

Requirements for the Ca²⁺-independent Component in the Initial Intercellular Adhesion of C2 Myoblasts

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Abstract. Using a sensitive and quantitative adhesion assay, we have studied the initial stages of the intercellular adhesion of the C2 mouse myoblast line. After dissociation in low levels of trypsin in EDTA, C2 cells can rapidly reaggregate by Ca²⁺-independent mechanisms to form large multicellular aggregates. If cells are allowed to recover from dissociation by incubation in defined media, this adhesive system is augmented by a Ca²⁺-dependent mechanism with maximum recovery seen after 4 h incubation. The Ca²⁺-independent adhesion system is inhibited by preincubation of cell monolayers with cycloheximide before dissociation. Aggregation is also reduced after exposure to monensin, implicating a role for surface-translocated glycoproteins in this mechanism of adhesion. In coaggregation experiments using C2 myoblasts and 3T3

fibroblasts in which the Ca²⁺-dependent adhesion system was inactivated, no adhesive specificity between the two cell types was seen. Although synthetic peptides containing the RGD sequence are known to inhibit cell-substratum adhesion in various cell types, incubation of C2 myoblasts with the integrin-binding tetrapeptide, RGDS, greatly stimulated the Ca²⁺-independent aggregation of these cells while control analogs had no effect. These results show that a Ca²⁺-independent mechanism alone is sufficient to allow for the rapid formation of multicellular aggregates in a mouse myoblast line, and that many of the requirements and perturbants of the Ca²⁺-independent system of intercellular myoblast adhesion are similar to those of the Ca²⁺-dependent adhesion mechanisms.

MYOGENESIS *in vivo* is a complex cellular process initially involving a series of cell-cell interactions whereby myoblasts fuse to form multinucleate myotubes (38, 77). Of fundamental importance in this process, before fusion, are the mechanisms involved in myoblast recognition and adhesion (36). Indeed, myoblast recognition can be thought of as a primary stage of myogenesis, and aggregation studies using cultured myoblasts present a convenient model for studying many of these early interactions (28, 36, 37). From the use of myoblast cultures, much has been learnt about the conditions required for myoblast adhesion. For example, the existence of two independent, non-complementing adhesive systems has been identified (21, 36) and a role for glycoprotein secretion has also been suggested (9, 35). Although myogenesis begins with myoblast recognition and adhesion, these events themselves are multistep phenomena (19). Initially, interactions probably occur between surface-associated ligands and their corresponding receptors (42). These interactions may involve homophilic binding, as in neural cell adhesion molecule binding to neural cell adhesion molecule (58), or heterophilic binding, as with integrin-ligand binding (8). The integrins (57) are a family of structurally related receptors which bind many adhesive glycoproteins that contain the tripeptide arginine-

glycine-aspartic acid (RGD) as their cell recognition site. Such proteins include fibronectin (50) and vitronectin (54).

It has long been recognized that early stages of intercellular adhesion do not require metabolic energy and cell aggregates are formed by weak, reversible associations. These are then supplemented by mechanisms which are energy dependent and which produce stable intercellular adhesions (43, 56, 72). Both stages occur within minutes of initial cell contact (56) and, in many systems, such adhesions are later further strengthened by a variety of junctional mechanisms (19).

The effects of the RGD tripeptide, and longer oligopeptides containing this sequence, on cell-substratum adhesion in nonmyogenic cells have recently received much attention (1, 2, 5, 30, 41, 46, 49, 55, 59, 61, 64). However, the only data at present on the effects of RGD on intercellular (in contrast to cell-substratum) adhesion in suspension relate to embryonic chick segmental plate and somite cells (39) and to the role of fibronectin and thrombospondin in platelet function (23, 29).

In this report we examine the conditions required for the formation of early intercellular adhesions in the C2 mouse muscle line (78). We show that the formation of such early adhesions is suppressed by the monovalent carboxylic iono-

phore monensin, and is enhanced by exposure to an RGD-containing tetrapeptide (RGDS). Furthermore, although a trypsin-sensitive mechanism is present in the early stages of murine myoblast adhesion, a component which is resistant to low levels of trypsin, and is Ca^{2+} -independent, is also prominent. These findings are discussed in relation to earlier reports of separate Ca^{2+} -dependent and Ca^{2+} -independent adhesive mechanisms in embryonic chick myoblasts (21, 35–37).

Materials and Methods

Cell Culture

The mouse C2 muscle cell line (78) was obtained from Dr. H. Blau, Stanford University, Stanford, CA. They were grown on uncoated 150-mm culture dishes in DME containing 2 mM L-glutamine, 20 U/ml gentamicin, and supplemented with 10% FCS. Cultures were maintained at 37°C in 8% CO_2 and used between passages 21 and 33. Only subconfluent cultures of replicating myoblasts were used for experiments and then only if all cells in the cultures were mononucleate. Mouse L cells and 3T3 fibroblasts were both obtained from Gibco Ltd., Paisley, UK and maintained under the same conditions as C2 myoblasts.

Pretreatments and Cell Dissociation

For the standard aggregation assays, cells were not pretreated before dissociation. However, in some cases cultures were preincubated in full medium with either 0.5 μM monensin (Calbiochem-Behring Corp., La Jolla, CA) or 50 $\mu\text{g}/\text{ml}$ cycloheximide before trypsinization. Generally, cultures were washed briefly in 7.0 mM EDTA at 4°C and incubated with 3.0 ml 0.5% trypsin in 7.0 mM EDTA for 6 min at room temperature with gentle agitation. Trypsin was purchased from Gibco Ltd. and had an activity such that 1 g trypsin will hydrolyse 250 g casein under standard conditions. It is important to note that the specific activity of recrystallized trypsin (10,000–13,000 U/ml Na-benzoyl-L-arginine ethyl ester) was found to be 10 times that of the trypsin used here, and was used in a similar dissociation protocol (17). This method of dissociation is also similar to that of Gibraltar and Turner (21) to yield LTE cells in their terminology. Cultures were discarded if <75% of cells had not dissociated by this time. Trypsinization was stopped by the addition of 5 $\mu\text{g}/\text{ml}$ leupeptin and the cells were collected into 20 ml $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks basal salts' solution (HBSS) at 4°C.

For some experiments, cells were trypsinized with 0.5% trypsin in HBSS. Although this is listed as a Ca^{2+} -free solution, measurements using a Ca^{2+} -specific electrode, showed the free Ca^{2+} concentration to be 18.0 μM . Addition of trypsin to HBSS then raised this concentration to 66.0 μM . Under these conditions, the time required for 0.5% trypsin to detach the cells was much greater and the reaction was stopped by leupeptin after 15, 20, and 25 min of incubation.

Recovery after trypsinization was investigated by two methods. First, cultures which had been treated as described above were centrifuged at 400 g for 10 min at 4°C and then washed in HBSS. These cells were then centrifuged as before and resuspended in 25 ml SATO medium and replated at a concentration of 2×10^5 cells/ml on 150-mm bacteriologic-grade dishes. Serum-free SATO medium (4) was the same as described by us previously for use in PC12 cultures (14), with the addition of 5 $\mu\text{g}/\text{ml}$ leupeptin. Cultures were incubated in SATO medium for up to 24 h at 37°C in 8% CO_2 . The loosely attached cells were removed from the substratum by gentle trituration with 10 ml MEM at 4°C and then prepared for the aggregation assay as described below. The second method used for trypsin recovery was once again to resuspend the HBSS-washed cells in SATO medium, but in this case the cells were kept in suspension by rotating them at 60 rpm for up to 24 h at 37°C.

Adhesion Assay and Aggregation Kinetics

All cell suspensions (with or without a recovery period) were washed in HBSS at 4°C and then resuspended at 4°C in MEM containing 5% ficoll and 5 $\mu\text{g}/\text{ml}$ leupeptin. Leupeptin was added to the final aggregation medium since it has been shown that residual trypsin can remain with trypsin-dispersed cells even after extensive washing (26). The cell suspension was then triturated, passed through a 19-gauge syringe, filtered through

a 20- μm mesh, and then passed through a 19-gauge syringe once more. All these steps were carried out at 4°C and ensured that the cell suspension contained >95% single cells. Cells were then diluted to either $1.4\text{--}1.8 \times 10^6$ cells/ml or $0.4\text{--}0.7 \times 10^6$ cells/ml. To this final aggregation medium, the following known or suspected perturbants of intercellular or cell-substratum adhesion were added: (a) 5–50 mM EDTA was added and no difference in effects on adhesion were noted between these concentrations, (b) monensin and cycloheximide at the concentrations described above, (c) 10% FCS, and (d) the tetrapeptides arginine-glycine-aspartic acid-serine (RGDS) or glycine-lysine-glycine-aspartic acid (GLGD). Both peptides were synthesized following standard procedures of fluorinyl methylene oxycarbonyl synthesis and purified to >90% homogeneity using an analytical C18 reverse-phase HPLC column. Amino acid analysis and fast atom bombardment mass spectrophotometry were used to confirm the sequences synthesized. In addition, the control tetrapeptide arginine-glycine-glutamic acid-serine (RGES) obtained from Peninsula Laboratories (Liverpool, UK) was also used. The tetrapeptides were added to the cell suspensions at a final concentration of 0.1–1.0 mg/ml. The suspensions were then kept at 4°C for 10 min before the aggregation assay.

The aggregation assay is based on measurements of the collision efficiency of cells using couette viscometry, in which a dissociated cell suspension is allowed to reaggregate under carefully controlled microenvironmental conditions (11). The technique permits calculations of both the total number of intercellular collisions in a cell suspension and the number of these collisions which produce adhesions as a function of time. The probability that a collision between two particles results in an adhesion is given by: $\ln N_{\infty}/N_{\infty 0} = -4G\alpha t/\pi$ where $N_{\infty t}$ and $N_{\infty 0}$ are the total number of particles at time t and time zero respectively; G is the shear rate of the suspending medium; ϕ is the volume fraction of the particles in suspension; and α is the collision efficiency or "adhesiveness" of the cells. If the cells are allowed to reaggregate in a laminar flow of a known constant shear rate, α can be calculated directly by measuring the change in particle number with time. Values ≤ 1 can be regarded as reflecting zero adhesiveness. The technique of couette viscometry provides for a shear rate to be selected and held constant during the course of the assay. In the present study, the speed of rotation of the viscometers was set such that a shear rate of 10^{-1} was generated. The inner surfaces of the viscometers were coated with silicon every 2 wk to prevent cell attachment during the assay. Further details of this assay have been reported by us previously (31–34, 51).

1.0 ml of cell suspension was transferred to the well of the viscometer and reaggregation was allowed to occur at 37°C. At 5-min intervals, 15- μl aliquots were removed and the total particle count measured using a hemocytometer. During the initial stages of adhesion, when aggregate size is small and the number of cells within them can still be accurately determined, it was confirmed that although the total number of particles in suspension decreased (as cells formed adhesions and were then recruited into larger aggregates), the total number of cells present did not change significantly, for example as a result of lysis. The viability of the cells was also confirmed by nigrosin exclusion in aliquots taken at the beginning and end of each of the assays. In all of the experiments described here, cell viability was found to be >80%.

Measurements of Adhesive Specificity

Adhesive specificity between C2 and 3T3 cells was measured based on modifications (51) of the technique described by Sieber and Roseman (60). Briefly, HBSS-washed C2 and 3T3 cells were resuspended in HBSS containing either 0.01% acetone (control) or 10 $\mu\text{l}/\text{ml}$ 6-carboxyfluorescein diacetate (CFDA).¹ CFDA is a novel fluorescein ester (7) which is more sensitive and stable than the fluorophore, fluorescein diacetate (24), and it also allows for a clear delineation between labeled and unlabeled fixed cells even after several months of storage at -20°C (51). CFDA was obtained from Molecular Probes Inc., (Junction City, OR) and kept as a 0.5% stock solution in acetone. Cells were incubated for 15 min at 37°C and then centrifuged at 400 g for 10 min at 4°C. The cells were washed once more in HBSS, spun down at 4°C, and finally resuspended in the aggregation medium at a final concentration of 0.5×10^6 cells/ml. The stained and unstained cell suspensions were carefully triturated to ensure that >99% of the cell suspensions consisted of single cells and the two suspensions were mixed immediately before aggregation.

After 20 min of aggregation, aliquots were transferred to hemocytometers and poly-L-lysine-coated multitest slides. In the latter case, cells and aggregates were allowed to attach for 5 min at room temperature and then rinsed in 0.1 M sodium cacodylate buffer (pH 7.2) before fixation in 4%

1. *Abbreviation used in this paper:* CFDA, 6-carboxyfluorescein diacetate.

paraformaldehyde in buffer for 30 min at room temperature. Aggregates containing three or four cells were scored and the distribution of labeled and unlabeled cells within them was recorded.

Statistical Analyses

Collision efficiencies were compared using Student's *t* test. In addition, the initial rates of aggregation (<15 min) were compared. During this period, the rate of particle decrease (i.e., aggregate formation) is either linear or approximates to linearity and thus allows for the comparison of regression lines by a two-tailed *F* test (62).

Adhesive specificity was tested as described previously (51). Briefly, if two cell populations coaggregate with no adhesive specificity then the distribution of the two cell types within the aggregates is binomial (60). The deviation from the observed distribution of cells within the aggregates from that calculated on the basis of no adhesive specificity can be measured by a chi-square test. In a sample of *N* aggregates (of a constant cell number) and consisting of A and B cell types, then the mean number of A cells per aggregate (\bar{A}) can be calculated, and the variance (s^2) is given by: $s^2 = [E(A - \bar{A})^2] / (N - 1)$. The relative frequencies of A (*m*) and B (*n*) cells in the cell suspension after aggregation were determined, and thus the binomial distribution which assumes nonspecific adhesion between the two cell types in, for example, the four-cell aggregate class is given by: $P(A) = N! / [m^A (n^{N-A})!]$, and the variance (σ^2) is given by $4mn$. The difference in variances between the two distributions is then tested by, $\chi^2 = Ns^2/\sigma^2$ with ν ($\nu = N - 1$) degrees of freedom.

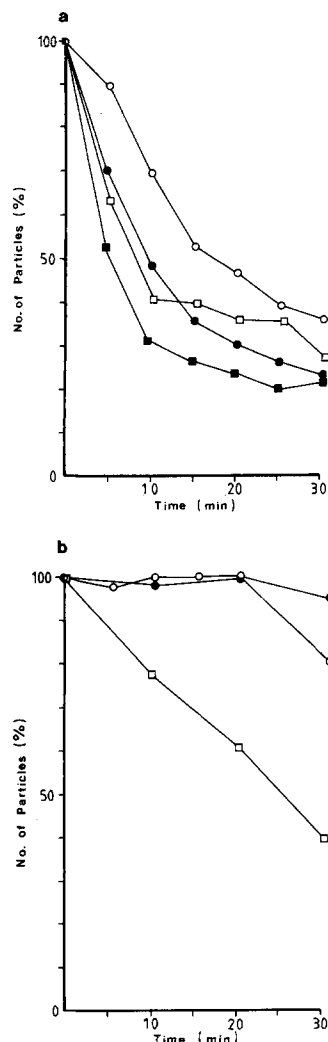


Figure 1. Aggregation kinetics of freshly dissociated C2 myoblasts in medium containing 2.8 mM Ca²⁺. (a) Cells were dissociated in 0.5% trypsin/5 mM EDTa and allowed to aggregate with (■, □) or without (●, ○) 10% FCS. Cells were incubated at $0.4\text{--}0.6 \times 10^6$ cells/ml (○, □) or $1.4\text{--}1.8 \times 10^6$ cells/ml (●, ■). In this and subsequent figures each data point represents the mean of four experiments and for each point the SEM was <5% of the mean. (b) Cells were dissociated by incubating monolayers with 0.5% trypsin/70 μM Ca²⁺ for 15 (□), 20 (○), and 25 (●) min. In all cases cells aggregated at a slower rate than in a, and the formation of multicellular aggregates was completely inhibited after 25-min trypsin incubation.

Results

Aggregation Kinetics of Freshly Trypsinized Myoblasts

C2 myoblasts, which were dissociated in 0.5% trypsin/7.0 mM EDTa and finally resuspended at $1.4\text{--}1.8 \times 10^6$ cells/ml, formed many doublets and triplets within 5 min of the aggregation assay. By 15 min, many multicellular aggregates were seen and the rate of aggregation was such that at this cell concentration there was relatively little change in particle number between 15 min and the end of the aggregation assay (Fig. 1 a), or up to 60 min of aggregation (data not shown). However, this rate of aggregation was too rapid for some of the assay criteria. For example, it was only possible to confirm that the initial cell number did not change for the 5-min time point; at later stages the aggregates were too large to identify individual cells. Furthermore, if the rate of cellular recruitment into the aggregates is hyperbolic, then the logarithm of the number of particles remaining will yield a straight line from which initial rates of adhesion (in addition to total adhesiveness as described below) can be calculated. When the cell density was decreased to $0.4\text{--}0.7 \times 10^6$ cells/ml, many multicellular aggregates were still formed within 15 min (Fig. 2 a) although a slower rate of aggregation was observed (Fig. 1 a). Furthermore, this time a logarithmic transformation generated a slope that was linear over the time course of the assay and not just for the first 15 min as was the case for the higher cell concentration (Fig. 3).

The rate of aggregation was also increased by the addition of 10% FCS to the suspensions containing cells at both high and low concentrations (Fig. 1 a). This is in contrast to our earlier findings using freshly trypsinized human skin fibroblasts in which no difference in adhesion was found during the first 30 min of aggregation in the presence (31) or absence (32, 33) of FCS. This may be related to the greater adhesiveness of these cells compared to C2 myoblasts such that the augmentative properties of FCS on adhesion are relatively small in the former case. The adhesiveness of C2 aggregation relative to human skin fibroblasts and to the weakly adhesive mouse L cell line (45) is shown in Table I where it can be seen that they exhibit intermediate intercellular adhesiveness.

It should be noted that although the initial rates of adhesion are different for C2 cells aggregated at high density (with and without FCS) and at low density with FCS, the number of particles remaining in suspension at 30 min is similar at ~30% (Fig. 1 a).

For the reasons given above, the conditions used to investigate the perturbants of C2 adhesion were those using the lower cell density and in the absence of FCS (to avoid adsorbed serum components masking any difference in adhesion).

Calcium and Trypsin Sensitivity of Myoblast Aggregation

Many reports have studied the Ca²⁺-dependent and Ca²⁺-independent components of intercellular adhesion by inactivating one or other mechanism by varying the calcium and trypsin content of the dissociation medium (see Discussion). In experiments in which we dissociated C2 cells in 0.5% trypsin in HBSS, the cultures required a minimum of 15 min at room temperature for >75% of cells to be detached from the substratum. This increased time for cell dissociation may

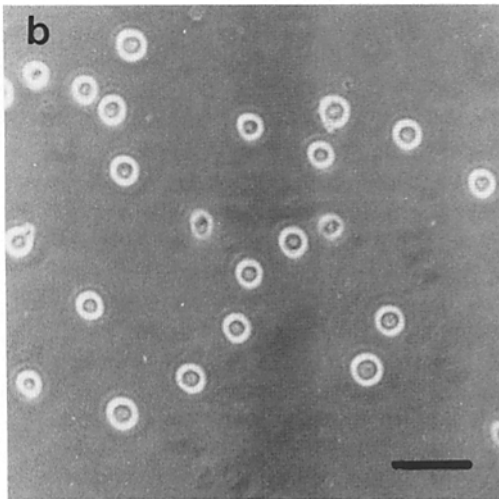
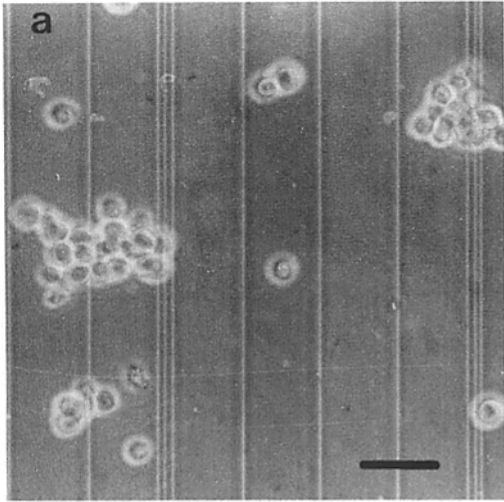


Figure 2. Representative micrographs of C2 myoblasts dissociated in trypsin/EDTA and allowed to aggregate for 15 min in 2.8 mM Ca^{2+} . Multicellular aggregates are frequently seen by this time in control media (a) although their formation is inhibited after the same period after a 2-h preincubation with 0.5 μM monensin (b). Bars, 50 μm .

have been a consequence of adventitious Ca^{2+} (66 μm) in nominally Ca^{2+} -free medium.

Cells exposed to trypsin in HBSS for 15 min aggregated much slower than cells trypsinized with EDTA for 6 min, and aggregation was further inhibited by increasing the exposure to trypsin (Table I, Fig. 1 b). In all cases, cell viability was not significantly affected by the longer exposure to trypsin. This was unexpected since if the presence of Ca^{2+} in the dissociating medium was sufficient to affect the proteolytic activity of the trypsin treatment, it might be expected to increase subsequent myoblast aggregation by a partial protection of the Ca^{2+} -dependent adhesion system (21). However, it is possible that although the adventitious Ca^{2+} in HBSS is sufficient to partially inhibit the action of trypsin in dissociating the cells from the substratum, it is not sufficient to protect the Ca^{2+} -dependent adhesion system.

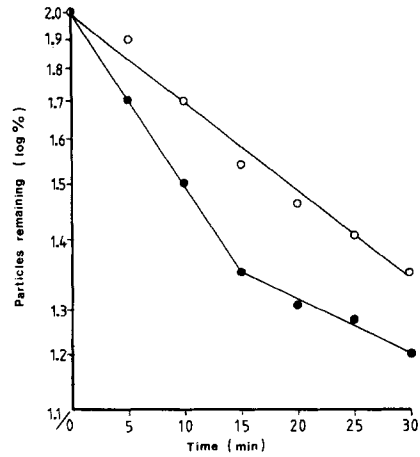


Figure 3. Aggregation rate of C2 myoblasts incubated at low (\circ) and high (\bullet) cell densities (see text for details). The lower cell density was selected as the concentration for all experiments described here since the logarithmic decrease in particles was constant throughout the time course of the assay. At higher cell concentrations, discontinuities were found at, or near, the 15-min time point.

The effect of trypsin on myoblast aggregation was also studied by allowing cells to recover from trypsinization. This was performed by either incubating them in suspension in FCS-supplemented or SATO media, or by seeding them onto bacteriologic-grade dishes. Trypsinized cells seeded onto bacteriologic-grade dishes and incubated at 37°C for 2–5 h, did not subsequently reaggregate; $\sim 70\%$ remained as single cells 20 min after the start of the assay (Fig. 4 a). The decrease in particle number was confirmed to be due to loss of the cells through lysis rather than to recruitment into aggregates. However, cells incubated in FCS-supplemented media for 2 h rapidly formed large, multicellular aggregates (Fig. 4 a). Although these cells were washed before aggregation, it is possible that serum components remaining adsorbed to the cell surface were affecting the kinetics of adhesion. Therefore, SATO medium was used as a recovery medium after trypsinization. No significant difference was seen in rates of adhesion (or the number of particles remaining at the end of the assay) between freshly trypsinized cells and those allowed to recover for 1 h in SATO (Table I). However, after recovery for 2 h, the rate of aggregation was much greater than for unrecovered cells, although the proportion of particles remaining after a 30-min aggregation was similar (Fig. 4 b). When the recovery period was extended to 3–4 h, both the rate of aggregation and the proportion of particles remaining at the end of the assay were affected. In the latter case only 20% of the original number of particles remained after a 20-min aggregation (Fig. 4 b). No differences were found between the kinetics of cells allowed to recover for 4–5 h. When cells were recovered in suspension in SATO for longer than 6 h, large histotypic aggregates (26) were formed which were stable against trituration and required trypsinization to disrupt them. Thus, although full adhesive recovery from trypsin treatment may not have been achieved by 4 h, it is clear that the intercellular adhesiveness of these cells has greatly increased (Table I). It is also evident that a major component of the mechanisms responsible for C2 intercellular adhesion, as measured by this assay, is resistant to mild

trypsin treatment in the absence of divalent cations (Table I, Fig. 4 b).

The Ca^{2+} content of the aggregation medium was measured to be 2.8 mM. Addition of 5 mM EDTA to the aggregation medium had no significant effect on the adhesiveness of freshly trypsinized C2 myoblasts; the values were similar to those aggregated in the presence of Ca^{2+} (Table I) and in both cases $\sim 40\%$ of particles remained after 30-min aggregation (Fig. 4 c). In contrast, for cells given a 4-h recovery period in SATO after trypsinization, no significant increase in adhesion (compared to unrecovered cells) was observed (Fig. 4 c). Thus similar kinetics of adhesion were seen in freshly trypsinized cells with and without EDTA, and recovered cells with EDTA. These data suggest major differences in the Ca^{2+} requirements of the trypsin-sensitive and trypsin-resistant components of C2 adhesion. They indicate that the Ca^{2+} -independent adhesion mechanism on C2 cells is relatively resistant to trypsin, as has been shown for neural retina cells (40).

Requirements for Protein Synthesis and Glycoprotein Secretion

The requirement for protein synthesis in the formation of histotypic aggregates over a 24-h aggregation assay by Ca^{2+} -independent (but not by Ca^{2+} -dependent) adhesive mechanisms has been demonstrated previously (25). We performed a similar study to test for the requirement of protein synthesis in the initial adhesions of C2 myoblasts with and without a 4-h trypsin recovery period. Incubating C2 monolayers with 50 $\mu\text{g}/\text{ml}$ cycloheximide for 2 h before dissociation in trypsin/EDTA resulted in no significant aggregate formation (Table I). Similarly, the aggregates formed after cycloheximide was added to cells allowed to recover in SATO for 4 h were very small and infrequent (data not

shown). In both cases cycloheximide was also present during the aggregation assay.

In a separate series of experiments, the requirement for glycoprotein secretion in C2 aggregate was also tested. This was performed by incubating cells with the monovalent carboxylic ionophore, monensin, which inhibits the translocation to the cell surface and secretion of glycoproteins that are transported through the Golgi system (68). C2 cell monolayers were incubated for 2 h with 0.5 μM monensin. The drug had a significant effect on the intercellular adhesiveness of C2 cells; when C2 monolayers were incubated with 0.5 μM monensin for 2 h at 37°C before dissociation and re-aggregating them with monensin, the rate of aggregation was much slower than for the corresponding controls. By 15 min, $>80\%$ of the cells existed as singlets (Fig. 2 b) and no aggregates could consistently be seen until 20 min after the start of the assay. At later stages the aggregates were still very small, $\sim 60\%$ of the original number of particles were still present after 30-min aggregation (Fig. 5 a). The α values for these cells were $\sim 70\%$ of those for untreated freshly trypsinized cells (Table I). Similarly, cells allowed to recover for 4 h in SATO in the presence of monensin (and after a 2-h monensin preincubation) also exhibited reduced aggregation (Table I). In this case, aggregates were again slower to form and although the measured intercellular adhesiveness of these cells was greater than for unrecovered monensin-treated cells, it was much less than for 4-h-recovered C2 cells. We interpret these results as reflecting a reduction, but not total suppression, of the translocation of functional adhesive secretory glycoproteins to the cell surface.

Mediation of Adhesiveness by RGDS

The possibility that adhesive proteins containing RGD as their cell-recognition site (integrins) are involved in the early

Table I. Intercellular Collision Efficiencies (α) of Treated Cells

Treatment	α	Treatment	α
L cells	1.08 \pm 0.53		
Skin fibroblasts	4.54 \pm 0.93		
C2 myoblasts			
– FCS	2.99 \pm 0.49	0.005% Cycloheximide	
+ FCS	3.56 \pm 0.46	Freshly trypsinized	1.23 \pm 0.33*
Recovery (h)		0.2 mM RGDS	
1	3.71 \pm 0.56	Freshly trypsinized	7.46 \pm 0.88*
2	5.20 \pm 0.85*	4-h recovered	7.49 \pm 1.18*
3	5.70 \pm 0.61*	0.2 mM RGES	
4	7.14 \pm 1.02*	Freshly trypsinized	2.11 \pm 0.44
0.5 μM monensin		0.2 mM GLGD	
Freshly trypsinized	2.18 \pm 0.26	Freshly trypsinized	3.60 \pm 0.15
4-h recovered	3.84 \pm 0.79†		
5 mM EDTA		Trypsin/ Ca^{2+} Dissociation (min)‡	
Freshly trypsinized	2.67 \pm 0.31	15	1.11 \pm 0.08*
4-h recovered	4.20 \pm 0.51‡	20	0.06 \pm 0.03*
		25	0.48 \pm 0.12*

All values represent cell suspensions which were dissociated with 0.5% trypsin/7 mM EDTA (except where otherwise indicated) and allowed to aggregate for 30 min. Details of treatments and pretreatments are given in Materials and Methods. All significance values refer to cell monolayers dissociated with trypsin/EDTA.

* Significantly different from untreated freshly dissociated C2 cell suspensions, $P < 0.05$.

† Significantly different from untreated, 4-h recovered C2 cell suspensions, $P < 0.05$.

‡ Cell monolayers were incubated with 0.5% trypsin/66 μM Ca^{2+} for the times shown.

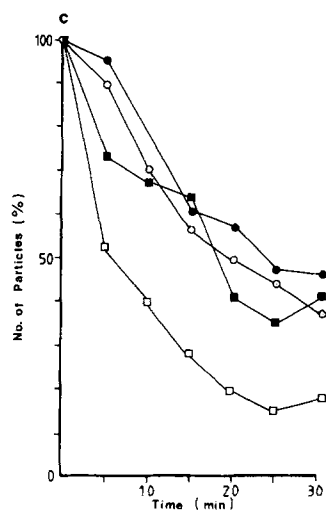
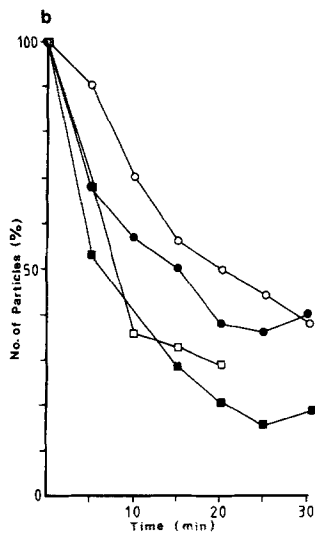
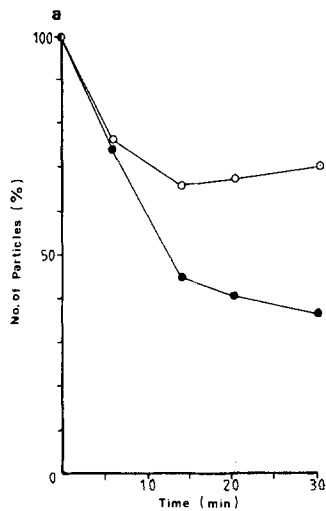


Figure 4. Aggregation kinetics of recovered and EDTA-treated C2 myoblasts. (a) Cells were allowed to recover from trypsinization for 2 h before reaggregation. Cell suspensions were either seeded onto bacteriologic-grade plates (○) or kept in suspension in DME supplemented with 10% FCS (●). The former recovery treatment did not allow for rapid reaggregation. Indeed, the rate was lower than for freshly trypsinized C2 monolayers. (b) C2 cells were allowed to recover for 0 (○), 2 (●), 3 (◐), and 4 h (◑) after trypsinization. The rate of reaggregation increased with longer periods of recovery and, under the conditions of the assay, maximal adhesiveness was seen after 4 h recovery. The kinetics for the 0 (○) and 4 h (◑) recovered cells are shown in c, together with the effects of 5 mM EDTA on the aggregation of freshly trypsinized cells (●). EDTA had no effect on the aggregation of these latter cells. However, the addition of EDTA to 4-h trypsin-recovered cells abolished the increased aggregation seen in untreated, recovered cells (◒).

adhesions measured here was investigated by aggregating C2 cells with the tetrapeptide RGDS and control analogs, RGES and GLGD. These studies were conducted both on the trypsin-sensitive and trypsin-resistant components of adhesion (i.e., with and without a recovery period) as identified by this

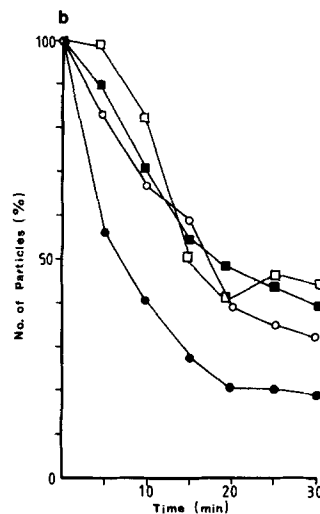
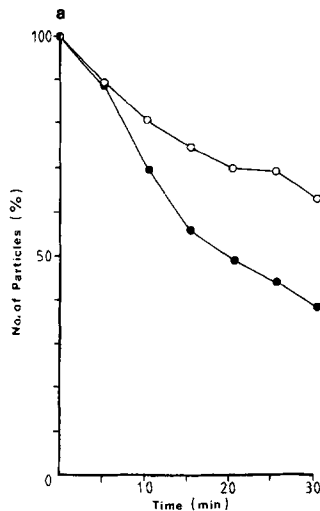


Figure 5. Effect of monensin treatment on the aggregation kinetics of C2 myoblasts. C2 myoblasts were preincubated with 0.5 μ M monensin, trypsinized, and then allowed to reaggregate in the presence of the ionophore (○). The rate of aggregation was much reduced compared to untreated cell monolayers (●). (b) Freshly trypsinized C2 myoblasts were allowed to aggregate in the presence of 0.2 mM RGDS (◐), 0.2 mM GLGD (○), or 0.2 mM RGES (◑). Incubation with GLGD or RGES had no significant effect compared to the aggregation kinetics of untreated cell suspension (●). However, incubation with RGDS greatly increased the rate of formation of multicellular C2 aggregates.

assay. C2 cells which were aggregated in the presence of RGDS rapidly formed very large multicellular aggregates (Figs. 5 b, 6 a). Using RGDS at a concentration of 100 μ g/ml (0.2 mM) produced maximal stimulation of cell adhesion, and aggregating C2 myoblasts with 100 μ g/ml of the control analogs had no effect on freshly trypsinized cell adhesion (Figs. 5 b, 6 b). Comparison of collision efficiencies of RGDS-incubated cells show that they demonstrate an enhanced adhesiveness indistinguishable from trypsin-recovered cells (Table I).

The effect of RGDS on the aggregation of trypsin-recovered cells was also investigated to determine if their rapid rate of aggregation could be yet further increased in the presence of the tetrapeptide. However, the collision efficiencies show (Table I) that the α value of cells given a 4-h recovery in SATO and incubated with 100 μ g/ml RGDS was 7.49 ± 1.18 which is not significantly different from the values of untreated trypsin-recovered cells (7.14 ± 1.02) or freshly trypsinized cells aggregated with RGDS (7.46 ± 0.88). Although the collision efficiencies and final particle number are similar in these three cases, the initial rate of successful cell adhesions is much greater for the trypsin-recovered cells aggregated in the presence of RGDS. In these cell suspensions,

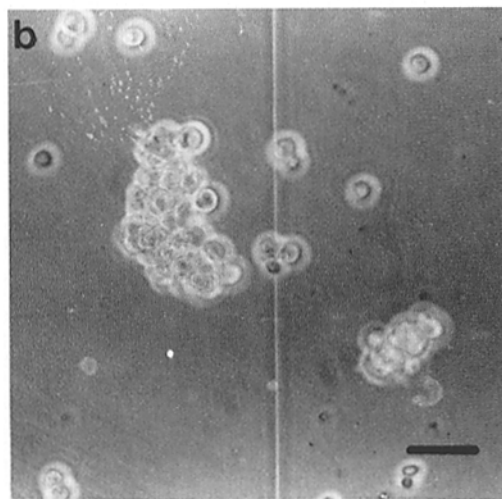
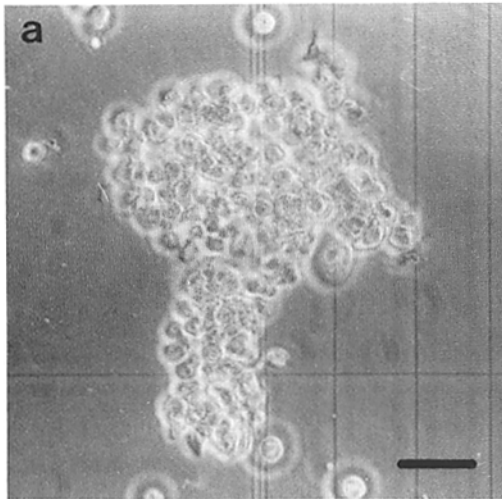


Figure 6. Micrographs of C2 myoblasts freshly dissociated in trypsin/EDTA and allowed to aggregate for 15 min. Suspensions were incubated with 100 µg/ml (0.2 mM) RGDS or GLGD. Incubation with RGDS (a) consistently produced larger aggregates than were seen in untreated suspensions whereas incubation with 0.2 mM GLGD (b) or RGEs (not shown) resulted in aggregates which were similar in size and frequency to those produced from untreated cell suspensions. Bars, 50 µm.

cell aggregation was so rapid that 60% of the original number of particles in suspension were recruited into aggregates within only 5 min of the start of the assay. No further significant change in the number of particles remaining was seen between 10 min of aggregation and the end of the assay period (Fig. 5 b).

Specificity of Early C2 Myoblast Adhesion

Experiments were conducted to determine whether the early adhesions formed during the aggregation of C2 myoblasts by the Ca^{2+} -independent, trypsin-resistant mechanism identified here is cell specific. The method used was that of analyzing the distribution of fluorescently labeled and unlabeled cells in aggregates and comparing these distributions to calculated

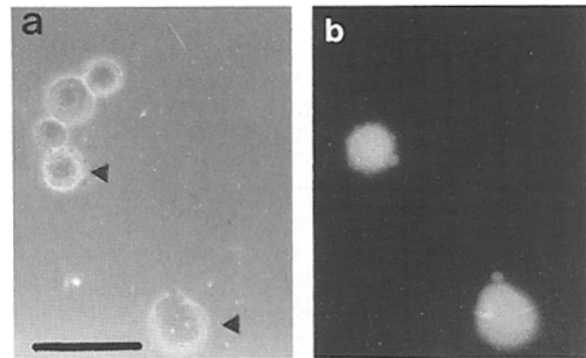


Figure 7. Micrographs of a four-cell aggregate formed by the coaggregation of CFDA-labeled C2 cells and unlabeled 3T3 cells. (a) Two C2 cells can be seen (arrowheads), one of which is incorporated into the 4-cell aggregate. (a) Phase contrast micrograph; (b) epifluorescence micrograph. Bar, 50 µm.

binomial distributions which assume nonspecific (random) adhesion (21, 51, 60). We have previously shown that 0.01% CFDA, the fluorophore used here, neither affects the viability of human skin fibroblasts nor their ability to aggregate in our assay (51). This was also confirmed for C2 myoblasts in the present study.

C2 cells were incubated with CFDA and allowed to coaggregate with unlabeled mouse 3T3 fibroblasts for 20 min. Both cell types rapidly cooperated in aggregate formation (Fig. 7). These aggregates were analyzed for the distribution of C2 and 3T3 cells within the three-cell and four-cell aggregate class since, (a) most aggregates were found to fall into these two aggregate classes and (b) the number of cells in aggregates consisting of more than four cells could not be consistently determined. Analysis of both the three- and four-cell aggregate distributions show that their compositions were not significantly different from those predicted on the basis of nonspecific adhesion (Fig. 8).

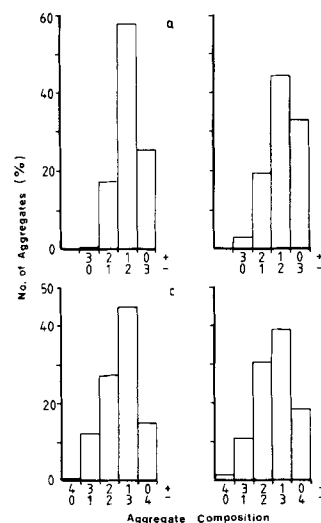


Figure 8. Aggregation of CFDA-labeled C2 myoblasts (+) and unlabeled 3T3 fibroblasts (-). In each distribution, the first class represents the proportion of aggregates which contain (or are predicted to contain) only labeled cells, the second class represents the aggregates with one unlabeled cell, and so on. (a and b) The three-cell aggregate class; (c and d) the four-cell aggregate class analyzed. The observed distribution of cells within the three-cell (a) and four-cell (c) aggregate classes were not significantly different ($P > 0.05$) from those binominal distributions calculated

on the basis of nonspecific adhesion (b and d) even though the unlabeled cells were much more common in the aggregates in this experiment. For the three-cell aggregate class: $\chi^2 = 34.7$, $\nu = 50$; and for the four-cell aggregate class: $\chi^2 = 43.6$, $\nu = 39$.

Discussion

Intercellular adhesion is a multistep process and although many methods exist for measuring such adhesions (18, 19, 27), these assays often measure different stages of the adhesion sequence. This particularly applies to assays which measure (directly or indirectly) the formation of multicellular aggregates with time in a single cell suspension subjected to a fluid shear. Therefore, it should be emphasized that the C2 myoblast adhesions measured here are those which occur rapidly (within 30 min) and under a very low, but constant, laminar shear. Furthermore, the short time-course of this assay also ensures that measurements are preferentially made on the early, reversible stages of recognition and cell adhesion rather than the subsequent events which are irreversible (36, 72).

It is also important to note that measurements of adhesive mechanisms in most vertebrate cells are limited by the necessity to dissociate them into a single-cell suspension. This will invariably cause either some selection of the adhesive systems present or damage to the adhesive apparatus in general. This applies to cells which are mechanically dissociated (76), dissociated with chelating agents (10), or proteolytic enzymes. In spite of these limitations, several reports have established that many of the events which occur in myogenesis *in vitro* can also be identified in myoblast aggregates unattached to a noncellular substratum (21, 28, 36, 37, 47).

A consistent observation which has been reported for embryonic chick myoblasts has been the existence of separate Ca^{2+} -dependent and Ca^{2+} -independent adhesion systems on their surfaces (21, 37) similar to those seen on a variety of other cell types (6, 65–67, 69, 73–75). Both systems have been shown to be inactivated by dissociating chick myoblasts in high concentrations of trypsin in the presence of EDTA, and that such cells subsequently fail to aggregate as a result of the loss of both Ca^{2+} -dependent and Ca^{2+} -independent adhesive mechanisms (21). However, the requirement for Ca^{2+} in the dissociation medium such that any Ca^{2+} -dependent adhesion mechanism remains intact varies greatly in different tissues and is probably also dependent upon the conditions of trypsinization. For example, for embryonic chick neural retina cells, it has been shown that 10 mM Ca^{2+} is required to maintain an intact Ca^{2+} -dependent adhesion system (67, 74), while others have found that under their conditions of dissociation 0.2 mM Ca^{2+} is sufficient (26).

Gibraltar and Turner (21) have also observed that when the concentration of trypsin was lowered, embryonic chick myoblasts aggregate well and that they only possess a Ca^{2+} -independent adhesion system. This treatment also inactivates only the Ca^{2+} -dependent adhesion system in Chinese hamster V79 fibroblasts (74). Furthermore, Magnani et al. (40) have shown that for the dual systems of adhesion (in terms of Ca^{2+} -dependence) that exist in embryonic chick neural retina cells, the Ca^{2+} -dependent mechanism is more sensitive to trypsin than the Ca^{2+} -independent system. It is clear that the dissociation procedure routinely used by us initially only exposes a Ca^{2+} -independent adhesion system since addition of EDTA to the aggregation medium had no obvious effect on the adhesiveness of the cells. The present study represents the first measurements of recognition and early intercellular adhesion by a Ca^{2+} -independent adhesive mechanism in mammalian myoblasts.

Since, in some other systems, the components of Ca^{2+} -dependent adhesion are more sensitive to proteolysis than those involved in Ca^{2+} -independent adhesion (40), the increase in adhesiveness in trypsin-recovered C2 cells may have been due to the replacement of the former at the cell surface. Alternatively, trypsinization may have partially damaged the Ca^{2+} -independent adhesive mechanism, although clearly much remains functional, and the increased adhesion after recovery may be due to replacement of some elements of this system. It is also possible that elements of both systems are replaced.

These possibilities were tested by aggregating recovered cells with EDTA. It was found that in this case, the adhesiveness of these cells was much less than recovered cells which were allowed to aggregate in the presence of Ca^{2+} ions, and thus we conclude that the increased adhesiveness of trypsin-recovered cells is mainly a consequence of the restoration of a functional Ca^{2+} -dependent adhesive mechanism. This is consistent with the findings that (a) after trypsinization in EDTA, embryonic chick myoblasts require a period of 2–3 h in complete medium for the recovery of Ca^{2+} -dependent adhesion (37) and (b) after similar dissociation, V79 cells start to recover their Ca^{2+} -dependent adhesiveness after 1–2.5 h incubation (65). However, the period required for recovery of full adhesiveness after dissociation will vary according to cell type and the methods used to assess recovery. For example, in studies on the turnover of surface proteins, it has been shown that although fibroblasts only regain full surface rigidity 5 d after trypsinization (12), these cells recover to aggregate maximally after only 70 min (63). Thus, although in our assay C2 myoblasts achieve maximal adhesiveness after 4-h recovery, there may still be surface-associated components of the Ca^{2+} -dependent and Ca^{2+} -independent adhesive systems that are not replaced within this time.

The requirement for protein synthesis in the dual mechanisms of intercellular adhesion has been investigated previously, but differences in experimental protocols often make it difficult to detect any general patterns that may be present. For example, it has been shown (26) that in 24-h cultures, the Ca^{2+} -dependent (but not the Ca^{2+} -independent) adhesion system of embryonic chick neural retinal cells is capable of forming large, histotypic aggregates in the absence of protein synthesis. Similarly, in an aggregation assay measuring earlier adhesions, it was found that 100 $\mu\text{g}/\text{ml}$ cycloheximide had only a slight effect on the Ca^{2+} -dependent aggregation of V79 cells (65), but in that study the cycloheximide preincubation was only for 30 min and thus the possibility exists that adhesion occurred as a result of residual pools of endogenous adhesion-associated proteins. In contrast, it has also been found that cycloheximide treatment before trypsinization of embryonic chick myoblasts growing in low levels of calcium inhibits their Ca^{2+} -dependent aggregation (37). In our assay, we have established that the Ca^{2+} -independent component of C2 myoblast adhesion also requires protein synthesis since following 2 h incubation with 50 $\mu\text{g}/\text{ml}$ cycloheximide, C2 myoblasts dissociated with trypsin/EDTA remained as a single cell suspension when allowed to aggregate. In addition, these cells also failed to aggregate when given a 4-h trypsin-recovery period during which protein synthesis was still inhibited.

It has been suggested that since fusion of embryonic chick (35), quail (48), and rat (9, 22) myoblasts is inhibited by

tunicamycin, glycoproteins may be important in mediating this process. However, there are some apparent species differences in that this inhibition is reversed by leupeptin in avian, but not rat myoblasts. It is also well established (3) that myoblast fusion requires the presence of Ca^{2+} ions (although probably in all cases where fusion has been studied the Ca^{2+} -independent adhesion system is also intact), and in chick myoblasts, inhibition of fusion by tunicamycin is accompanied by inactivation of Ca^{2+} -dependent myoblast adhesion (35). Our findings suggest that surface glycoproteins may also be involved in Ca^{2+} -independent C2 adhesion since incubation of cells with 0.5 μM monensin for 2 h before dissociation in trypsin/EDTA greatly inhibited aggregate formation. We suggest that this may be a consequence of the known effects (68) of monensin on inhibiting the translocation of nascent glycoproteins which may be involved in recognition and adhesion at the cell surface. We have previously shown that only 30 min exposure to 0.5 μM monensin is sufficient to reduce cell-substratum adhesive interactions in human skin fibroblasts (52, 53).

Cell aggregation in monensin-treated, trypsin-recovered cells was also greatly reduced compared to the recovered control cells although there was no difference in aggregation between these cells and freshly trypsinized untreated C2 myoblasts. This could indicate that although the trypsin-recovered cells have had time to replace the Ca^{2+} -dependent adhesion system, the presence of monensin has inhibited this process and the aggregation kinetics observed are due to recovery of the Ca^{2+} -independent system only. This would be consistent with previous findings which show that after exposure to the same monensin concentration, embryonic chick myoblasts fail to fuse (13) and would further suggest that the failure of these cells to fuse is a consequence of the inhibition of early glycoprotein-mediated adhesion rather than to any effect of monensin on later fusion events. However, the results of monensin treatment on trypsin-recovered cells are equivocal and the increased adhesion, compared to monensin-treated unrecovered cells may simply be a consequence of the partial inhibition of the drug on intracellular transport (70). Thus, since glycoprotein translocation occurs at a constant but reduced rate in the presence of monensin (71), the 4-h recovery time may simply allow for a relatively greater amount of adhesive glycoproteins (of both adhesive systems) to be translocated to the cell surface.

The observation that following dissociation in low concentrations of trypsin in EDTA, myoblasts and fibroblasts do not segregate into homogeneous aggregates consisting of only one cell type, suggests a lack of tissue specificity in the Ca^{2+} -independent adhesion systems on C2 and 3T3 cells. In similar coaggregation studies, a lack of adhesive specificity has also been reported between chick neural retina cells and V79 (67) or chick limb bud (69) cells, and between embryonal carcinoma cell variants (66). Thus it has been concluded that, in general, cells will cross-adhere, regardless of their tissue origin if they share at least one of the categories of their adhesive systems in an active state; i.e., Ca^{2+} -dependent adhesion systems will interact with Ca^{2+} -dependent systems on other cells, and the same is true for Ca^{2+} -independent systems (69). However, although adhesive specificity exists between these two mechanisms (21, 69), true cell-specific adhesion is a relatively rare phenomenon (19, 20).

It was initially shown that the general nature of nonspecific

adhesion also extended to Ca^{2+} -dependent adhesion between chick fibroblasts and myoblasts. However, this was later shown to be due to fibroblast contamination of the myoblast cultures before fluorescent labeling (21). When pure myoblast and fibroblast cultures were used, in contrast to the general findings mentioned above on the nonspecific nature of early intercellular adhesion, coaggregation experiments demonstrated that when these cells are dissociated with EDTA or with trypsin/2.5 mM CaCl_2 ; they exhibit specific (nonrandom) aggregation (21). A similar result was also seen in fusion-blocked chick myoblasts after equivalent dissociation procedures (36). However, no data were presented on the adhesive specificity between cells dissociated in low levels of trypsin in EDTA (equivalent to LTE cells in Gibraltar and Turner [21]). Therefore the differences in adhesive specificity between previous results (21, 37) and those reported here may be due to the different adhesive systems exposed by the two dissociation procedures, or to differences in some of the early stages of avian and mammalian myoblast adhesion (9, 22, 48).

Many molecules are known or suspected to be involved in the early stages of intercellular adhesion (8, 15, 16, 42). Although the aims of the current study were to characterize the conditions for Ca^{2+} -independent adhesion in C2 myoblasts rather than to attempt to characterize the molecules involved, it was considered important to investigate the possible involvement of a major class of receptors which recognize the RGD sequence (integrins) in adhesive glycoproteins. Recently, it has been shown that RGDS and antibodies to the integrin receptor complex will inhibit myogenic differentiation and fusion in embryonic chick myoblast monolayers (44), although whether this was a consequence of an inhibition of early intercellular adhesion was not studied. Indeed, although many recent studies have shown that synthetic peptides containing the RGD sequence inhibit cell-substratum adhesion (see Introduction), there have been very few reports on the effect of RGD on other aspects of cell adhesion. However, it has recently been shown that although an RGD-containing pentapeptide inhibits cell-substratum adhesion of embryonic chick segmental plate and somite cells, it promotes intercellular adhesion between these cells (39). Our results, which show the stimulation of C2 myoblast aggregation by RGDS, confirm these results and together they represent the first reports of the promotion of intercellular adhesion by integrin-binding peptide fragments. Furthermore, in the study by Lash et al. (39), cell-cell and cell-substratum adhesion were measured in the same culture dishes and the possibility could not be excluded that increased cell-cell adhesion was a consequence of inhibition of cell-substratum adhesion either by increased intercellular collisions or by competition between the two processes. Since there was no provision for cell-substratum adhesion in our assay, we can conclude that RGD-containing oligopeptides stimulate intercellular adhesion directly.

The mechanism whereby a small monovalent competitive inhibitor of many adhesive glycoproteins can stimulate intercellular adhesion is unclear. However it has recently been shown, in a cell-substratum assay, that murine mammary epithelial cells contain at least two classes of molecules that bind fibronectin and which show differential competition by an RGD-containing pentapeptide (59). Clearly, investigations of the multiple receptor classes within the integrin su-

perfamily, together with the possibility of binding sites of different affinity for different adhesive functions (39) will be required to elucidate the apparent paradoxical effect of RGD on the adhesive behavior of myoblasts and other cell types.

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