noise ratios and permits collection of decay curves from single cells in reasonably short times.

The suitability of this probe to serve as a donor to fluorescein (FITC) in RET experiments can be seen from Figure 5 which shows the spectral overlap between pyrene emission and fluorescein absorption. The R_0 for the pyrene–fluorescein pair is 43 Å which is sufficiently large to make this donor–acceptor combination useful for studies of cell surface receptor clustering [see following paper (Herman & Fernandez, 1982)]. In addition, the large Stokes shift of the pyrene fluorescence results in little self-overlap between pyrene's own emission and absorption bands. Thus, the R_0 for pyrene–pyrene self-transfer is only 22 Å (compare to 48 Å for fluorescein self-transfer). This constitutes an additional advantage of employing pyrene as a donor, since potential artifacts due to donor self-transfer are significantly reduced. Finally, the rapid local relaxation of the pyrene moiety relative to the Con A molecule and the fact that there is little direct excitation of fluorescein at the excitation maximum of the pyrene further add to the usefulness of this pyrene–Con A derivative as a probe for resonance energy transfer studies.

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Dynamics and Topographical Distribution of Surface Glycoproteins during Myoblast Fusion: A Resonance Energy Transfer Study†

Brian A. Herman‡ and Salvador M. Fernandez*

ABSTRACT: We have investigated changes in topography and lateral translational mobility of concanavalin A (Con A) receptors on the surface of cultured chick muscle cells during the period of myoblast fusion. A temporal correlation between these phenomena and the alteration in membrane fluidity known to occur during this time period is established. Receptor topography and mobility are studied by means of a resonance energy transfer technique employing pyrene- and FITC–Con A conjugates. All measurements are performed through a microscope on single cells. Our results reveal that during the period of myoblast fusion Con A receptors undergo a dramatic redistribution on the cell surface. Furthermore, our data suggest that the changes in membrane fluidity observed during muscle differentiation serve to modulate the lateral mobility of these receptors.

One of the earliest events in the process of muscle differentiation is the fusion of myoblasts into multinucleated myotubes. This phenomenon has been the subject of a great deal of attention not only because of its fundamental importance as a developmental process but also because it provides a good model for the study of membrane fusion in general. A number of observations now indicate that cell surface lectins and the lipids of the plasma membrane play important roles in the process of myoblast fusion. Lectins, on the one hand, are envisaged as serving a function in cellular recognition and adhesion (Rosen et al., 1975) while membrane lipids, on the other hand, are considered to be more intimately involved with the fusion event proper (Horwitz et al., 1979; Kalderon & Gilula, 1979).

Several lines of evidence have implicated lectins in the process of myoblast fusion. First, cultured muscle cells elaborate surface lectins as a prelude to fusion activity (Gartner & Podleski, 1976; Nowak et al., 1976; Teichberg et al., 1975). Second, exogenous addition of certain lectins to myogenic cultures results in marked inhibition of fusion (Den et al., 1975; Sandra et al., 1977). More recently (Parfett et al., 1981) it has been shown that Con A[1] resistant myoblast lines defective in their ability to synthesize surface mannosylated glycoproteins do not undergo fusion. Suggestions for a lipid in-

† From the Physiology Department, University of Connecticut Health Center, Farmington, Connecticut 06032. *Received January 12, 1982.* Supported by U.S. Public Service Research Grant GM 23235.

‡ Present address: Anatomy Department, Harvard Medical School, Boston, MA 02115.

[1] Abbreviations: Con A, concanavalin A; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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volvement in the fusion process include the following: (a) myoblast fusion is preceded and accompanied by an increase in membrane fluidity (Prives & Shinitzky, 1977; Herman & Fernandez, 1978); (b) experimental alteration of the membrane fatty acyl and/or polar head group composition of cultured muscle cells can markedly affect the rate and extent of myoblast fusion (Horwitz et al., 1978); and (c) ultrastructural analysis of muscle cells reveals particle-free membrane regions at sites of fusion (Kalderon & Gilula, 1979).

In spite of these key observations, the basic mechanisms by which two cells recognize each other and subsequently fuse are not fully understood. In particular, the functional significance of the alterations in surface lectin composition and of the transient increase in membrane fluidity observed during muscle differentiation remains unclear. The former may be involved in recognition and adhesion phenomena while the latter may regulate fusion through lipid–lipid interactions. In addition, both of these phenomena may be related; i.e., the alteration in lipid dynamics may serve to modulate the mobility and topographic display of cell surface proteins involved in recognition and adhesion.

In order to test these hypotheses, we have investigated the topographical distribution and the lateral mobility of Con A receptors on the surface of muscle cells at different times during development in vitro. To this end, we employ a resonance energy transfer technique (Fernandez & Berlin, 1976) which permits quantitation of the average proximity of these receptors on the surface of single cells. Information on Con A receptor mobility is obtained by following the kinetics of ligand-induced receptor clustering. This phenomenon reports on translational diffusion on the plane of the membrane, and its use as a sensitive indicator for this kind of motion has been suggested previously (Edidin, 1974).

Materials and Methods

Resonance Energy Transfer (RET) Method for the Study of Receptor Topography. The method employed in this work is essentially the same as that previously described (Fernandez & Berlin, 1976) with some minor modifications. Briefly, the RET method is founded on the fact that a fluorophore (donor) in an excited state may transfer its excitation energy to a neighboring chromophore (acceptor) nonradiatively through dipole–dipole interactions (Forster, 1965). This process requires some spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and for a given donor–acceptor (D–A) pair, the efficiency of the transfer process is dependent on their relative orientation and on the distance between them. The dependence of the energy transfer efficiency on D–A separation provides the basis for the utility of this phenomenon in the study of cell surface topography.

In essence, the experiments are done as follows: Substrate-attached cultured cells are labeled in a solution of Con A which contains a mixture of two populations of Con A separately conjugated to donor and acceptor fluorophores. Pyrene– and fluorescein–Con A conjugates are employed as donor and acceptor, respectively (Herman & Fernandez, 1982). The ratio of donor to acceptor (1:6.8) is empirically chosen such that at the surface density of bound Con A an approximation of receptors is manifested as an increase in energy transfer.

Since resonance energy transfer (RET) results in an increase in acceptor emission (sensitized fluorescence) and a decrease in donor fluorescence, we have taken the ratio of acceptor to donor fluorescence (I_A/I_D) as a convenient experimental measure of RET. The value of this ratio depends on the average distance between D–A pairs, and thus on the state of aggregation of Con A receptors. As the average distance between D–A pairs increases, this ratio approaches a limiting value which corresponds to the absence of RET. For constant donor/acceptor ratios, this limiting value of I_A/I_D depends on the shape of the donor emission spectrum and on the amount of direct excitation of the acceptor at the exciting wavelength of the donor. Likewise, an upper limit for I_A/I_D exists which corresponds to a maximal packing density of Con A receptors.

In order to interpret the value of I_A/I_D obtained from single cell studies, we employed the following procedure to empirically determine the magnitude of the limiting values of I_A/I_D.

Solutions of Con A containing donor and acceptor conjugates in a 1:6.8 ratio were diluted with native Con A to vary the relative proportions of labeled and unlabeled species. These mixtures of Con A were then precipitated with anti-Con A antibody and examined in the microscope. I_A/I_D values were then determined for the various samples. The maximum value of I_A/I_D obtained was 1.75, and as the average D–A distance increased by the presence of greater amounts of unlabeled Con A, I_A/I_D decreased asymptotically to a value of 0.75. This empirical calibration curve thus provides a semiquantitative basis for interpreting the cellular RET data.

Energy transfer also results in a decrease in the fluorescence lifetime of the donor fluorophore. Thus, we have carried out donor lifetime determinations from single cells as an alternative method to quantitate the extent of RET.

For studies of inherent Con A receptor topography at different times during development, cells of the appropriate age were prefixed in 2% paraformaldehyde at 37 °C for 20 min and then labeled with Con A, at 20 μg/mL (D/A ratio of 1:6.8), for 1 h at 4 °C. The cells, on cover slips, were washed and examined in the microscope.

For studies of Con A receptor mobility, intact live cells were labeled in the usual manner with donor– and acceptor–Con A at 4 °C and subsequently incubated for varying periods of time at 37 °C. At the end of each incubation period the cells were fixed and examined for RET. Alternatively, the live labeled cells were placed on the microscope stage at 25 °C, and changes in RET were monitored in real time.

Membrane Lipid Dynamics. Information about membrane lipid dynamics is based on measurements of the apparent rotational correlation time of the amphipathic probe 8-anilino-1-naphthalenesulfonate (ANS) bound to substrate-attached intact living cells. Measurements are performed on single cells as previously described (Herman & Fernandez, 1978).

Cell Cultures. Cultures were prepared by nonenzymatic dispersion of 12-day-old chick embryo pectoral muscle in calcium- and magnesium-free Puck's saline. The cells were centrifuged, resuspended in 5% MEM (Eagle's minimal essential medium with 10% horse serum and 5% chick embryo extract), filtered through a Swinex unit, preplated twice for 15-min periods, and then seeded onto collagenized coverslips at a density of 2×10^5 cells mL^{-1}. Cultures were grown at 37 °C in a humidified 5% CO_2 atmosphere. After 36 h they were treated for 48 h with 2% MEM (2% chick embryo extract and 20% horse serum) containing 10^{-5} M cytosine arabinoside. Maintenance medium thereafter consisted of 2% MEM and was replaced every 48 h. Fusion-arrested cells are grown in 2% MEM containing 1.8 mM EGTA (Shainberg et al., 1969).

Fusion Kinetics. At appropriate time intervals the medium is removed, and the cells are washed twice with phosphate-buffered saline (PBS) and then fixed and stained for 1 min with 0.1% orcein in methanol (Knudsen & Horwitz, 1978).

The fixed cells are coated with glycerol, and fusion is scored as the fraction of total nuclei in cells containing three or more nuclei.

Effect of Colchicine and Nocodazol. The effect of these agents on the topographical distribution of Con A receptors was examined as follows: Fusion-arrested cells were grown for 68 h in medium supplemented with 1.8 mM EGTA. At this time the medium was replaced with medium containing calcium and either colchicine or nocodazol at a concentration of 1×10^{-7} M. Cells were then examined for RET at varying times after addition of the drug.

Radiolabeling of Intact Cells with [^{125}I]Con A. For exploration of the nature of the Con A binding sites, unfixed cells at 24, 48, and 72 h in culture were labeled at 4 °C with [^{125}I]Con A by employing the same procedure described for fluorescent Con A derivatives. The radiolabeled cells were then subjected to mild trypsinization in 20 μg/mL trypsin for 10 min at 37 °C. The amount of radioactivity remaining bound to the cells and that released into the medium after this treatment were determined with a γ counter by a procedure adapted from Den et al. (1975). A similar protocol was employed to investigate possible internalization of the Con A label in experiments that required incubation of unfixed labeled cells at 37 °C for varying periods of time.

Fluorescence Measurements on Single Cells. Fluorescence measurements were performed on substrate-attached single cells (or portions thereof) with a photon-counting microspectrofluorometer assembled in this laboratory. This apparatus has been previously described (Fernandez & Berlin, 1976; Herman & Fernandez, 1978) and essentially consists of a modified Zeiss research microscope equipped with a vertical illuminator (Schott UG1 excitation filter with a Zeiss FL 395 dichroic mirror) and coupled to an RCA 8850 photomultiplier tube through an f/4 200-mm focal length symmetrical Czerny–Turner monochromator. All spectral data are corrected for the photocathode response of the photomultiplier tube and for dark count base line. Time-resolved measurements from single cells are carried out with an Ortec 9200 nanosecond fluorometer interfaced to the microscope, and deconvolution of fluorescence decay curves is carried out by the method of moments (Isenberg & Dyson, 1969).

Spectral measurements are performed on three morphologically distinct regions of cells: perinuclear, myotube, and fusion or cell contact regions (Herman & Fernandez, 1978). Diaphragms select the portion of the cell to be observed. In this study a rectangular aperture of variable size selected the field of view (typically a 4 μm × 4 μm area). All measurements are made with a 100× oil immersion, pH 3 Zeiss objective (N.A. 1.25) at 25 °C.

Results

The excitation and emission maxima for pyrene–Con A at pH 7.4 are 373 and 463 nm, respectively; those for FITC–Con A are 483 and 513 nm. Dye to protein ratios were 2.0 for the FITC and 2.2 for the pyrene conjugates. The critical Forster radius, R_0, for this donor–acceptor pair is 43 Å as calculated from the spectral overlap integral assuming a value of $^2/_3$ for the κ^2 orientation factor (Dale et al., 1979). The R_0 value for pyrene–pyrene self-transfer is 22 Å. Typical spectra from cells labeled with either pyrene–Con A or a combination of pyrene–Con A and FITC–Con A at a donor:acceptor ratio of 1:6.8 are shown in Figure 1. Also shown is the background spectrum obtained from an unlabeled cell.

Trypsinization of cells labeled with [^{125}I]Con A resulted in complete release of cell-bound radioactivity into the medium at all ages investigated, i.e., 24, 48, and 72 h in culture. This indicates that the Con A receptors being probed in this study are predominantly glycoprotein in nature. There appears to be negligible binding of Con A to glycolipids under the conditions employed.

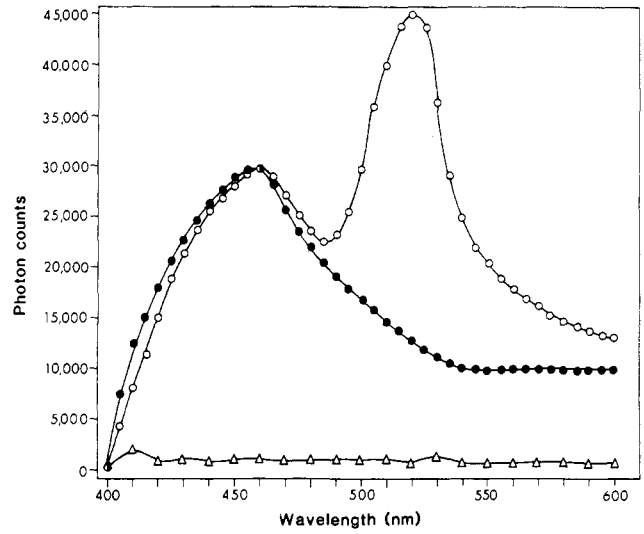

FIGURE 1: Fluorescence emission spectra from single cells labeled with pyrene–Con A (●), pyrene–Con A:FITC–Con A at a donor: acceptor ratio of 1:6.8 (○), and an unlabeled cell (△). The total concentration of Con A in the labeling medium was 20 μg/mL. The spectra of the labeled cells are normalized at 460 nm. I_A/I_D ratios in the work refer to the ratio of intensities at 513 and 463 nm collected with a 20-nm slit. Pyrene–Con A exhibits some emission at 513 nm, and this amounts to 32% of the intensity found at 463 nm.

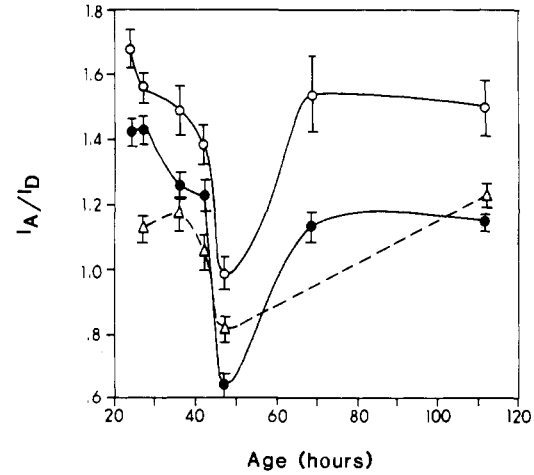

FIGURE 2: Resonance energy transfer from myogenic cells at different times during development. The extent of RET is quantitated as the ratio I_A/I_D of acceptor (FITC) to donor (pyrene) fluorescence. Cells of the appropriate age were fixed and labeled as described in the text and examined for RET in the microscope. Open circles represent values obtained from regions of cell contact or fusion, whereas the solid circles and the triangles represent data obtained from perinuclear and myotube regions, respectively. In this and subsequent figures, the values are presented as the mean ± standard error of the mean. Each point represents the average of 48 individual measurements (duplicates on 24 cells).

Results from studies of the inherent Con A receptor topography of cultured muscle cells at sequential times during differentiation are shown in Figure 2. Three morphologically distinct cellular regions were examined: perinuclear, myotube, and fusion regions, which at early times represent areas of cell–cell contact. Several findings become apparent upon examination of these data. Most prominent is the marked decrease and subsequent increase in RET that takes place between 30 and 70 h in culture. The time at which the steep decline in RET begins lags the onset of myoblast fusion activity

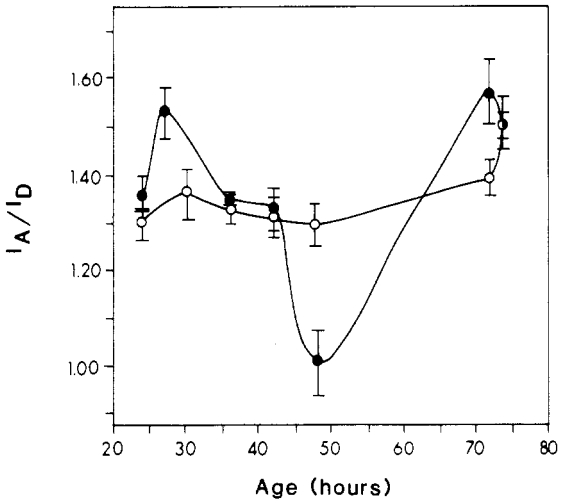

FIGURE 3: Resonance energy transfer from perinuclear regions of control cells (●) and fusion-arrested cells grown in medium supplemented with 1.8 mM EGTA (○). Cells were prefixed in 2% paraformaldehyde before labeling with donor acceptor/Con A mixtures.

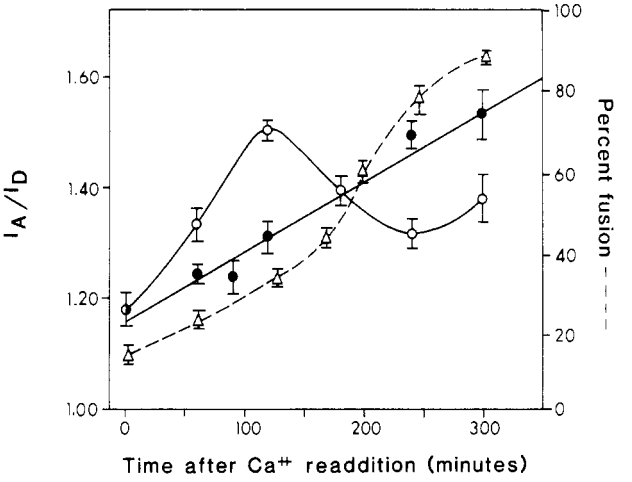

FIGURE 4: Resonance energy transfer in synchronously fusing cells after replacement of low calcium medium with regular medium. Time zero in this study corresponds to 68 h in culture and represents the time at which the medium containing EGTA is replaced with normal medium. The open symbols represent RET values from cells released from fusion block, whereas the closed symbols refer to cells which remained in EGTA-supplemented medium. The percent of nuclei in multinucleated myotubes (an index of fusion activity) in the synchronized cultures is shown for comparison (△). Data from cells remaining in EGTA were fit to a straight line by using a linear regression analysis.

by a few hours (see Figure 8). This temporal variation in RET suggests that surface Con A receptors in cultured muscle undergo a marked topographical rearrangement in the time period during which myoblasts are undergoing fusion. More specifically, it appears that the average proximity of Con A receptors decreases during the period of fusion and then increases again to a level somewhat lower than that found at prefusion times. It is noteworthy that qualitatively the same temporal variation in RET is observed at all three cellular regions examined although there appears to exist spatial heterogeneity in the actual I_A/I_D ratios. For example, at prefusion times (36 h) regions of cell contact exhibit the highest level of RET followed by perinuclear and myotube regions.

In order to probe further the relationship between changes in Con A receptor topography and the fusion process, we followed changes in RET from fusion-arrested cells (grown in the presence of 1.8 mM EGTA) and cells released from fusion block which undergo synchronized fusion with accelerated kinetics.

Figure 3 shows the effect of fusion arrest on RET. The solid symbols represent RET values observed during normal development (comparable to those shown in Figure 2), whereas the open symbols refer to the fusion-arrested cells. The cells grown in low calcium medium (and not undergoing fusion) do not exhibit the marked temporal alteration in RET seen in the control cells. Instead, RET in these cells remains relatively constant up to about 68 h after which time it begins an upward trend.

Addition of calcium at 68 h to these fusion-arrested cultures results in a period of synchronized fusion activity which leads to 90% fusion in 5 h. The effect of calcium readdition on RET is shown in Figure 4. This figure reveals that addition of calcium (and thus release from fusion block) results in an initial increase in RET followed by a decrease and a subsequent increase to a value intermediate between the high and low values seen at earlier times. RET values from cells that remained in EGTA do not exhibit this oscillation but rather continue to increase at an approximately linear rate.

The oscillation in RET values seen in the synchronized cells is analogous to that seen in the control cells allowed to develop normally (Figure 3), namely, an initial increase followed by a decrease in RET. Furthermore, although the kinetics of fusion are accelerated in the synchronized cells, the observed changes in RET and the time course of fusion maintain a

Table I: Energy Transfer and Percentage Fusion of Normal and Fusion-Arrested Cells during Myogenesis in Vitro (Perinuclear)

age (h)	I_A/I_D (control)	I_A/I_D (EGTA)[a]	fusion (%) (control)	fusion (%) (EGTA)[a]
28	1.38 ± 0.05[b]		33 ± 2	
30			52 ± 1	
48	1.15 ± 0.03	1.50 ± 0.06	73 ± 2	52 ± 3
72	1.44 ± 0.05	1.73 ± 0.08	78 ± 4	56 ± 5

[a] Cells were placed in medium containing EGTA (1.8 mM) at 30 h. [b] Mean ± standard error of the mean.

temporal correlation similar to that observed in normal developments; i.e., the changes in RET in this case also occur on a proportionately faster time scale.

A further demonstration of the close coupling among the presence of calcium, fusion activity, and changes in RET is provided by the following experiment: Cultures were allowed to grow in normal media for 30 h at which time half of the plates received media supplemented with EGTA. At 30 h in culture, a number of the cells have already undergone fusion or are in contact but the decrease in RET is just beginning to take place (Figure 8). The extent of fusion and of RET were then followed in both sets of cultures up to 72 h. The results of this study are presented in Table I. Alterations in RET in the control cells follow the usual pattern, i.e., an initial high I_A/I_D ratio which decreases to a minimum value at about 48 h and then increases again. On the other hand, addition of EGTA at 30 h resulted in both prompt arrest of fusion activity and abolition of the decrease in RET normally observed. Instead, I_A/I_D ratios increase steadily after chelation of calcium with EGTA.

One additional important aspect of our results stems from our microscopic approach that enables us to make measurements on single cells. Thus, at any one time during the course of fusion activity we can selectively examine isolated single cells that are not in contact with others, cells which at the light microscope level still appear as separate entities but are in contact or fusing with another cell, or multinucleated myotubes

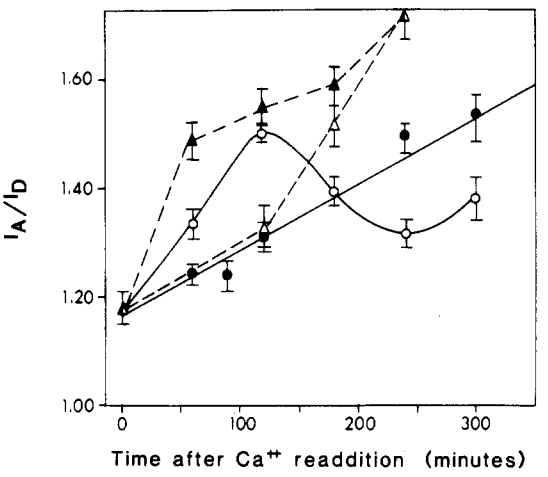

FIGURE 5: Effect of nocodazol (▲) and colchicine (△) on resonance energy transfer in synchronously fusing cells. Media containing calcium and either nocodazol or colchicine at 10^{-7} M concentration were added to the cells at 68 h in culture (time zero). Cells were fixed and labeled at various subsequent times and examined for RET. The triangles represent RET values from the drug-treated cells, while the open and closed circles are the same as in Figure 4.

which have resulted from the fusion process. We have found that at any given time in culture the values of I_A/I_D ratios from perinuclear and myotube regions are similar regardless of whether the cell being examined is an isolated cell or one that has undergone cell contact or fusion.

Effect of Colchicine and Nocodazol on Con A Topography. Considerable evidence indicates that the cytoskeleton plays an important role in the regulation of the topographic display of surface Con A receptors in a number of cell lines. We thus examined the effect of the pharmacological agents colchicine and nocodazol (10^{-7} M) (whose action is the disruption of microtubules) on the topographical distribution of Con A receptors during myoblast fusion activity. We also investigated the effect of these drugs on the fusion activity proper.

During the course of these studies, it was found that prolonged incubation in colchicine resulted in fragmentation of the cells. In order to minimize exposure time to the drugs and thus deleterious effects, we examined the effect of these agents on cells that had been synchronized by previous incubation in low calcium medium. Under these conditions, few gross morphological changes were induced by nocodazol treatment, although colchicine caused some rounding of the cells after 5 h of incubation.

The effect of nocodazol and colchicine on RET in synchronized cells is illustrated in Figure 5. In cultures treated with either drug, the biphasic change in RET normally seen after addition of calcium is not observed. Instead, a monotonic increase in RET takes place. The time course of this increase is significantly faster with nocodazol, a behavior that is consistent with the higher affinity of this drug for microtubules. It is interesting that these effects are seen at drug concentrations of 10^{-7} M and that similar final ratios of I_A/I_D are obtained in both cases in spite of the different morphology of the cells after treatment. Additionally, during the course of these studies, it was observed that while myogenic cells undergo morphological alterations in the presence of colchicine, fibroblast morphology seems unchanged.

The cause of the high RET values observed in the drug-treated cells becomes apparent upon examination of these cells in the fluorescence microscope. In both cases, the drug-treated cells exhibit a punctate or patchy pattern of fluorescence, indicating that Con A receptors are aggregated into clusters (Herman, 1980). It is unlikely that these punctate areas of fluorescence arise from internalized label since the cells in these studies were fixed prior to labeling. Nevertheless, to test for this possibility, nocodazol-treated cultures were fixed and sequentially labeled with rhodamine–Con A and FITC–anti-Con A antibody. The patchy distributions of fluorescence from both labels were always superimposable, suggesting that these fluorescent clusters represent surface accumulations rather than internalized compartments of Con A (Herman, 1980).

Addition of nocodazol and colchicine was found to inhibit fusion when added to normal medium containing calcium. Nocodazol inhibits fusion by approximately 65%, whereas colchicine appeared to cause much less inhibition. Scoring fusion in these latter cultures, however, is difficult due to the altered morphology of the colchicine-treated cells.

In order to ascertain whether the variations in RET observed in all of the above experiments could be due to changes in the total amount rather than to alterations in the distribution of a relatively fixed number of receptors, we measured the amount [^{3}H]Con A bound to muscle cultures at different times during development and during release from fusion arrest, both in the presence and absence of colchicine and nocodazol. The concentration of [^{3}H]Con A used for these binding studies was chosen to be the same as the total concentration of fluorescent Con A employed for energy transfer experiments, i.e., 20 μg/mL. The results were as follows (Herman, 1980): The amount of [^{3}H]Con A bound per milligram of protein at different times during normal development increases from 24 h (earliest time point examined) until a plateau is reached at about 50 h in culture. When cells are released from fusion arrest at 68 h in the presence or absence of nocodazol or colchicine, no statistically significant change in the amount of [^{3}H]Con A per milligram of protein bound to these cells is observed in the 5-h period during which changes in RET are observed.

Fluorescence Anisotropy from Fixed Cells Labeled with Con A. Anisotropy measurements of pyrene and FITC fluorescence from cells labeled only with either of these fluorophores were collected and compared with the anisotropy of FITC fluorescence from doubly labeled cells excited at the excitation maximum of pyrene–Con A. Pyrene-labeled cells yield anisotropy values ranging from 0.17 to 0.19, FITC labeled cells had values of 0.11–0.13, and doubly labeled cells ranged from 0.05 to 0.08.

Time-Resolved Studies of RET in Single Cells. RET data presented thus far are based on spectral measurements of the ratio of acceptor sensitized fluorescence to donor fluorescence. Alternatively, RET efficiencies can, in principle, be calculated from measurements of the donor mean radiative lifetime (Schiller, 1975). It is also possible, in principle, to obtain information about the distribution of donor–acceptor distances by analysis of the donor fluorescence decay (Grinvald et al., 1972; Haas et al., 1975). The analysis of donor fluorescence decay in the present case is complicated by the fact that the donor (in the absence of acceptor) does not decay with first-order kinetics and, second, because on the cell surface each donor is surrounded by a unique distribution of acceptors. This leads to emission kinetics which deviate increasingly from single-exponential decay as the extent of energy transfer increases. In spite of these analytical difficulties, decay curves were collected from single cells labeled only with donor or doubly labeled with donor and acceptor Con A, at different times during development. Figure 6 shows some typical data. At 48 h (Figure 6a), when RET is at a minimum, pyrene decay curves from donor and (donor + acceptor)-labeled cells are

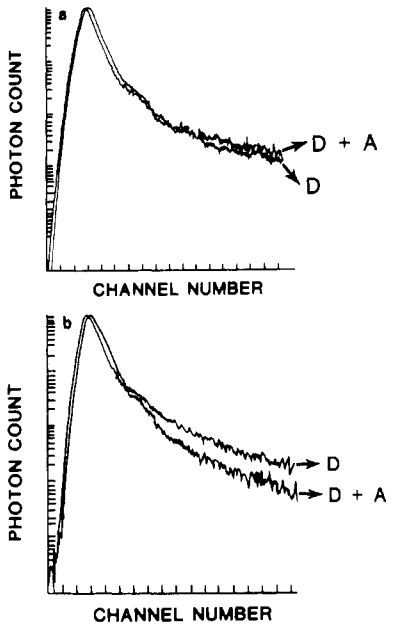

FIGURE 6: Nanosecond fluorescence decay curves of pyrene–Con A obtained from single cells labeled only with pyrene–Con A (D) or with both pyrene– and FITC–Con A (D + A) at a 1:6.8 ratio. The decay curves in (a) and (b) were obtained from cells cultured for 48 and 72 h, respectively. Both curves were collected to 20 000 counts in their peak channels. The time base calibration in this figure is 0.20 ns/channel. Each abscissa marker corresponds to 20 channels.

similar, although the decay of intensity is slightly faster in the doubly labeled cells. For the data shown in Figure 6a the average donor lifetimes from singly and doubly labeled cells at 48 h were respectively 4.51 and 4.39 ns. Better fits to the decay curves are obtained with biexponential analyses which typically yield lifetimes of about 2.5 and 20 ns. The amplitude of the short lifetime component accounts for about 98% of the total signal. At 24 and 72 h (Figure 6b) when RET is significant, the decay curves differ markedly with the intensity from doubly labeled cells decaying more rapidly. The curves in Figure 6b yield average lifetimes of 4.73 and 3.54 ns. In general, two-exponential analysis of decay curves from doubly labeled cells at 24 and 72 h reveals that the long lifetime component remains relatively constant whereas the magnitude of the short lifetime decreases significantly relative to that obtained in control cells labeled only with donor Con A.

Information on donor–acceptor distance distributions can also be obtained, in principle, from time-resolved emission spectra (TRS) which permit determination of the kinetics of the energy-transfer process. This, however, would require successful deconvolution of decay data from both donor and acceptor, which is a very difficult problem. Nevertheless, we have reconstructed TRS from (D + A)-labeled cells from undeconvoluted decay curves. A series of such TRS are shown in Figure 7. The behavior seen in this graph, namely, that the I_A/I_D ratio decreases rapidly in the nanosecond time range, is consistent with an energy-transfer situation in which there exists a statistical distribution of donor–acceptor distances. This is what would be expected on the cell surface.

Ligand-Induced Alteration of Con A Receptor Topography. We have previously shown that significant changes in the apparent rotational mobility of membrane-bound ANS occur in cultured myogenic cells in the time period during which these cells undergo fusion (Herman & Fernandez, 1978). More specifically, coincident with the onset of fusion activity a significant decrease in the apparent rotational correlation time of this probe takes place. This increase in ANS rotational

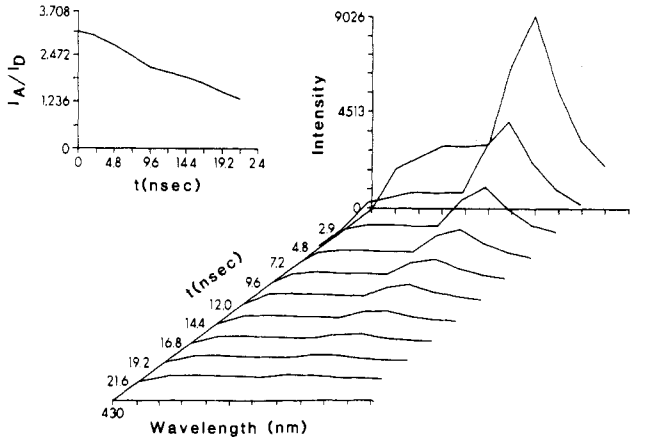

FIGURE 7: Nanosecond time-resolved spectra from a single cell labeled with pyrene– and FITC–Con A at a donor acceptor ratio of 1:6.8. Fluorescence decay curves were collected at various wavelengths, and the time-resolved spectra were constructed from the undeconvoluted decay curves. Time zero is arbitrarily defined to be the peak channel of the decay curve collected at 463 nm.

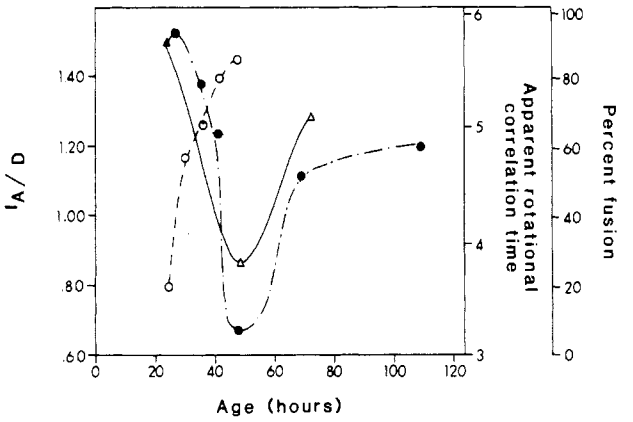

FIGURE 8: Composite graph illustrating the time course of fusion (○), the apparent rotational correlation time of membrane-bound ANS (△), and the extent of RET (●) in myogenic cells at different times during development in vitro.

mobility seems to be associated with an increase in membrane fluidity. Prives & Shinitzky (1977) have also reported similar changes in membranes microviscosity in these cells. The temporal relationship among the changes in ANS rotational mobility, Con A RET, and fusion activity are shown in Figure 8.

The functional role served by this observed developmental variation in membrane "fluidity" is unknown. It is tempting to speculate that the diffusional mobility in the plane of the membrane and the topographical display of membrane components may be controlled or at least modulated by such alterations in lipid dynamics. We have investigated this possibility by studying the kinetics of ligand-induced clustering of Con A receptors by tetravalent Con A at three different times during development when differences in lipid dynamics are known to exist, namely, at 24, 48, and 72 h in culture.

Figure 9 illustrates the result of one such experiment. These data show that ligand-induced aggregation of Con A receptors, as evidenced by the time course of increase in RET, occurs most rapidly at 48 h in culture, a time when membrane fluidity is highest. The kinetics of aggregation at 24 and 72 h are significantly slower and do not reach the high plateau level seen at 48 h. The time course of this phenomenon is followed only until 25 min of incubation at 37 °C. Internalization of Con A was negligible in this time range. This was verified by performing a similar experiment with [^{125}I]Con A labeled

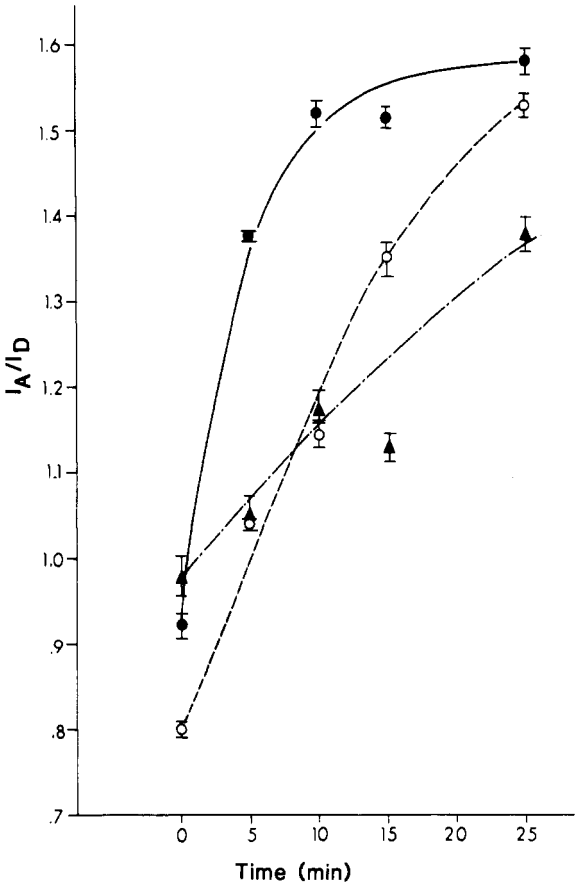

FIGURE 9: Time course of changes in RET (I_A/I_D) during ligand-induced aggregation of Con A on the surface of myogenic cells at 24 (○), 48 (●), and 72 h (▲). Cells were labeled with pyrene- and FITC–Con A at a D:A ratio of 1:6.8 for 1 h at 4 °C. The total concentration of Con A was 20 μg mL^{-1}. At the end of the 1-h labeling incubation, the cells were placed at 37 °C for varying periods of time and subsequently fixed with 2% paraformaldehyde for 20 min at 37 °C. Time zero in the abscissa refers to cells fixed immediately after 1-h incubation at 4 °C. Similar experiments performed by examining unfixed living cells in real time yield similar results, although the data are somewhat noisier.

cells and subjecting the cells to trypsinization after 25 min of incubation at 37 °C. No significant amount of radioactivity remained bound to the cells after this procedure.

Discussion

Type of Information Provided by the RET Method. Our main approach has been to quantitate the extent of RET by measuring the intensity ratio of sensitized acceptor fluorescence to donor fluorescence (I_A/I_D). The magnitude of this ratio depends on the average distance between donor and acceptor fluorophores, and thus, in the present case, changes in I_A/I_D can be interpreted in terms of the average proximity of Con A receptors on the cell surface.

The main advantage of this approach for the study of single cells is simplicity, experimental convenience, and speed. Its main drawback is that energy-transfer efficiencies cannot be calculated from these data. Therefore only relative changes in average proximity can be monitored without relating these changes to absolute distances.

Energy-transfer efficiencies, however, can be calculated from analysis of the donor lifetime. For a given donor–acceptor pair the energy-transfer efficiency E can be calculated according to (Schiller, 1975)

$$E = 1 - \frac{\tau_D}{\tau_D^0} \quad (1)$$

where τ_D and τ_D^0 are the donor lifetimes in the presence and absence of acceptor, respectively. Once the efficiency is known the D–A separation R can be calculated according to (Schiller, 1975)

$$R = (E^{-1} - 1)^{1/6} R_0 \quad (2)$$

where R_0 is the critical Forster radius.

The present situation, unfortunately, does not lend itself to the simple analysis provided by eq 1 and 2. First, each donor on the cell surface sees a unique surrounding distribution of many possible acceptors; second, the Con A molecule has dimensions equivalent to a sphere with a diameter of 30–40 Å (Quiocho et al., 1971) which is commensurate with the R_0 for the pyrene–fluorescein pair; third, the spatial distribution of the fluorophores on the surface of the Con A molecule is unknown. These problems make rigorous quantitative analysis of our results in terms of absolute distances difficult. Some semiquantitative estimates, however, can be made.

A theoretical model for calculation of energy-transfer efficiencies between proteins randomly labeled with fluorescent donor and acceptor probes developed by Gennis & Cantor (1972) is of interest in this regard. In one such model these authors calculate the energy-transfer efficiency between spheres of equal radii (R) as a function of the average number of acceptors per pair (assuming a Poisson distribution of label) for different values of R/R_0. In this model the donors are localized on one protein while the acceptors are on the other.

We can apply this model to the present situation by employing a value of 0.70 for R/R_0 and an acceptor dye to protein ratio of 2. In the absence of any other information we assume the label to be distributed completely at random on the surface of the Con A molecule. Extrapolating from the results of Gennis & Cantor (1972) for two such proteins which are touching, we obtain an energy-transfer efficiency of about 20%. Of course, if the donor is surrounded by more than one acceptor protein, as in the present case, the transfer rate is the sum of the individual rates for all donor–acceptor pairs, and the efficiency would be correspondingly higher.

According to this model, as the proteins move apart the energy-transfer efficiency drops rapidly to values of a few percent for center-to-center distances greater than two molecular diameters. These values can be compared with the average efficiences obtained from eq 1 by employing the short lifetime component in the calculation. Lifetimes from 48-h cells, for which I_A/I_D values are at a minimum, yield efficiences of 1–6%; those from 72-h cells yield values ranging from 57% to 80%. The high efficiences at 72 h therefore indicate that Con A receptors at this time exist predominantly in clusters, i.e., either in contact or at a distance of closest approach of the order of one molecular diameter.

This conclusion based on donor lifetime analysis is consistent with that obtained from the spectral data. I_A/I_D ratios in Figure 2 range from a maximum of about 1.74 at 24 h to a minimum of about 0.75 at 48 h. The value of 1.74 is within experimental error of the maximum value of 1.75 derived from the calibration curve obtained from Con A–anti-Con A antibody precipitates. The value of 0.75 corresponds to absence of energy transfer.

It may be noted at this point that the fluorescence anisotropy data obtained from single cells are also consistent with a resonance energy transfer interpretation of the spectral data. Pyrene–Con A from singly labeled cells yielded the highest anisotropy values followed by FITC–Con A from singly and doubly labeled cells, respectively. The lower anisotropy values of FITC–Con A relative to those of pyrene–Con A (in singly

labeled cells) are expected from (a) the longer mean lifetime of the FITC–Con A, (b) the longer R_0 for FITC self-transfer (47 Å vs. 22 Å for pyrene), and (c) the greater number of FITC–Con A molecules on the cell surface (1:6.8). Finally, the fact that FITC–Con A from doubly labeled cells exhibits the lowest anisotropy of all cases is what would be expected from an RET situation since some reorientation of the excited donor fluorophores takes place prior to transfer.

Surface Con A Receptors Undergo a Marked Reorganization during the Period of Myoblast Fusion. As Figure 2 shows, a marked increase in average Con A receptor separation on the developing muscle cell surface takes place between 24 and 48 h in culture. This interval corresponds to the time period in which these cells have ceased to proliferate and are undergoing fusion (Figure 8) or becoming ready to do so. Figure 2 also shows that this increase in average receptor separation is transient, since after 48 h—a time when fusion is essentially complete—energy transfer increases rapidly and levels off at values somewhat lower than those seen at 24 h. Finally, the data in this figure show that this transient reorganization of Con A receptor topography occurs over the entire cell surface, although significant spatial heterogeneity in average receptor proximity can be readily discerned between regions of cell contact, perinuclear regions, and distal peripheral areas.

The discussion presented in the preceding section suggests that these changes in average Con A receptor proximity represent a dramatic reorganization of the cell surface from a state in which receptors are predominantly in microclusters to one in which they are essentially dispersed. Furthermore, after 48 h, once the fusion process is completed, a reversal of this pattern back to a clustered state takes place. During the time period in which the decrease and subsequent increase in RET are observed, the overall density of surface accessible Con A receptors does not change significantly, as evidenced by autoradiographic analysis (Sandra et al., 1977). This further supports the interpretation that changes in RET are not due to alterations in overall receptor density but rather to a transition from a clustered to disperse distribution or vice versa.

It should be noted that the degree of clustering revealed by the RET technique is a subtle phenomenon that cannot be readily seen by simple fluorescence microscopy. Except for cells treated with nocodazol and colchicine which generally displayed easily discernible patches of fluorescence, high RET cells do not look appreciably different than low RET cells when examined in the microscope. The reorganization of Con A receptors that take place, thus, is not one that results in the formation of gross aggregates.

Other investigators (Rash & Fambrough, 1973) have employed freeze–fracture and thin-section electron microscopy to examine fusion regions of myogenic cells in culture and fusion sites of isolated sarcolemmal vesicles. These workers have found membrane specializations resembling gap junctions at regions of cell–cell contact and a high density of IMPs at contact regions between isolated vesicles. While no direct link between IMPs and Con A receptors was demonstrated, work from other laboratories has shown that exogenous addition of Con A to cultured muscle cells will induce a redistribution of IMPs (Sandra et al., 1977), suggesting a connection between the two. The high density of IMPs found at cell contact regions by electron microscopy correlates well with the high RET values that we observed in these areas.

It should be noted here that the "Con A receptor" in this study represents a heterogeneous class of glycoproteins. The pronounced changes in RET observed thus suggest that the surface reorganization that takes place during fusion or in response to ligand binding entails the concerted redistribution of a variety of surface components. These changes in RET, therefore, reflect a generalized, albeit subtle, alteration of surface architecture.

Regulation of Receptor Topography. The mechanisms by which the described alterations in Con A receptor surface topography are effected are not at present well understood. In general, translational diffusional mobility of membrane components is thought to be modulated by membrane lipid fluidity (Edidin, 1974; Singer & Nicolson, 1972). In addition, a large body of circumstantial evidence supports the notion that cytoskeletal components play a role in the control of the topographical display of surface Con A receptors (Albertini et al., 1977; Berlin et al., 1978; Schlessinger et al., 1976). We have, therefore, investigated how membrane fluidity affects the lateral translational mobility of Con A receptors in intact cultured muscle cells, and we have examined the effect of the microtubule disrupting agents nocodazol and colchicine on the changes in Con A receptor topography that are normally observed in synchronized cells released from fusion block.

The effects of colchicine and nocodazol on receptor topography are presented in Figure 5 which shows that the normal biphasic change in RET seen in the control group does not occur in the drug-treated cells. Instead, in the presence of these drugs there is a monotonic increase in RET to very high values. Thus disruption of microtubules not only prevents the normally observed clustered-to-disperse transition in Con A receptor topography but also results in increased receptor clustering, as evidenced by fluorescence microscopy observations and RET measurements.

These results suggest a role for microtubules in the regulation of Con A surface topography during myoblast fusion. The mechanisms by which such surface alterations are effected and the role of microtubules in this phenomenon, however, remain unclear. As previously discussed, an increase or a decrease in RET can be best interpreted as due to the formation or disappearance of receptor clusters. Removal of the clusters could be accomplished by internalization or lateral dispersal. Evidence for reversible microclustering of lectin receptors in other cell types has been found (Schreiber et al., 1980). Which of these (or other) possibilities actually accounts for the observed behavior cannot be ascertained from the available data, and elucidation of this problem will require further study.

A possible role for membrane fluidity in the regulation of Con A receptor mobility and topography emerges from the data shown in Figure 9. To obtain these results, we took advantage of the transient decrease in membrane fluidity that occurs in developing muscle cells prior to and during the period of fusion activity. The experimental strategy was to follow the kinetics of ligand-induced receptor clustering at different times during this cycle of change in fluidity. Our results show that at 48 h in culture, a time when the apparent rotational diffusion of lipid-bound ANS is highest (see Figure 8), the ligand-induced aggregation of Con A receptors occurs with the fastest time course. At 24 and 72 h, the kinetics of ligand-induced clustering are significantly slower than at 48 h, a behavior that correlates with the slower rotational diffusion of ANS at these time points. These results thus provide a correlation between the rotational diffusional mobility of a lipid-bound probe and the lateral translational mobility of a surface glycoprotein and give credence to the notion that the endogenous developmentally regulated change in membrane fluidity associated with myoblast fusion may serve to modulate

the mobility and display of cell surface components.

As an aside, it may be noted that the starting values of the I_A/I_D ratios (at $t = 0$) in Figure 9 are different than those corresponding to 24, 48, and 72 h for perinuclear regions in Figure 2. There may be several causes for this apparent discrepancy. First, larger cellular regions were examined to obtain the data shown in Figure 9; second, different preparations of labeled Con A with slightly different dye/protein ratios were employed for the experiments on ligand-induced redistribution; third, and most important, the RET measurements shown in Figure 2 were performed on cells that were prefixed prior to labeling, whereas for the experiments shown in Figure 9 the cells were labeled for 1 h at 4 °C prior to fixation or further incubation at 37 °C. It would appear that incubation at 4 °C for 1 h may induce subtle alterations of the normal surface topography.

Relationship between Changes in Surface Topography and the Fusion Event. Our results show that between 24 and 72 h in culture a profound reorganization of the myogenic cell surface takes place, which includes an alteration in membrane lipid dynamics as well as a dramatic change in the topographical distribution of Con A receptors—a heterogeneous class of cell surface components. The time period from 24 to 72 h is the interval during which the cells have ceased to proliferate and undergo a burst of fusion activity. Our data also show that the observed changes in lipid dynamics and Con A receptor distribution maintain a close temporal correlation with the period of fusion activity, even when the onset and kinetics of the latter are experimentally manipulated by alteration of the calcium concentration in the media.

This temporal correlation suggests a causal connection between the observed cell surface changes and the process of fusion. Two observations are of interest in this regard. First, the alterations in lipid dynamics and Con A receptor topography are both global phenomena which occur over the entire cell surface; second, these changes proceed with the same time course regardless of whether the cell being examined has undergone contact or fusion with another cell. In other words, the transition from a clustered to a disperse distribution of Con A receptors is not triggered by cell contact and/or fusion. Rather, it would appear that these changes are part of a developmental program; i.e., the cells develop, for a certain period of time, a surface architecture which is conducive or necessary for recognition, adhesion, or fusion to occur. After this period of time, the duration of which is developmentally regulated, these cell surface characteristics which render the cells "fusion competent" are realtered.

In summary, our results suggest that plasma membrane fluidity and microclustering of surface glycoproteins are part of the sequence of events that lead to myoblast fusion and that microtubules may play a role in the latter phenomenon. Furthermore, our data suggest that the endogenous change in lipid dynamics (fluidity) that is associated with myoblast fusion may serve to modulate the lateral mobility of cell surface glycoproteins.

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