoblasts. By 72 h, these cultures incorporated 90% of the nuclei into myotubes in the presence of Ca++. Myoblast cultures were grown in Ca++-free DME plus 10% FCS and, when indicated, 5% embryo extract. EGTA was added during the first 24 h to achieve a final concentration that prevented myotube formation but did not disturb cell–substratum adhesion (i.e., ~65 µM). Under these conditions, the cultures displayed a maximal ability to form myotubes at ~52 h culture age (Knudsen and Horwitz, 1977). Therefore, fusion-competent myoblasts are defined as fusion-blocked, 52-h-old cells.

Myoblasts were harvested either by pipette or by the addition of CSAT monoclonal antibody (Neff et al., 1982) which recognizes CSAT-antigen (Neff et al., 1982; Knudsen et al., 1985), an integrin–glycoprotein complex involved in cell–substratum adhesion (for review see Buck and Horwitz, 1987). CSAT binds to both myoblasts and fibroblasts; however, the antibody predominantly perturbs the adhesion of the myoblasts and thus permits the collection of a population of cells highly enriched in myoblasts (Neff et al., 1982; Knudsen, 1985). While the addition of CSAT to muscle cultures at the time of initial plating has been shown by Menko and Boettiger (1987) to inhibit myogenic differentiation, addition of the antibody to 52-h cells has no effect on myoblast fusion (Neff et al., 1982) or myoblast interaction (Knudsen, 1985).

Myoblast Aggregation

Aggregation of fusion-competent myoblasts was performed essentially as described (Knudsen and Horwitz, 1977). Briefly, 52-h, fusion-competent myoblasts grown in low Ca++ medium were harvested, washed once, and mixed in suspension at a concentration of 3–5 × 10^6 cells/ml for 18 min at 37°C in serum-free HHBSS in the presence of either 1 mM EGTA or 1.5 mM Ca++. Using the above nondenzytatic methods of harvesting cells, the myoblasts presumably expressed both Ca++-independent and -dependent adhesion mechanisms since they aggregated in both the absence and presence of Ca++. However, in the presence of Ca++, both the number of cells in aggregates and the size of aggregates were larger than in the absence of Ca++. Thus, under our conditions, aggregation in the presence of Ca++ can be considered to result from a combination of Ca++-independent and -dependent mechanisms.

Aggregation was scored microscopically by counting cells as described (Knudsen and Horwitz, 1977). Aggregation increased with time (Knudsen and Horwitz, 1977), but after ~20 min the aggregates in the presence of Ca++ became too large to accurately score the number of cells in an aggregate. Therefore, the assay was routinely terminated after 18 min. The values for percent aggregation varied somewhat from day to day, perhaps due to variation in the cell concentration or, possibly, to the differentiation state of the cells: i.e., the number of the myoblasts expressing the aggregation- and fusion-competent phenotype. The Ca++-independent and -dependent aggregation varied in concert, however; that is, if the percent aggregation in the presence of Ca++ was high, so was the percent aggregation in its absence. (Note that for unknown reasons that may have to do with embryos from a different flock and/or FCS from a different lot, we recently have had to include embryo extracts in the cell culture medium to achieve good myotube formation. Using cells grown under these conditions, we noted that longer exposure to PI-PLC (30–60 min) was required to see an inhibitory effect on myoblast aggregation.)

Myoblast Fusion

Myoblast fusion was initiated by the addition of 1.5 mM Ca++ to 52-h cultures grown in low Ca++ medium. In the studies presented here, fusion was scored qualitatively by microscopic examination of the cultures for the presence of multinucleate myotubes and the results were recorded photographically.

SDS-PAGE

SDS-PAGE of NP-40 extracts or immunoprecipitates was performed under reducing conditions as described by Laemmli (1970). The acrylamide con-
centration of the resolving gel was 7-7.5%. Molecular mass markers included myosin (205 kD), beta-galactosidase (116 kD), phosphorylase b (97 kD), BSA (66 kD), ovalbumin (43 kD), and carbonic anhydrase (30 kD). When indicated, cell supernates or cell extracts were exposed to 1 U/ml neuraminidase (C. perfringens) in sodium acetate buffer, pH 5.5, for 30 min at 37°C to remove sialic acid residues from proteins before SDS-PAGE.

Immunoblot Analysis of Cell Extracts

Immunoblot studies were performed according to Towbin et al. (1979) and Burnette (1981) except that enzyme-coupled antibodies and dyes were used to detect antigen. Similar numbers of cells (e.g., myoblasts and fibroblasts and control and PI-PLC-treated cells) were harvested by scraping and extracted with 0.5% NP-40 nonionic detergent as described (Knudsen et al., 1981). Extracted proteins were resolved by SDS-PAGE, transferred electrophoretically to nitrocellulose, incubated with 3% BSA to block nonspecific protein-binding sites, exposed to first antibody (i.e., pooled mouse antisera to PLC-released glycoproteins or rabbit anti-N-CAM), and developed with the appropriate alkaline phosphatase-conjugated second antibody. The substrates included both nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results

The Effect of PLC from B. cereus on Myotube Formation

To determine whether or not PI-PLC treatment affected myotube formation we removed the medium from 52-h cultures grown in low Ca++ medium containing 10% FCS, exposed the cells to PLC from B. cereus (Sigma Chemical Co.) for 10 min at 37°C, removed the enzyme, returned the medium to the cells, and added 1.5 mM Ca++ to initiate myoblast fusion. Control cultures were treated similarly except that no enzyme was added. Exposure to PLC had a noticeable inhibitory effect on myotube formation observed 20 h later. Control cultures in Ca++-repleted medium formed large multinucleate myotubes (Fig. 1 A), while PLC-treated cultures in Ca++-repleted medium formed no large myotubes and looked more similar to cultures in low Ca++ medium (Fig. 1, C and B, respectively). Exposure to PLC under the above conditions had no noticeable effect on cell–substratum adhesion, suggesting that the effect of the enzyme was not due to contaminating protease activity. In addition, the lack of any obvious effect on cell–matrix adhesion suggested that the inhibition of myotube formation was not due to generalized membrane perturbations by PLC.

Exposure to PLC did not appear to be toxic since the PLC-treated myoblasts formed large myotubes and looked similar to Ca++-repleted control cultures after an additional 24 h in culture (Fig. 1, E and D, respectively). Taken together the results suggested that PLC had a specific, nontoxic, and reversible effect on cell surface events involved in myoblast fusion. However, because the formation of myotubes in culture

Figure 1. The effect of PLC on myotube formation. Myoblast cultures were prepared from 11-d chick embryos and grown in low Ca++ DME plus 10% FCS for 52 h. The medium was removed and the cells were treated with PLC (2 U/ml; Sigma Chemical Co.) in Ca++-free HHBSS for 10 min at 37°C. The enzyme was then removed, the conditioned medium returned, and 1.5 mM Ca++ added to initiate fusion. Control cells were treated similarly except that the enzyme was omitted. 20 (A–C) or 44 h later (D and E), the cells were fixed, stained, and photographed. (A and D) Control cells in the presence of Ca++. (B) Control cells in the absence of Ca++. (C and E) PLC-treated cells in the presence of Ca++. Bar, 200 μm.

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includes the combined events of cell migration, cell–cell adhesion, and lipid bilayer union and because the enzyme preparation might affect more than one aspect of myoblast fusion, we decided to focus our studies on the effect of PLC on the intercellular adhesion of fusion-competent myoblasts.

The Effect of PLC from B. cereus on Myoblast–Myoblast Adhesion

We investigated the possibility that PI-PLC treatment of fusion-competent myoblasts perturbed the cell–cell adhesion step of myotube formation. 52-h, fusion-blocked chick myoblasts were harvested with CSAT antibody, washed, adjusted to a concentration of 3–5 × 10⁶ cells/ml, exposed to PLC (B. cereus) for 10 min at 37°C, and then diluted 10-fold with HBSS to minimize the enzyme’s activity during the subsequent aggregation assay. Control cells were treated similarly except that the enzyme was omitted. The aggregation assay was performed by mixing cells in suspension (3–5 × 10⁵ cells/ml) in the presence or absence of Ca²⁺ for 18 min at 37°C and scoring the extent of aggregation by counting cells using a phase–contrast microscope.

Treatment of fusion-competent myoblasts with PLC from B. cereus inhibited myoblast aggregation in a dose-dependent manner (Fig. 2). The enzyme’s effect appeared to be maximal at a PLC concentration of ~1–2 U/ml. It is to be noted that myoblast aggregation was not completely inhibited. Increasing the enzyme concentration to 10 U/ml resulted in a somewhat greater inhibition of myoblast aggregation but also resulted in membrane damage to an increasing number of cells, as indicated by the cells’ inability to exclude trypan blue dye. At no enzyme concentration was the effect of the PLC on myoblast aggregation altered by the addition of protease inhibitors such as serum, soy bean trypsin inhibitor, leupeptin, or PMSF, however, indicating that the enzyme effect, even at high concentrations, was not likely due to protease activity in the PLC preparation.

The effect of PLC treatment on myoblast adhesion was similar whether or not Ca²⁺ was present during the 10-min incubation with the enzyme before the aggregation assay. However, if the myoblasts were permitted to form aggregates in the presence of Ca²⁺ for 18 min before adding the enzyme, PLC was unable to dissociate the aggregated cells, suggesting that some event(s) occurred that resulted in cell–cell adhesion no longer being susceptible to the action of PLC. This result further suggested that the enzyme activity was not likely due to protease contamination since, after 18 min, the myoblast aggregates could be dispersed by trypsin (Knudsen and Horwitz, 1977).

Specificity of the Effect of PI-PLC on Myoblast Adhesion

Two lines of evidence suggested that the adhesion of fusion-competent myoblasts was inhibited specifically by the activ-

Table I. The Effect of PI-PLC on Cell–Cell Adhesion of Fusion-competent Myoblasts

| Treatment | Ca²⁺ | Aggregation | Ca²⁺ control* |
|-----------|------|-------------|---------------|
|           | mM   | %           | %             |
| Control   | –    | 9 ± 1%      | 37            |
| Control   | 1.5  | 24 ± 2%     | 100           |
| PI-PLC    | –    | 5 ± 2%      | 21            |
| PI-PLC    | 1.5  | 13 ± 3%     | 54            |

Fusion-competent myoblasts were harvested with CSAT after growing the cells in low Ca²⁺ medium for 52 h. Cells at a concentration of 3–5 × 10⁶ cells/ml were treated in Ca²⁺-free HBSS for 10 min at 37°C with 3 U/ml PI-PLC purified from culture supernatants of B. thuringiensis (gift of M. Low). 

Control cells were treated similarly except that no enzyme was added. The mixture was diluted 1:10 with HBSS and the aggregation assay was performed as described in Materials and Methods.

Figure 3. The effect of PLC on cell–cell adhesion of fusion-competent myoblasts in the presence of IP₁. Muscle cultures were prepared from 11-d chick embryo pectoral muscle and grown in Ca²⁺-free DME plus 10% FCS for 51 h. The myoblasts were harvested with CSAT monoclonal antibody, adjusted to a concentration of 3–5 × 10⁶ cells/ml, and treated with various concentrations of PLC from B. cereus (type III; Sigma Chemical Co.) in the presence of various concentrations of IP₁. The PLC and IP₁ were mixed together before adding the cells and incubating the mixture for 10 min at 37°C. The mixture was then diluted 1:10 and the aggregation assay was performed as described in Materials and Methods.
Adhesion-inhibiting Activity of an Antiserum to Myoblasts

PI-PLC from \textit{B. thuringiensis} (and from \textit{B. cereus}) inhibited the aggregation of myoblasts occurring in either the absence or presence of Ca\textsuperscript{++} (Table I). The effect of the enzyme on myoblast aggregation in the presence of Ca\textsuperscript{++}—which in our system presumably includes both Ca\textsuperscript{++}-independent and -dependent mechanisms (see Aggregation Assay in Materials and Methods)—did not appear to be due solely to the enzyme's effect on the Ca\textsuperscript{++}-independent system. That is, an inhibitory effect on aggregation in the presence of Ca\textsuperscript{++} (Ca\textsuperscript{++} independent plus dependent) by PI-PLC was noted even when aggregation in the absence of Ca\textsuperscript{++} was mathematically subtracted from aggregation in the presence of Ca\textsuperscript{++} (Table I). Like PLC from \textit{B. cereus}, PLC from \textit{B. thuringiensis} did not completely abolish myoblast adhesion even when the enzyme concentration was increased 10-fold to 30 U/ml. The enzyme from \textit{B. thuringiensis} had no effect on the cells' ability to exclude trypan blue even at incubation times up to 60 min or at high concentrations (i.e., 30 U/ml). Lastly, PI-PLC treatment similarly inhibited the aggregation of myoblasts regardless of whether the cells were harvested by pipette or with CSAT antibody.

Table II. Effect of Material Released from Fusion-competent Myoblasts with PLC on the Adhesion-inhibiting Activity of an Antiserum to Myoblasts

| Treatment                  | Aggregation | Control* |
|----------------------------|-------------|----------|
| Preimmune serum            | 34          | 100      |
| Anti-myo                   | 15          | 44       |
| Anti-myo + PLC + IP\textsubscript{T} | 14          | 41       |
| Anti-myo + PLC-R + IP\textsubscript{T} | 35          | 103      |

Anti-myo is an adhesion-inhibiting antiserum that was generated by injecting a rabbit with fusion-competent myoblasts and has been described previously (Knudsen, 1985). Preimmune serum and anti-myo were used at dilutions of 1:30. IP\textsubscript{T} (4 mM) was added to prevent PLC from acting on the myoblasts during the aggregation assay. PLC-released material (PLC-R) was from 10 times the number of cells in the assay. The values reported for percent aggregation represent the average of duplicates that varied from the mean by \(<15\%\). All assays were performed in the presence of 1.5 mM added Ca\textsuperscript{++}.

Table III. The Effect on Myoblast Aggregation of Mouse Antisera to Glycoproteins Released by PLC from Fusion-competent Myoblasts

| Treatment                  | Ca\textsuperscript{++} Aggregation | Ca\textsuperscript{++} control |
|----------------------------|-----------------------------|-----------------------------|
| Control                    | 12                          | 36                          |
| Control                   | 1.5                        | 33                          |
| Preimmune serum           | 1.5                        | 38                          |
| Anti-PLC-R (GP)           | 1.5                        | 16                          |

The mice were immunized with glycoproteins released by PLC (\textit{B. cereus}) from fusion-competent myoblasts and absorbed on immobilized \textit{L. culinaris} lectin as described in Materials and Methods. Preimmune serum and antisera to PLC-released glycoproteins (anti-PLC-R/GP) were used at dilutions of 1:30. The aggregation assay was performed as described in Materials and Methods. The numbers reported for percent aggregation for controls and preimmune serum groups represent the average of two numbers that varied from the mean by \(<18\%\). The number for percent aggregation of the group treated with antisera to PLC-released glycoproteins represents the average of four different antisera that varied from the mean by \(<20\%\).

Evidence that Treating Fusion-competent Myoblasts with PI-PLC Released Adhesion-related Glycoproteins into the Supernatant

We postulated that PI-PLC acted by releasing a form of cell surface glycoproteins involved in the adhesion of fusion-competent myoblasts rather than by nonspecifically perturbing the myoblast surface. The detection and identification of the released form(s) of myoblast adhesion molecules was therefore of interest. Whereas we speculated that the adhesion molecules in material released from fusion-competent myoblasts by PI-PLC might directly perturb cell–cell adhesion of the myoblasts, we observed no significant effect on cell aggregation when PLC-released material from 10 times the number of cells present in the assay was added to aggregating cells. This might have resulted from a number of complications, including insufficient soluble adhesion-related glycoproteins or a loss of physiological (i.e., adhesion) activity of the PLC-released molecules. Since the possibilities appeared difficult to approach experimentally, we turned to indirect methods to detect the presence of adhesion-related molecules in the material released from myoblasts by PI-PLC.

First, we assayed for the presence of adhesion-related molecules by using a competitive immunoassay and observed that material released by PI-PLC was able to inhibit the effect of anti-myo antiserum previously shown to perturb myoblast aggregation (Knudsen, 1985) (Table II). This result suggested that antigen in the material released by PI-PLC neutralized the effect of adhesion-perturbing antibodies in anti-myo antiserum, which presumably inhibited myoblast aggregation by binding to molecules on the surface of the myoblasts involved in adhesion.

Secondly, we assayed for the presence of adhesion-related molecules in PLC-released material by testing its ability to generate adhesion-perturbing antisera. To minimize the amount of PLC in the material to be injected into animals we prepared a glycoprotein fraction from the material released by PI-PLC as the immunogen. Using this approach we observed that glycoproteins in the PLC-released material stimulated adhesion-inhibiting antisera when injected into mice (Table III). To generate these antisera we found we had to immunize mice with immobilized \textit{L. culinaris} lectin that had been exposed to material released by PI-PLC and washed free of nonabsorbed material as described in Materials and Methods. Not surprisingly the antisera also contained antibodies to \textit{L. culinaris} lectin and PLC, as detected by immunoblot analysis (data not shown). However, antisera from mice injected with Affi-Gel 10, lectin-conjugated Affi-Gel 10, or PLC had no effect on myoblast aggregation (data not shown). We took the approach of immunizing mice in this way because we were unable to efficiently elute material bound to the lectin, suggesting that glycoproteins in the PLC-released material had a high affinity for the lectin, which may not be surprising considering that myoblast adhesion-related molecules are glycosylated at asparagine-linked sites (Knud-
Antigens Expressed by Fusion-competent Myoblasts That are Recognized by the Mouse Antisera to PLC-released Glycoproteins

To probe for candidate adhesion molecules expressed by fusion-competent myoblasts, detergent extracts of chick cells were immunoblotted with pooled adhesion-perturbing antisera to the PLC-released glycoprotein fraction from fusion-competent myoblasts. Six to eight bands in the molecular mass region of 116,000 to >200,000 D, with a major band at 140 kD, were observed in the extract of fusion-competent myoblasts but were not observed in the extract of fibroblasts (Fig. 4). Similar bands were detected in immunoprecipitates of NP-40 extracts of [125I]-surface labeled, fusion-competent myoblasts (data not shown). The antisera did not appear to be entirely muscle specific, however, since they also detected material in the extract of fibroblasts. This was not surprising since considerable protein was shed from myoblasts in suspension in the absence of PI-PLC and some of this likely represented glycoproteins expressed by chicken cells other than myoblasts. Nevertheless, the immunoblot data using antisera to the PLC-released glycoprotein fraction from fusion-competent suggested that the bands of interest as candidates for myoblast adhesion molecules were in the molecular mass region of 116–200 kD.

The Relationship of the PI-PLC-released Molecules to Other Known Phosphatidylinositol-anchored Adhesion Molecules

We were interested in knowing whether the molecules released by PI-PLC from the fusion-competent myoblasts were related to any other known phosphatidylinositol-linked proteins. We focused on N-CAM for a number of reasons. First, N-CAM is known to have an isoform linked to the cell surface membrane via glycosyl-phosphatidylinositol. Second, N-CAM mediates cell–cell adhesion by virtue of its ability to self-associate (i.e., N-CAM on one cell binds to N-CAM on an adjacent cell). Lastly, N-CAM is expressed by muscle as multiple polysialylated isoforms with molecular masses in the region of the bands detected in myoblasts by our antisera to PLC-released glycoproteins.

Fig. 5 shows that N-CAM was present in the supernate of PI-PLC-treated fusion-competent myoblasts, but not in the supernate of control cells. Neuraminidase treatment of the supernate to remove sialic acid residues from N-CAM before immunoblot analysis caused N-CAM to migrate as a single band with a molecular mass of ~120,000 D, instead of as a smear with molecular masses from 120 to >205 kD. The 120-kD desialo N-CAM band was greatly reduced in the neuraminidase-treated detergent extract of the PI-PLC-treated cells when compared with the extract of control cells, indicating that this isoform of N-CAM was released by PI-PLC and, therefore, linked to the surface of fusion-competent myoblasts via phosphatidylinositol. One major desialo isoform of N-CAM was detected in the extract of the cells, but not in the supernate of PI-PLC-treated cells. This N-CAM...
isofrom is presumably anchored to the cell surface via a transmembrane peptide domain and likely explains why PI-PLC did not completely inhibit myoblast interaction even in the absence of Ca++. Both major desialo isoforms of N-CAM, including the 120-kD lipid-linked form, were detected in the detergent extract of control myoblasts not treated with PI-PLC.

Discussion

The data presented here suggest that a portion of the glycoproteins involved in the formation of myotubes is linked to the cell surface lipid bilayer through a phosphatidylinositol anchorage. Cell–cell, but not cell–matrix, adhesion of fusion-competent myoblasts was inhibited by treating the cells with PI-PLC. The effect of the enzyme appeared to be specific, nontoxic, reversible, and not due to contaminating protease activity. While inhibition of myotube formation by PLC from B. cereus (Sigma Chemical Co.) may have resulted from the inhibition of myoblast–myoblast adhesion, the enzyme preparation used in these studies could have affected another step in myotube formation, such as lipid bilayer union.

PI-PLC treatment of fusion-competent myoblasts inhibited cell–cell adhesion in both the absence and presence of Ca++. In our system and appeared to have an inhibitory effect on both the Ca++-independent and -dependent aspects of myoblast adhesion. The enzyme appeared to act by releasing adhesion-related glycoproteins from the surface of fusion-competent myoblasts since such molecules were detected indirectly by (a) the ability of PLC-released material to block the effect of an adhesion-perturbing antisera to intact myoblasts; and (b) the ability of PLC-released glycoproteins to stimulate adhesion-perturbing antisera when injected into mice.

Myoblast adhesion was inhibited only partially by PI-PLC treatment of the cells, suggesting that only a portion of the molecules mediating myoblast interaction is linked to the myoblast surface via phosphatidylinositol. This might be explained in several ways. First, PI-PLC-resistant adhesion could involve PI-PLC-insensitive isoforms of the PI-PLC-releasable adhesion molecule, as is the case with N-CAM. Alternatively, the PI-PLC-resistant myoblast adhesion might involve peptide-anchored molecules biochemically unrelated to the PI-PLC-releasable adhesion glycoprotein. Most likely, myoblast interaction involves a combination of the two scenarios, especially when Ca++ is present.

PI-PLC released an isoform of N-CAM from the surface of fusion-competent myoblasts. Assuming that our antisera to PI-PLC-released glycoproteins included antibodies to N-CAM, it is understandable that this antisera recognized multiple bands of molecular masses 116–200 kD in the extract of myoblasts since N-CAM is expressed in polysialylated forms with molecular masses ranging from 120 kD to much higher than 200 kD in chicken muscle (Rutishauser et al., 1983).

Our observation that fusion-competent myoblasts express a lipid-linked isoform of N-CAM is consistent with those of Moore et al. (1987) and Barton et al. (1988) demonstrating phosphatidylinositol-linked forms of N-CAM in mouse and human muscle cells, respectively. A role for N-CAM in neuromuscular interactions has been demonstrated (Grunet et al., 1982; Rutishauser et al., 1983), but a role for N-CAM in myogenesis has only been postulated. Our results suggest a role for N-CAM in the cell–cell adhesion step of myotube formation. N-CAM self-associates in the absence of Ca++ (Edelman et al., 1987) and is expressed by chick muscle cells (Grunet et al., 1982; Rutishauser et al., 1983), making it possible that N-CAM participates in myoblast–myoblast adhesion and is likely the predominant, or only, adhesion mechanism mediating myoblast interaction in the absence of Ca++. In support of this hypothesis, experiments conducted in our lab (Knudsen, K. A., S. A. McElwee, and L. Myers, manuscript submitted for publication) show that antibodies to N-CAM inhibit myoblast interaction.

The inhibitory effect of PI-PLC on Ca+-dependent, as well as Ca+-independent, adhesion may result directly from the release of an as yet unidentified lipid-linked Ca+-dependent adhesion molecule(s) from the myoblast surface. Alternatively, the PI-PLC effect on Ca+-dependent myoblast adhesion may be indirect and reflect the result of a loss of N-CAM molecules on the Ca+-dependent adhesion mechanism. The latter interpretation is supported by the observations of Rutishauser et al. (1988) which indicate N-CAM can influence cell–cell interaction mediated by other mechanisms.

The physiological significance of the inositol phospholipid-anchored N-CAM in myogenesis is not understood at this time. The significance of such a lipid anchorage has been speculated on for other proteins, however (see Low, 1987; Low and Saltiel, 1988; Sefton and Buss, 1987; Cross, 1987; Ferguson and Williams, 1988). The glycosyl-phosphatidylinositol moiety might simply serve to anchor the protein to the lipid bilayer or might confer on the protein unique properties. For instance, an increased lateral mobility might play a role in the protein’s function. In addition, the protein portion of the molecule might be released by an endogenous PLC, resulting in consequences such as termination of a cell surface event, rapid production of cell polarity, or release of 1,2-diacylglycerol, a known activator of protein kinase C (Nishizuka, 1986).

In the presence of Ca++, when both Ca+-independent and -dependent adhesion mechanisms are intact (as in the in vivo situation and in our in vitro system), N-CAM most likely functions in combination with a Ca+-dependent adhesion molecule(s) that is likely a member of the cadherin family. Together, N-CAM and the Ca+-dependent adhesion molecule(s) function to mediate myoblast recognition and adhesion during myogenesis.

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