

Properties of Extracellular Adhesion-mediating Particles in Myoblast Clone and Its Adhesion-deficient Variant

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ABSTRACT Both the skeletal muscle myoblast cell line L6 and an adhesion-deficient variant of L6 released glycoprotein complexes, termed adherons, into their culture medium. The adherons from the variant, M3A, differed from those of L6 in a number of properties. M3A adherons were much less effective in promoting the cell-substratum and cell-cell adhesion of myoblasts than L6 particles. The adherons from the two cell lines also differed in their relative sedimentation velocities in sucrose gradients and had different chemical compositions. The M3A particle lacked chondroitin and contained relatively less collagen and fibronectin than the L6 adheron. Both L6 and M3A particles adhered to plastic surfaces and cells equally well in the absence of calcium ions. Neither cell-cell adhesion nor particle aggregation occurred in calcium-free medium. However, in the presence of calcium, the L6 adherons aggregated completely and M3A particles aggregated poorly. These data suggest that at least two sets of interactions are required for adheron-mediated adhesion: a calcium-independent binding of the adheron to the cell, and a calcium-dependent interaction between particles that is directly responsible for adhesion. The M3A variant is blocked at the calcium-dependent step, resulting in an adhesion deficiency.

Myogenic cells release into their culture medium a glycoprotein complex which aggregates myoblasts and alters the adhesion of cells to the substratum of plastic petri dishes (13). When adsorbed onto culture dish surfaces, the material from clonal L6 skeletal muscle myoblasts stimulated the adhesion of myoblasts and inhibited the adhesion of a clone of sympathetic nerverlike cells. The adhesion-mediating activity had a sedimentation value of 16 S in sucrose gradients in the absence of calcium; it aggregated in the presence of calcium. This 16 S myoblast particle, termed an adheron, was composed of glycosaminoglycans (GAGs) and several proteins, including collagen and fibronectin.

We selected a variant of the L6 skeletal muscle myoblast cell line which adheres poorly to the surface of culture dishes (14, 15). The variant line, designated M3A, was selected from an unmutagenized L6 population for its ability to grow on a nonadhesive agar surface; the parental L6 line was anchorage dependent for growth. A comparison of the protein and GAG composition of these two cell lines and the material they release into the culture medium showed that the M3A variant had lost the ability of its L6 parent to synthesize extracellular chondroitin and complete collagen α chains (14). A further analysis of this pair of cells showed that the substrate-attached material adsorbed to the culture dish surface from growth-conditioned

medium of L6 cells was very effective in stimulating the adhesion of myoblasts, while the substrate-attached material from M3A was relatively less active (15). Since the adhesion-promoting activity in the conditioned medium of the parental L6 cell line was a 16 S glycoprotein complex, it was possible that the diminished adhesiveness of M3A cells was due to the synthesis of a defective adhesion-mediating particle. The following data show that the adhesion-promoting particle of M3A has a number of binding and structural properties which are different from that of L6. This information is used to characterize some of the interactions which are responsible for the function of the normal 16 S myoblast adheron.

MATERIALS AND METHODS

Cells and Culture

The L6 myoblast line was obtained from Yaffe (22) and maintained in 10% fetal calf serum in Dulbecco's modified Eagle's medium (DME). M3A was selected for its ability to grow over a nonadhesive agar surface and has been described in detail (14, 15). It was grown on petri dishes in DME and 10% fetal calf serum.

Protein and Glycosaminoglycan Assays

Cells were labeled in serum-free DME with reduced methionine with ^{35}S methionine, and slab gel electrophoresis was done in gels containing 10% acryl-

amide and 0.1% SDS (1). Cytoskeletons were prepared according to Starger et al. (17) using 1% Triton X-100. GAG analysis was carried out by labeling cells with [³H]glucosamine or [³⁵S]sulfate, chromatographing the sample on a DEAE-cellulose column (21), and identifying the GAGs in the individual peaks by enzymatic methods (11). *Streptomyces* hyaluronidase (B grade; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was used at 50 U/ml for 24 h at 37°C. Chondroitinase ABC and AC (Miles Laboratories, Elkhart, IN) were used at 0.5 U/ml for 24 h at 37°C. GAG breakdown was monitored by column chromatography in Sepharose CL6B. Hydroxyproline was determined by labeling cells with [¹⁴C]proline. The fractions of interest were isolated, hydrolyzed in 6 M HCl, chromatographed on an amino acid analyzer, and the radioactivity in the 4-hydroxyproline and proline peaks was determined.

Preparation of Conditioned Medium and Substrate-attached Material

Conditioned medium was prepared from exponentially dividing cells by washing the cells twice in serum-free HEPES-buffered DME medium (HEPES-medium) and incubating the cells in the same medium for 15 h at 37°C. To prepare substrate-attached material coated dishes, growth-conditioned medium was placed in 35-mm plastic petri dishes (Falcon Labware, Oxnard, CA) for 18 h, and then the dishes were washed twice with 0.5 mM EGTA, three times with water, and once with HEPES medium. After the final wash, 2 ml of HEPES medium containing 0.2% bovine serum albumin was added.

Adhesion Assays

To assay cell-substratum adhesion, exponentially dividing cells were labeled with [³H]leucine (5 μCi/ml) for 15 h. The cells were washed three times with HEPES medium containing 0.2% albumin, and 0.2-ml aliquots were pipetted into 35-mm coated plastic petri dishes containing 2 ml of the same medium. At indicated times the dishes were swirled 10 times, the medium was aspirated, and the remaining attached cells were dissolved in Triton X-100 and their isotope content was determined. The data were plotted as the fraction of input cells that adhered as a function of time. Variation between duplicates was <5%. Cell-cell aggregation was determined by measuring the disappearance of single cells from a single-cell suspension (10). Cells were washed twice in HEPES buffer and added to 0.5-ml aliquots of the test medium. The cells were agitated on a rotary shaker at 100 rpm and the disappearance of single cells was monitored with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

RESULTS

Adhesion-mediating Activities of L6 and M3A Adherons

The clonal L6 skeletal muscle myoblast cell line grows as a monolayer on plastic tissue culture dishes (22). A clonal variant of L6, designated M3A, adheres poorly to most substrata, and does not attach to plastic bacteriological petri dishes which have not been exposed to cells. M3A can, however, attach to petri dishes on which cells have been grown; the extent of adhesion depends upon the type of cell. To assay for cell-substratum adhesion, M3A cells were isotopically labeled and plated into 35-mm petri dishes. The unattached cells were removed at different times, and the isotope remaining on the dish was assayed. When the initial kinetics of adhesion were determined, <0.5% of the input cells attached to new petri dishes over a period of 1 h. If, however, the M3A cells were plated into petri dishes on which L6 cells were grown for 1 wk and then removed by EGTA, they rapidly adhered. They adhered poorly, however, to petri dishes which had previously contained the same number of M3A cells (Fig. 1A).

To further study these adhesive responses, it is advantageous to have a system where the material responsible for the cell-substratum adhesion is in a soluble form. This was accomplished by briefly growing M3A or L6 cells in serum-free HEPES medium. The "conditioned" culture medium was then incubated in 35-mm petri dishes to allow adsorption of conditioned medium components, and cell-substratum adhesion was measured as described above. Fig. 1B shows that when isotop-

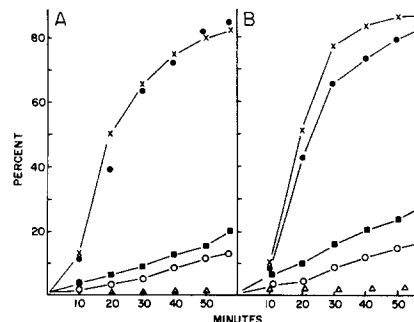


FIGURE 1 Adhesion of L6 and M3A cells to substrate-attached material prepared from L6 and M3A. Exponentially growing M3A or L6 myoblasts were incubated overnight in serum-free HEPES medium. The growth-conditioned medium was then placed in 35-mm petri dishes overnight at 37°C. Alternatively, L6 and M3A cells were grown for 7 d to a final density of 1×10^8 cells/dish and removed by EGTA. Both sets of dishes were washed with EGTA, water, and HEPES medium. Exponentially dividing L6 or M3A cells were isotopically labeled, dissociated with EGTA (L6), washed, and plated at 3×10^5 cells/dish into the coated dishes containing 2 ml of HEPES medium and 0.2% BSA. The data are presented as the percentage of input cells that adhered at each time point. (A) Δ , M3A to new petri dishes; \circ , adhesion of M3A to petri dishes on which M3A were grown; \blacksquare , adhesion of L6 cells to petri dishes on which M3A were grown; \times , M3A to petri dishes on which L6 myoblasts were grown; \bullet , L6 cells to petri dishes on which L6 myoblasts were grown. (B) Δ , adhesion of L6 cells to petri dishes; \circ , M3A cells to material adsorbed from M3A conditioned medium; \blacksquare , L6 cells to material from M3A conditioned medium; \bullet , L6 cells to material from L6 conditioned medium; \times , M3A cells to material from L6 conditioned medium.

ically labeled M3A cells were plated into dishes containing material adsorbed from the growth-conditioned media of equal numbers of L6 or M3A cells, the kinetics of adhesion were very similar to those obtained by growing cells on the culture dishes used in the adhesion assays. To assay the adhesion of the parental L6 line, cell monolayers were dissociated with EGTA, washed extensively in HEPES medium, and their adhesion to material adsorbed from L6 and M3A conditioned media was determined. Fig. 1 shows that L6 cells, like M3A cells, adhered rapidly to substrata derived from L6 but poorly to those from M3A. The treatment of M3A cells with EGTA reduced their adhesion to L6 substrate-attached material by <10%.

When growth-conditioned media of myoblasts were centrifuged at 100,000 g for 2 h, all of the adhesion-promoting activity was removed from suspension and recovered in the pellets (13). 0.4% of the total cell protein was in the particulate material of both L6 and M3A conditioned medium. To determine if this particulate material accounted for the difference in the adhesion of L6 and M3A cells to substrate-attached material derived from growth-conditioned media, serum-free growth-conditioned media were prepared from $\sim 3 \times 10^8$ L6 and M3A cells, and the media were centrifuged at 100,000 g for 2 h. The pellets were resuspended and incubated overnight at different protein concentrations on plastic petri dishes. By the inclusion of isotopically labeled material prepared from the growth-conditioned medium of the same cells, it was shown that all of the input protein and carbohydrate adhered to the culture dish surface. The adhesion of L6 and M3A cells was then assayed at the various concentrations of particulate material. Fig. 2 shows that at 1 μg/dish the material derived from M3A was ~20% as active as that from L6 in promoting the adhesion of L6 and M3A cells. The L6 material stimulated

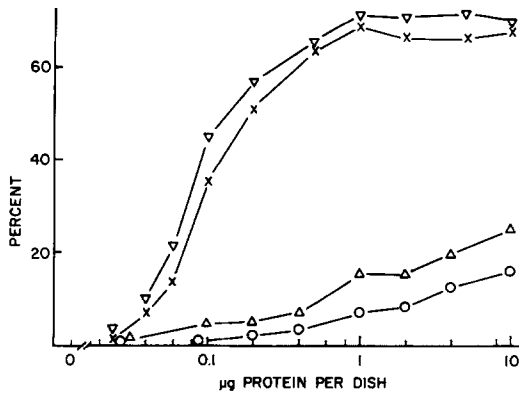


FIGURE 2 Quantification of myoblast adhesion to L6 and M3A adherons. Serum-free growth-conditioned medium from L6 and M3A cells was centrifuged at 100,000 g for 2 h to pellet the adhesion-promoting particles. The particles were then resuspended in HEPES medium and incubated at various concentrations overnight on petri dishes. The next day, the dishes were washed twice with HEPES medium and the adhesion of M3A and L6 cells was determined at 10-min intervals over a period of 1 h as described in Fig. 1. A smooth curve was drawn through the adhesion curves for protein each condition, and the percent of the adhered input cells was determined at 30 min. This percent is plotted against the amount of protein in the particles adhered to the culture dish. The adhesion of M3A or L6 cells to petri dishes alone at 30 min was <2.0% of the input. Δ , L6 cells to M3A particle; \circ , M3A cells to M3A particle; \times , L6 cells to L6 particle; ∇ , M3A cells to L6 particle.

maximum adhesion at 1 $\mu\text{g}/\text{dish}$, while the adhesion of cells to the M3A derived substratum was still linear up to 10 $\mu\text{g}/\text{dish}$.

All of the adhesion-promoting material from L6 growth-conditioned medium sediments in calcium-free sucrose gradients as a 16 S peak (13). The 16 S particle was composed primarily of collagen, fibronectin, and several GAGs (13). To determine if the adheron from M3A cells is structurally different from that of L6, L6 and M3A cells were labeled with [^{35}S] sulfate in serum-free medium; unlabeled growth-conditioned media from 5×10^7 L6 and M3A cells were also prepared. The conditioned media were centrifuged at 100,000 g for 2 h; the pellets were washed with HEPES medium twice, resuspended in 0.01 M HEPES buffer, pH 7.1, and centrifuged into 5–20% sucrose gradients. Each gradient fraction was assayed for isotope and its ability to promote cell-substratum adhesion of M3A cells. Fig. 3 shows that while the sulfate-labeled GAGs and adhesion-promoting activities of L6 sedimented at 16 S relative to ribosomal RNA markers, those of M3A sedimented as a pair of peaks at ~ 11 and 8 S.

Binding Properties of L6 and M3A Adherons to Plastic Substrate and Cells

The above data demonstrate that adherons from L6 differ from those of M3A with respect to their ability to promote the adhesion of M3A and L6 cells, and their relative size on sucrose gradients. There are a number of particle interactions which could be involved in the functional deficiencies of the M3A adherons: (a) The binding of the adheron to the plastic petri dish surface used in the adhesion assay; (b) binding of the particle to cells; and (c) the divalent ion requirement for cell-cell and particle-particle interactions. These alternatives will be dealt with sequentially.

Although particles from both L6 and M3A adhered completely to petri dish surfaces over a period of 18 h, it was

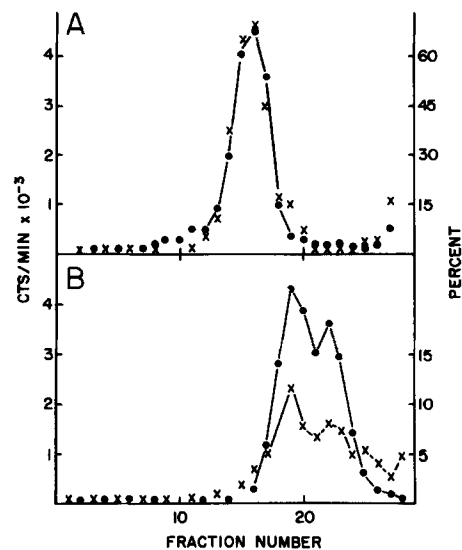


FIGURE 3 Sedimentation of L6 and M3A GAGs and adhesion activities in sucrose gradients. L6 and M3A cells were labeled with $^{35}\text{SO}_4$ in serum-free medium (5×10^6 cells) or were incubated in serum-free medium alone (5×10^7 cells). After 18 h the conditioned media were centrifuged at 100,000 g for 2 h. The two 100,000 g pellets were then centrifuged for 18 h at 35,000 rpm at 4°C in an SW 41 Beckman rotor (Beckman Instruments, Spinco Div., Palo Alto, CA) in 5–20% linear sucrose gradients containing 0.01 M HEPES buffer (pH 7.1). The data are presented as counts per minute (cpm) per fraction: 94% of the input counts in M3A and 88% in L6 were recovered in the gradient. Each gradient fraction with unlabeled cellular material was diluted into HEPES medium and incubated in petri dishes overnight at 37°C. The next day, the adhesion of isotopically labeled M3A cells was determined and these data are presented as the percentage of input cells that adhered at 30 min. The top of the gradient is at the right. \bullet , $^{35}\text{SO}_4$ -labeled material; \times , adhesion of M3A cells. (A) L6 adherons. (B) M3A adherons.

possible that their initial adhesion kinetics were different. Therefore, isotopically labeled L6 and M3A adherons were placed in petri and tissue culture dishes containing serum-free HEPES medium. The rate of adhesion of the particles to the two different plastic substrata was determined at 37°C. The binding of M3A and L6 adherons to the highly sulfonated tissue culture dishes was identical, with a half time of 5.5 h. The binding to the less charged petri dish surface was more rapid, and M3A adherons were more adhesive (half-time 2.2 h) to this surface than L6 particles (half-time 4 h). When the particle binding assay was done in HEPES medium at 4°C or in the absence of calcium and magnesium ions, the binding curves of L6 or M3A particles to both surfaces were very similar to those done at 37°C in the presence of calcium (data not presented). It follows that the initial kinetics of adhesion of L6 and M3A adherons to plastic surfaces are distinguishable, do not require calcium or magnesium ions, and are not temperature dependent.

To assay the binding of adherons to cells, the incubation temperature must be near 4°C to prevent internalization of the bound material. Sulfate-labeled adherons were incubated at 4°C with a constant number of cells, and the percent of total input particles which bound to the cells was determined as a function of time. Fig. 4 shows that L6 and M3A adherons bind to M3A and L6 cells equally well, with a half saturation time of ~ 1.5 h. The binding curves were similar in the presence and absence of 2 mM calcium. The leveling-off of the binding curve

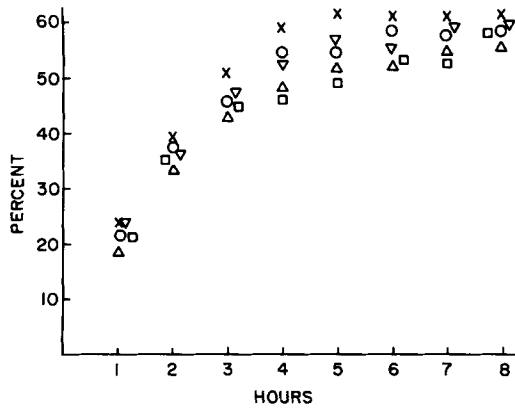


FIGURE 4 Binding of L6 and M3A adherons to cells. ^{35}S -labeled adherons were prepared from L6 and M3A cells as described in Fig. 3. L6 cells were removed from the dish by scraping with a rubber policeman. The particles were resuspended in 0.01 M HEPES buffer, pH 7.1, and 5,000 cpm added to plastic centrifuge tubes containing 2×10^6 cells in 2 ml of HEPES medium at 4°C (with or without 2 mM calcium and 1 mM magnesium). At various times the cells were pelleted and an aliquot of the supernatant was counted. The data are plotted as the percent of the input counts adsorbed by the cells as a function of time. There was no significant difference between the calcium and calcium-free samples. With the exception of L6 adheron binding to L6 cells, only those with calcium are presented. X, L6 adheron to M3A cells; O, M3A adheron to M3A cells; Δ , L6 adheron to L6 cells; ∇ , L6 adheron to M3A cells; \square , L6 adheron to L6 cells minus calcium and magnesium.

at 60% could be due to either equilibrium binding or because some of the adherons in the preparation were defective in their binding characteristics. To distinguish between these alternatives, the isotope remaining in the supernatant after the initial binding curve was incubated again with an equal number of cells at 4°C . After 4 h, the cells were removed and it was determined that the cells again bound $\sim 60\%$ of the input adherons. These data suggest that binding of adherons to L6 and M3A cells at 4°C is an equilibrium process, and that there is no major difference between the ability of L6 and that of M3A particle to bind to M3A or L6 cells.

Role of Calcium in Cell-Cell Adhesion

When L6 adherons were centrifuged in a sucrose gradient in the absence of calcium ions, they migrated as 16 S peaks; in the presence of 2 mM calcium, they aggregated and were pelleted in the gradient (13). It was possible that a calcium-dependent aspect of adheron-mediated adhesion was altered in the variant M3A cells. To assay the calcium dependence of adhesion, the ability of M3A and L6 cells to adhere to surfaces coated with L6 and M3A particles was assayed in the presence and absence of calcium. Fig. 5 shows that the adhesion of L6 cells to L6 particles was completely calcium dependent, while the adhesion of M3A cells to M3A adherons was independent of calcium. The adhesion of L6 cells to M3A adherons and M3A cells to L6 particles was reduced in the absence of calcium, suggesting that some interaction can take place between the calcium-dependent L6 adhesion mechanism and the calcium-independent M3A adhesion process. Since all of the interactions involving M3A cells or M3A adherons have an altered calcium dependency relative to L6, it follows that the M3A particle is at least partially defective in that part of the cellular adhesion process which requires calcium.

In addition to causing cell-substratum adhesion, adherons

from L6, but not from M3A, aggregated M3A cells; L6 cells aggregated spontaneously in the presence of calcium. The calcium dependency of these interactions was determined by measuring the disappearance of single cells from cell suspensions at different calcium concentrations (Fig. 6). In the absence of calcium, L6 cells did not aggregate, while in the presence of 2 mM calcium 50% of the single cells were eliminated within 15 min. M3A cells did not aggregate spontaneously, and the addition of M3A adherons to M3A cultures did not induce aggregation in 2 mM calcium. However, when L6 adherons were adsorbed to M3A cells at 4°C , the cells washed, and then their aggregation determined in the presence of calcium, the cells aggregated with a half-time of ~ 10 min (Fig. 6). In the absence of exogenous calcium, aggregation of M3A cells by L6 adherons was greatly reduced. If the cells were washed and then calcium was added to the medium, the cells aggregated, showing that the L6 particles remained bound to the cells in the absence of calcium (see also Fig. 4). Therefore, both the spontaneous aggregation of L6 myoblasts and the L6 adheron-

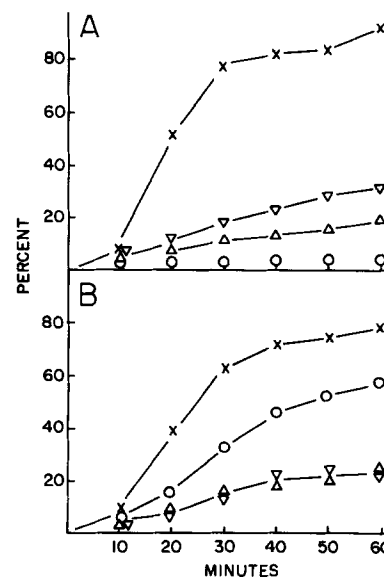


FIGURE 5 Calcium dependence of cell-substratum adhesion. L6 and M3A cells were isotopically labeled and washed in calcium and magnesium-free HEPES medium. Petri dishes were incubated overnight with $5 \mu\text{g}/\text{dish}$ of L6 or M3A particles, washed with EGTA, and then with calcium and magnesium-free HEPES medium. The cells were plated into the various dishes with or without 2 mM calcium chloride, and the percent of input adherent cells was determined as a function of time. (A) L6 Cells: X, L6 adheron coated dishes plus calcium; O, L6 coated dishes minus calcium; ∇ , M3A coated dishes plus calcium; Δ , M3A coated dishes minus calcium. (B) M3A cells: X, L6 adheron coated dishes plus calcium; O, L6 coated dishes minus calcium; ∇ , M3A coated dishes plus calcium; Δ , M3A coated dishes minus calcium.

mediated aggregation of M3A cells is calcium dependent.

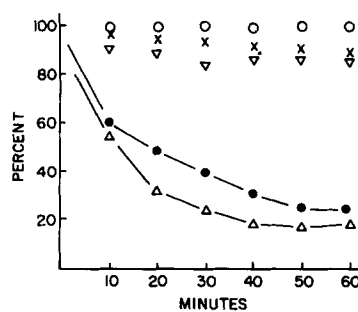


FIGURE 6 Aggregation of L6 and M3A cells. L6 myoblasts were dissociated with EGTA as described in Fig. 1. L6 and M3A cells were washed three times with HEPES medium minus calcium and magnesium, and diluted into medium with or without 2 mM calcium. In one sample, M3A cells were preincubated

with $5 \mu\text{g}/\text{ml}$ of L6 adherons at 4°C for 2 h before the adhesion assay, and washed in calcium-free medium. The disappearance of single cells was then monitored, and the data were plotted as the percent input cells which remain as single cells as a function of time. O, M3A cells alone plus calcium; X, L6 cells minus calcium; ∇ , M3A cells plus L6 adheron minus calcium; ●, L6 cells plus calcium; Δ , M3A cells plus L6 adheron plus calcium.

induced aggregation of M3A cells were calcium dependent (8), while M3A adherons were unable to cause any cell aggregation, although they did bind to M3A cells (Fig. 4).

The above data suggest that M3A adherons may be deficient relative to L6 adherons in their ability to be aggregated by calcium ions. To directly test this possibility, L6 and M3A adherons were resuspended in various concentrations of calcium ions and centrifuged into 5–20% sucrose gradients made up in the same calcium concentration. The percent of the input counts which were recovered as aggregates in the bottom of the gradient was plotted against ion concentration, and the data are shown in Fig. 7. Similar curves were generated when the disappearance of the nonaggregated adheron peaks was plotted against calcium concentration. These data show that L6 adherons aggregated in the presence of calcium, while M3A particles did so to a much lesser extent. In addition to particle aggregation, L6 cell aggregation and L6 particle-induced M3A aggregation were also calcium dependent. Increasing amounts of calcium in the assay media stimulated aggregation with about the same response curve as with the cell-free particles (Fig. 7). These data show that the aggregation of L6 adherons and L6 cell-cell aggregations were both calcium dependent. Since the dose-response curves were similar with cells and particles, the data are consistent with the possibility that adherons are involved in the cellular adhesive responses.

Chemical Composition of L6 and M3A Adherons

To assay the protein and GAG compositions of the adhesion-modulating particles, the cells were labeled for 16 h with [^{35}S] methionine, [^3H]glucosamine, or $^{35}\text{SO}_4$ in serum-free media. The culture media were then centrifuged at 100,000 g for 2 h, and the pellets were washed twice, and sedimented in sucrose

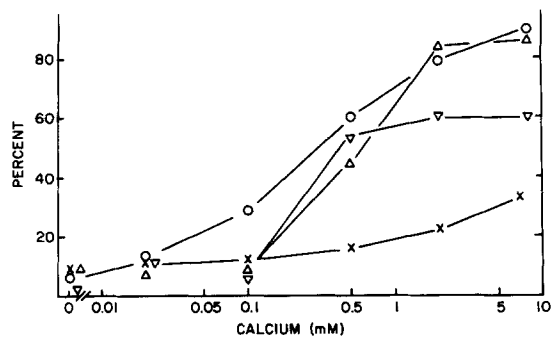


FIGURE 7 Calcium dependence of aggregation. To assay cell-cell aggregation, cells were washed in calcium- and magnesium-free medium and diluted into medium containing the indicated amount of calcium. M3A cells were incubated with L6 adherons (5 $\mu\text{g}/\text{ml}$) for 4 h at 4°C in calcium-free HEPES medium. The cells were then washed and diluted into HEPES medium containing the indicated amounts of calcium. The disappearance of single cells was monitored (Fig. 6), and the percent of single cells lost at 30 min for each calcium concentration was plotted as a function of calcium concentration. To assay particle aggregation, [^{35}S]sulfate-labeled L6 and M3A particles were incubated at the different calcium concentrations for 2 h at 37°C, and then centrifuged into 5% to 20% sucrose gradients made up in the same calcium concentration and 0.01 M HEPES buffer, pH 7.1 (Fig. 3). After 24 h at 36,000 rpm in a Beckman SW41 rotor, the gradients were fractionated and the amount of isotope associated with the bottom 2 cm of the tubes (pelleted material) was also determined. The percent of input isotope which was recovered in the pellet was plotted as a function of calcium concentration. \circ , L6 particles; \times , M3A particles; ∇ , aggregation of L6 cells alone; Δ , aggregation of M3A cells plus L6 particles.

gradients as described in Fig. 3. The isotopically labeled peaks were removed, the sucrose was diluted with HEPES medium, and the particles were repelleted by centrifugation overnight at 100,000 g. These pellets were assayed for proteins on polyacrylamide gels containing SDS, and the GAG content was determined by column chromatography and enzymatic analysis. When the leucine-labeled material was electrophoresed on a SDS acrylamide gel system which readily separates fibronectin and the collagens, the protein compositions of the particles from the two types of muscle cells were very different from each other and distinct from the Triton X-100-insoluble cytoskeletons prepared from each line (Fig. 8). Evidence presented elsewhere has shown that L6 band A is fibronectin and that bands B and C (Fig. 8) are collagen-related (13, 14). The identity of the remaining fractions is unknown. The adherons

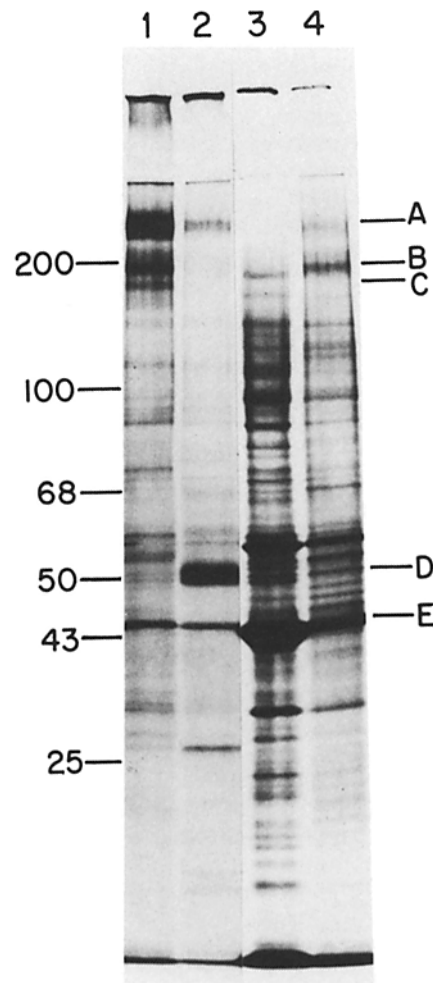


FIGURE 8 SDS-acrylamide gels of adheron proteins from L6 and M3A. Exponentially dividing cells were labeled with ^{35}S -methionine for 18 h and the culture supernatant was centrifuged (100,000 g for 2 h). The pellet was then sedimented on a 5–20% sucrose gradient as described in Fig. 3. The peaks were isolated (the two unresolved peaks of M3A were pooled) and electrophoresed in 10% polyacrylamide gels containing SDS. Cytoskeletons were prepared as described in Materials and Methods. (Lane 1) L6 adherons. (Lane 2) M3A adherons. (Lane 3) M3A cytoskeleton. (Lane 4) L6 cytoskeleton. 20,000 cpm were applied to the slab gels for each of the adherons, and 70,000 cpm were applied for each of the cytoskeletons. Lanes 1–4 were run on the same slab, but the lanes containing adherons were exposed to film longer than the lanes containing cytoskeletons. Molecular weight, $\times 10^3$.

from M3A have relatively less fibronectin and a very small amount of a collagen-related protein (Fig. 8, band C). Band C (Fig. 8) of M3A was identified as collagen on the basis of its high ratio of proline, glycine, and alanine to leucine, 4-hydroxyproline content, and susceptibility to collagenase (data not presented). The major band in M3A adherons (Fig. 8, band D) was not collagen-related by the above criteria. It migrates on SDS acrylamide gels with an apparent molecular weight of 50,000, it is not a major cytoskeletal protein, and its identity is unknown. Band E (Fig. 8) comigrates with actin, is present in cytoskeletons, and is probably actin.

The GAGs in the L6 myoblast adherons and those of M3A were also different. Fig. 9 shows the elution profile from a DEAE column of [³H]glucosamine-labeled GAGs prepared from the adherons of L6 myoblasts and M3A cells. Four classes of GAGs were separated, and each peak of radioactivity was identified by enzymatic degradation in addition to its comigration with known GAGs on the DEAE column. Peaks IA and IB in both cell lines were degraded by fungal hyaluronidase and contained no sulfate; they were both hyaluronic acid (Fig. 9). The double peak is due to size or charge heterogeneity. Peak II in L6 was degraded by chondroitinase ABC and testicular hyaluronidase, but not by nitrous acid or fungal hyaluronidase. It is therefore chondroitin. Since M3A lacks extracellular chondroitin but does accumulate chondroitin intracellularly, there may be a block in the secretion of this GAG (14). Peaks IIIA and IIIB contained sulfate and were degraded by nitrous acid but not by fungal hyaluronidase. They were tentatively identified as heparan sulfate, for heparin eluted after the chondroitin sulfates on the DEAE column. Peaks IVA and IVB also contained sulfate and were degraded by chondroitinase ABC but not by nitrous acid. They were the chondroitin sulfates. No distinction was made between the three classes of chondroitin sulfates.

DISCUSSION

The following conclusions may be drawn from the above data. (a) Both L6 and its less adhesive variant, M3A, released glycoprotein particles into their culture medium. (b) The particles, termed adherons, from the M3A variant were much less efficient in promoting cell-substratum adhesion of L6 and M3A cells than those from the parental L6 line (Figs. 1 and 2). (c) The adherons from the L6 myoblast line and its variant had different sedimentation velocities in sucrose gradients (Fig. 3) and different protein and GAG compositions (Figs. 8 and 9) (d) Particles from both of the cell lines were able to adhere to plastic surfaces and to cells equally well in the absence of calcium ions (Fig. 4). M3A adherons did not, however, possess the calcium-dependent adhesion mechanism which was required for the particles to aggregate in sucrose gradients and to stimulate cellular adhesion (Figs. 5, 6 and 7). These data suggest that the interactions which mediate cellular adhesion are distinct from those which are involved in the binding of adherons to cells.

A variety of molecules have been described which are involved in cell-cell and cell-substratum adhesion. Perhaps the best studied cellular adhesion mechanisms are the collagen-fibronectin interaction which mediates cell-substratum adhesion in fibroblastlike cells (for review see reference 23) and two molecules thought to be involved in chick neural retina cell aggregation (9, 19). In the case of neural retina, both protein molecules were isolated from growth-conditioned medium. Similarly, collagen, fibronectin, and GAGs are released into

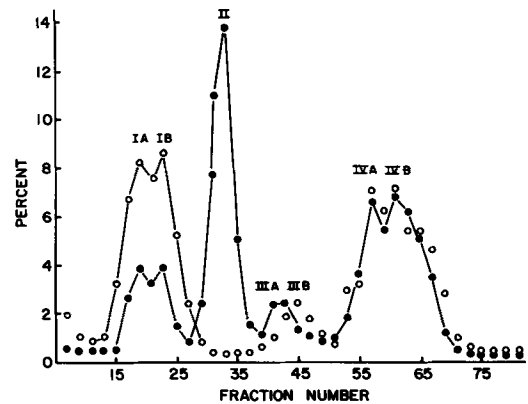


FIGURE 9 Elution profile of GAGs from L6 and M3A particles. Exponentially dividing cells were labeled with [³H]- or [¹⁴C]glucosamine and ³⁵SO₄ for 18 h and the culture medium supernatant was centrifuged. The pellet was applied to a 5–20% sucrose gradient in the absence of calcium. The peaks were isolated, digested with pronase, and the GAGs were run on a DE-52 column. The data are expressed as the percentage of the total recovered counts in each fraction. ○, [¹⁴C]glucosamine-labeled M3A; ●, [³H]glucosamine-labeled L6. Sulfate label was only associated with peaks IIIA, IIIB, IVA, and IVB (data not shown).

the culture medium by fibroblasts, but most attention has been directed toward the interaction of substrate-bound collagen and cell-surface fibronectin. The data presented above show that in skeletal muscle myoblasts, a subset of the extracellular macromolecules are organized into a structurally defined particle which is able to adhere to cells and increase their initial rates of adhesion. The 16 S adherons from skeletal muscle myoblasts promoted the cell-cell and cell-substratum adhesion of myoblasts, but inhibited the adhesion of the sympathetic nervelike cell line PC12. Conversely, adherons from all smooth muscle cell lines tested promoted the adhesion of PC12 cells (16). These results were consistent with the required specificity of sympathetic nerves interacting with muscle, for only smooth muscle is innervated by sympathetic ganglion cells. Since this type of limited tissue specificity is demonstrable, it follows that adherons may play a role in normal cell-cell interactions. In addition, an alteration in adheron structure may be associated with abnormal cellular differentiation and growth.

The mechanisms involved in the adhesion of L6 and its less adhesive M3A variant are functionally distinguishable by the following criteria. (a) Substrate attached fibronectin and collagen stimulated the adhesion of L6 cells but did not promote the adhesion of M3A cells (15). (b) Exogenous GAGs inhibited the adhesion of M3A cells to adherons prepared from L6 and M3A, while they did not block L6 adhesion to L6 particles (13, and unpublished observations). (c) The efficiency of L6 particles in promoting the adhesion of L6 or M3A cells was at least fivefold greater than that of M3A (Figs. 1 and 2). (d) L6 particles aggregated M3A cells, while M3A adherons were inactive (Fig. 6). These results suggest that the cell-substratum adhesion mechanism employed by L6 involves the collagen-fibronectin system and is therefore similar to that used by other mesodermally derived cells. The adhesion-deficient M3A variant, however, apparently lost the fibronectin-collagen adhesion system and employed a mechanism which more directly involves GAGs. It may be generally true that transformed mesodermal cells lose the primary fibronectin-mediated adhesion mechanism (5) and revert to a secondary, less efficient adhesion mechanism employing GAGs. In the pair of cells

described here, this change is reflected in the adhesive properties of their adherons. Both calcium-dependent and calcium-independent adhesion mechanisms have recently been described in two other cell types (18, 20). In both cases the calcium-independent adhesion mechanism was less sensitive to trypsin than the calcium-sensitive mechanism. It is possible that the trypsin-sensitive, calcium-dependent mechanisms are analogous to the fibronectin-mediated adhesion of L6, while the less calcium-sensitive adhesions are mediated primarily by GAGs as with M3A. What can the characterization of the M3A variant adheron tell us about the normal adhesive functions of L6 adherons?

The M3A particle aids in distinguishing two classes of adheron binding properties. The first interaction, which is shared by L6 and M3A particles, is the ability to bind to a variety of substrata and cells in the absence of exogenous calcium ions (Fig. 4). The second binding property of the normal L6 adheron is its ability to aggregate in a calcium-dependent manner. The apparent deletion of the calcium-dependent adhesion mechanism of M3A cells and adherons is reflected in both cell-substratum and cell-cell adhesion deficiencies (Figs. 5 and 6). It follows that the adherons of skeletal muscle myoblasts normally bind to cells and other surfaces by a calcium-independent mechanism, but once they are attached to the surface, they affect cellular adhesion by interactions requiring exogenous calcium ions. The type of adhesion mechanism used by rat myoblast adherons appears to be very similar to that of sponge aggregation factors, where there is a calcium-independent binding of the aggregation factor to surface receptors and a calcium-dependent interaction between factors on adjacent cells to promote aggregation (6).

There are several structural alterations in M3A adherons which were potentially responsible for the functional differences between M3A and L6 particles. M3A adherons had relatively less collagen and fibronectin than those of L6, and the major protein in M3A particles was of 50,000 mol wt and unknown identity (Fig. 8). L6 adherons were composed of 30% GAGs and 70% protein, while only 10% of the M3A particles were GAGs. In addition, the M3A adherons lacked unsulfated chondroitin, which was a major constituent of L6 adherons (Fig. 9). It follows that either chondroitin or collagen, or a combination of both, was involved in the normal L6 calcium-dependent adhesion process which is defective in M3A adherons. Since the adhesion of fibroblasts to collagen is calcium dependent (7), the relative lack of collagen in the M3A particle may have eliminated the most effective adhesion mechanism of its parental cell line.

The *in vivo* function of adherons remains to be defined. Particles sedimenting between 5 S and 18 S in sucrose gradients have been isolated from a variety of cell types, including skeletal and smooth muscle (13, 16), clonal nerve and glial cell lines, fibroblasts, and chick neural retina (unpublished observations). These particles either stimulate or inhibit cellular adhesion, contain GAGs and a limited number of proteins, and in some cases have the adhesive specificity which is required if they play a role during normal development (16). GAG-containing particles have been demonstrated along the outer surfaces of basement membranes (3), and the particles may be involved in basement membrane assembly. Substrate-

attached material containing GAGs and protein promote the neurite outgrowth of some cultured nerve cells (2). Since the adheron particles are sticky in a nonspecific way through the calcium-independent mechanism, they could form a limited gradient around a cell mass to either promote or inhibit the adhesive interactions with other cell types. The release of particles during the cell proliferative phase of development may promote nucleation phenomena, helping to keep cells together. Finally, adherons may serve as markers to show where cells have been. For example, regenerating frog nerves tend to regrow to the exact areas of muscle where the original synapse was located. Since only the muscle basement membrane but not the living cell need be present for the process to occur (12), it is likely that the active nerve terminal deposited a unique marker which enabled it to retrace its original path and terminate at a particular spot on the muscle basement membrane. Adheronlike particles released by the nerve could carry out both of these functions.

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