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Properties of hexose-transport regulatory mutants isolated from L6 rat myoblasts

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A hexose-transport regulatory mutant (D1/S4) was isolated from L6 rat myoblasts on the basis of its resistance to detachment and cell lysis in the presence of antibody and complement. Growth studies indicated that D1/S4 cells had a slower doubling time (29 h) compared with the parental L6 cells (22 h). Furthermore, after 9 days growth, less than 1% cell fusion was observed with D1/S4 cells, whereas 95% cell fusion was observed with the L6 cells. When the parental L6 cells were starved of glucose or treated with anti-L6 antibody, a significant increase in the $V_{max.}$ of 2-deoxy-D-glucose (dGlc) and 3-*O*-methyl-D-glucose (MeGlc) transport was observed. Although glucose-grown D1/S4 cells possessed normal hexose-transport activity, the above treatments had no effect on dGlc and MeGlc transport in these cells. Electrophoresis and immunoblotting studies revealed that D1/S4 cells possessed decreased amounts of a 112 kDa plasma-membrane protein. It is conceivable that this protein may play a role in triggering the antibody- and glucose-starvation-mediated activation of hexose transport and in myogenic differentiation. Unlike D1/S4, mutant F72, a mutant defective in the high-affinity hexose-transport system, was found to possess normal amounts of the 112 kDa protein. Although glucose starvation has no effect on the hexose-transport activity in this mutant, its hexose transport activity can be increased by antibody treatment. These studies with mutants suggest the involvement of regulatory components in the activation of hexose transport.

INTRODUCTION

Hexose transport in undifferentiated L6 rat myoblasts has been shown to occur by two systems (D'Amore & Lo, 1986*a*,*b*,*c*; D'Amore *et al.*, 1986*a*). 2-Deoxy-D-glucose (dGlc) was transported by both high- and low-affinity systems, whereas 3-*O*-methyl-D-glucose (MeGlc) was taken up predominantly by the low-affinity system. We have previously observed that hexose transport in glucose-grown L6 rat myoblasts can be stimulated by treatment with anti-L6 antibody (Lo & Duronio, 1984*a*,*b*; D'Amore *et al.*, 1986*b*). In addition, hexose transport in purified plasma-membrane vesicles could also be activated by anti-L6 antibody (D'Amore *et al.*, 1986*b*). This suggests that the cell-surface component(s) recognizable by the anti-L6 antibody are likely to play important roles in the regulation of hexose transport.

Glucose starvation has also been shown to increase hexose transport in L6 cells (D'Amore & Lo, 1986*a*; D'Amore *et al.*, 1986*b*), as well as in other cell lines (Ullrey *et al.*, 1975; Christopher *et al.*, 1976*a*; Rapaport *et al.*, 1979; Gay & Hilf, 1980). In rat myoblasts only the high-affinity hexose-transport system was observed to be increased by glucose starvation (D'Amore & Lo, 1986*a*; D'Amore *et al.*, 1986*b*). Unlike the antibody-mediated effects, this activation is dependent on protein synthesis (Christopher *et al.*, 1976*a*,*b*).

In an attempt to elucidate further the regulatory mechanism(s) of hexose transport in L6 rat myoblasts, the present paper reports the properties of two hexose-transport regulatory mutants.

MATERIALS AND METHODS

Materials

2-Deoxy-D-[G-^{3}H]glucose (8.3 Ci/mmol) and 3-*O*-[*methyl*-^{3}H]methyl-D-glucose (60–90 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). HP/b scintillation fluid was from Beckman Instruments (Irvine, CA, U.S.A.). 2-Deoxy-2-fluoro-D-glucose was purchased from Calbiochem (La Jolla, CA, U.S.A.). Goat anti-rabbit IgG was purchased from Cappel Laboratories (West Chester, PA, U.S.A.). All other chemicals were obtained from commercial sources and were of the highest available purity.

Methods

Cell lines and culture media. Yaffe's (1968) L6 rat skeletal myoblast line was maintained in Alpha medium (Flow Laboratories) supplemented with 10% (v/v) horse serum and gentamycin (50 μg/ml) as previously described (Lo & Duronio, 1984*a*). Glucose-starvation medium was Alpha medium prepared without glucose and supplemented with dialysed horse serum and 0.1% fructose. The antibody-resistant mutant (D1/S4) isolated from L6 cells was generously provided by Dr. B. D. Sanwal of this Department. Mutant F72 was maintained on fructose medium supplemented with 0.05 mM-2-deoxy-2-fluoro-D-glucose (D'Amore *et al.*, 1986*a*).

Preparation of antibody. Rabbit or sheep anti-(L6 rat myoblast) antibodies were raised against L6 cells or their plasma-membrane vesicles (PMVs), and IgG was purified

Abbreviations used: dGlc, 2-deoxy-D-glucose; MeGlc, 3-*O*-methyl-D-glucose; PMV(s), plasma-membrane vesicle(s); SMP, skimmed-milk powder; TBS, Tris-buffered saline (20 mM-Tris/140 mM-NaCl/0.02% NaN_3, pH 7.4); PMSF, phenylmethanesulphonyl fluoride.

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as described previously (Lo & Duronio, 1984*a*,*b*). Goat anti-rabbit antibody was iodinated with Iodogen (Pierce Chemical Co., Rockford, IL, U.S.A.) at an IgG/Iodogen ratio of 10:1 (w/w) (Markwell & Fox, 1975).

Transport studies. Cells were grown for 2 days in six-well Costar plates and 1 min uptake assays were performed as described previously (D'Amore & Lo, 1986*a*; D'Amore *et al.*, 1986*b*). Cells were treated with antibody for 35 min before the transport assays whenever required (Lo & Duronio, 1984*a*; D'Amore *et al.*, 1986*b*).

Preparation of PMVs. PMVs were prepared from L6 and mutant cell lines as described previously (Cheung & Lo, 1984). Rat myoblasts were grown in two (15 mm × 150 mm) tissue-culture dishes at 37 °C. Cells were harvested before reaching confluency (3 days) by incubating each plate with 4 ml of citrate/saline (134 mM-KCl/23 mM-sodium citrate) for 30 min. Cells were then pelleted by centrifugation at 4 °C at 1000 *g* for 5 min. The pellet was then suspended in 15 ml of ice-cold buffer containing 0.25 M-sucrose/1 mM-EGTA and 0.5 mM-PMSF in 10 mM-Hepes, pH 7.0. The cell suspension was homogenized with a tight-fitting Dounce homogenizer. The extent of cell breakage was monitored by phase-contrast microscopy; about 20 strokes/15 ml of suspension was generally required. The cell homogenate was then forced through a 27½-gauge needle several times to ensure cell breakage. This was then centrifuged at 700 *g* for 5 min to remove unbroken cells and nuclei (pellet). The supernatant was then centrifuged at 33000 *g* for 15 min to separate microsomes (supernatant) from the enriched plasma-membrane fraction (pellet) (Cheung & Lo, 1984).

Electrophoresis and western blots. Electrophoresis was performed with 0.1% SDS/9%-(w/v)-polyacrylamide slab gels by the method of Laemmli (1970). After electrophoresis, proteins in the gel were transferred electrophoretically on to nitrocellulose paper by using the method of Towbin *et al.* (1979). After staining with Amido Black, the lanes were cut out and incubated with 5% (w/v) powder SMP in TBS for 2 h to eliminate non-specific binding. Each strip was rinsed once with TBS and then incubated in 5%-SMP/TBS buffer containing about 1 μg of rabbit IgG/ml overnight at 23 °C. The strips were rinsed six times with TBS and incubated with 10^5 c.p.m. of ^{125}I-labelled goat anti-rabbit IgG in 5%-SMP/TBS buffer for 4 h. Finally, the strips were washed six times with TBS, air-dried and exposed to an X-ray film at −80 °C.

Affinity purification of the anti-L6 IgG. Glutaraldehyde-coupled horse serum immunoabsorbent was prepared by the method of Ternynck & Avrameas (1976). A portion (about 100 μg of protein) of anti-PMV antibody was incubated with 1 ml of immunoabsorbent for 1 h. The suspension was centrifuged and the supernatant was then frozen.

Other methods. Protein determinations were made by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Fusion indices were determined by the method of Morris & Cole (1972).

RESULTS

Growth properties of L6 and mutant cells

We have previously observed that prolonged incubation of L6 myoblasts with anti-L6 antibody and complement resulted not only in morphological changes, but also in eventual cell lifting and lysis (Lo & Duronio, 1984*a*). Mutant D1/S4 was isolated on the basis of its resistance to detachment from the plates upon incubation with anti-L6 antibody and complement. Fig. 1 shows that this mutant is unable to undergo myogenic differ-

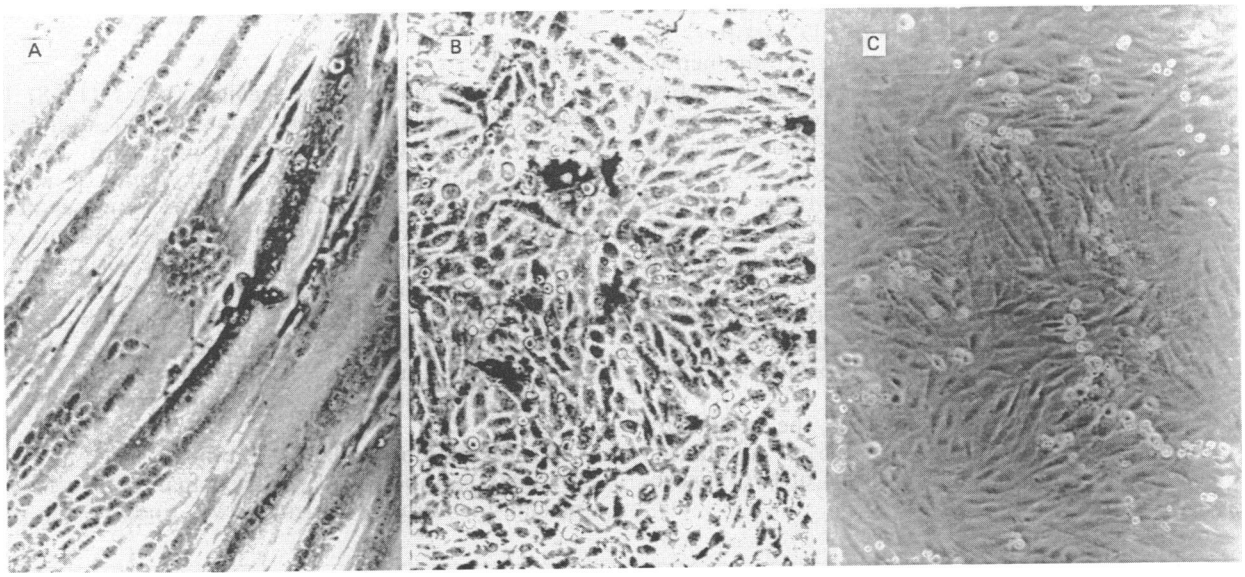

Fig. 1. Morphology of L6, D1/S4 and F72 myoblasts on day 9 after subculturing

Myoblasts were plated at a density of 10^5 cells/100 mm plate. The cells were grown on glucose for 9 days, at which time fusion indexes were determined. Panel A, L6 cells; panel B, D1/S4 cells; panel C, F72 cells.

entiation. Panel A (Fig. 1) shows the presence of multinucleated myotubes in glucose-grown L6 cells. Determination of the fusion index revealed that more than 90% fusion occurred after 6 days of growth and about 95% fusion after 9 days. Panel B (Fig. 1) shows that mutant D1/S4 is unable to form multinucleated myotubes; less than 1% fusion could be observed after growth for 9 days. No increase in cell fusion could be observed in D1/S4, even after 20 days, although after such long periods of growth, cells began to detach from the plates. Panel C (Fig. 1) shows that mutant F72, a previously characterized hexose-transport mutant (D'Amore *et al.*, 1986*a*), is also defective in myogenesis. Glucose-grown L6 cells were found to have a doubling time of about 22 h, whereas that of D1/S4 was about 29 h.

Mutants are defective in hexose-transport activation

We have previously demonstrated that hexose transport in L6 cells can be activated by anti-L6 antibody (Lo & Duronio, 1984*a*,*b*; D'Amore *et al.*, 1986*b*) and by glucose starvation (D'Amore & Lo, 1986*a*; D'Amore *et al.*, 1986*b*). The effect of these treatments on hexose transport in D1/S4 was therefore examined. As indicated in Table 1, hexose transport in L6 was increased about 1.6-fold after glucose starvation (fructose-grown cells) and also after treatment with anti-L6 antibody for 35 min. However, these two treatments had virtually no effect on D1/S4; less than 9% increase was observed in both cases. Thus mutant D1/S4 seems to be defective in some component(s) responsible for triggering the activation of hexose transport by specific antibody and by glucose starvation.

We extended these studies further by determining the kinetics of hexose transport. Table 2 shows the effect of antibody treatment and glucose starvation on both high- and low-affinity hexose-transport systems. As expected, glucose starvation and antibody treatment results in activation of both transport systems in L6 cells (D'Amore *et al.*, 1986*b*). Although the transport capacities of both systems in glucose-grown D1/S4 are not altered, the mutant hexose-transport systems cannot be activated by glucose starvation nor by antibody treatment. Thus mutant D1/S4 seems to be defective in element(s) involved in triggering the activation of hexose transport.

We have previously demonstrated that mutant F72 is defective in the high-affinity hexose-transport system (D'Amore *et al.*, 1986*a*). Unlike the case in D1/S4, this defective transport system in F72 can be restored to the untreated glucose-grown L6 level by treatment with anti-L6 antibody (Table 2). Thus the F72 high-affinity hexose-transport system differs from that of D1/S4 in its inability to function under physiological conditions; however, it can be activated by antibody treatment, a protein-synthesis-independent process (Lo & Duronio, 1984*a*,*b*; D'Amore *et al.*, 1986*b*).

The most important point about the above observation is that both F72 and L6 cells exhibit increased transport activity when treated with the anti-L6 antibody, even though the F72 cells have much lower transport activity than glucose-grown L6 cells. On the other hand, although glucose-grown D1/S4 exhibits transport activity similar to that of the L6 cells, this mutant does not respond to antibody treatment. Thus there are obvious differences between F72 and D1/S4.

Although not indicated in Table 2, the high- and low-affinity hexose-transport systems in L6 cells have apparent K_m values of 0.6 mM and 4.0 mM for dGlc and MeGlc uptake respectively (D'Amore & Lo, 1986*a*,*b*). The transport affinities of these systems are not altered in both mutants. Furthermore, antibody treatment and glucose starvation do not alter the transport affinities of the mutants and L6 cells (results not shown).

Table 1. Effect of antibody and glucose starvation on dGlc transport (v) in L6 and D1/S4 cells

Cells were grown on glucose or fructose for at least 2 days before the transport assays. The concentration of dGlc used was 0.06 mM (sp. activity of 8.3 mCi/mmol). Glucose-grown cells were treated with anti-L6 antibody for 35 min before the transport assays. Results are the averages ± S.E.M. for at least four trials.

	v (pmol/min per 10^5 cells)		
Cell line	Glucose-grown	Fructose-grown	Glucose-grown (+antibody)
L6	18.6±1.8	29.3±1.6	30.9±1.9
D1/S4	19.7±1.7	21.2±2.1	21.3±1.1

Table 2. Effect of antibody and glucose starvation on dGlc and MeGlc transport ($V_{max.}$)

The $V_{max.}$ values (pmol/min per 10^5 cells) were determined as previously described (D'Amore & Lo, 1986*a*). These values were determined from double-reciprocal plots of the initial rates versus substrate concentrations. The results represent the averages ± S.E.M. for at least three trials.

	$V_{max.}$ values					
	Glucose-grown		Fructose-grown		Glucose-grown (+antibody)	
Cell line	dGlc	MeGlc	dGlc	MeGlc	dGlc	MeGlc
L6	235±15	357±20	396±10	596±38	456±22	714±40
D1/S4	236±20	386±15	232±18	407±22	241±10	410±20
F72	128±10	370±30	143±12	362±35	233±15	400±30

Mutant D1/S4 is defective in a plasma-membrane protein

The lack of response of D1/S4 cells to antibody treatment suggested the absence or alteration of cell-surface antigenic site(s) recognizable by the anti-L6 antibody. This possibility was explored by immunoblotting studies. Fig. 2 shows that four major protein bands in L6 cells are recognized by the anti-PMV antibody. In these studies, 70 μg of protein were applied per lane. The D1/S4 preparation differs from that of L6 only in its much diminished amount of the 112 kDa protein. All the other bands appear to be labelled to the same extent and have M_r values similar to those of the major horse serum proteins labelled by the anti-PMV antibody. It is likely that the anti-PMV IgG may contain antibodies, directed against horse serum, which might be present in the PMV preparations originally used to raise antibodies. In order to resolve this problem, the anti-(horse serum) IgG was removed from the rabbit anti-PMV antibody by incubation with the glutaraldehyde-coupled horse serum immunoabsorbent for 60 min. This affinity-purified antibody was then used in a repeat experiment of Fig. 2.

Fig. 3 shows that the affinity-purified anti-PMV antibody can recognize only the 112 kDa protein present in cell extracts and in PMVs from L6 cells (lanes C and E). However, it can no longer bind to the horse serum proteins (lane G). This Figure also shows that PMV are free of contamination by horse serum, as only the 112 kDa protein is recognized by both crude and affinity-purified anti-PMV antibodies (lanes D and E). The horse serum proteins were removed during various washing steps in the preparation of the PMVs. Thus it seems likely that this 112 kDa protein is the only rat myoblast protein recognized by the anti-PMV antibody and it is associated with the plasma membrane.

In order to ascertain the presence of the 112 kDa protein in various mutants and in the glucose-starved cells, PMVs were prepared from these cells. Fig. 3 (lanes A and B) shows that the 112 kDa protein is present in similar amounts in both L6 and F72 cells. Fig. 4 shows that although this protein can be detected in vesicles from glucose-grown L6 cells and fructose-grown L6 and F72 cells, it is present in much diminished amounts in plasma membranes from D1/S4. In fact, after normalization of the amount of proteins applied, densitometer tracings of the gel indicates that fructose-grown L6 (103%) and F72 (112%) cells contain similar amounts of the 112 kDa protein when compared with glucose-grown L6 cells (100%). On the other hand, only 30% of this protein was observed in D1/S4 cells when compared with glucose-grown L6 cells. This is in agreement with findings obtained with whole-cell homogenates. Both Figs. 3 and 4 show that mutant F72 has amounts of the 112 kDa protein similar to those in L6. This may explain why the hexose-transport system in this mutant is still responsive to antibody treatment.

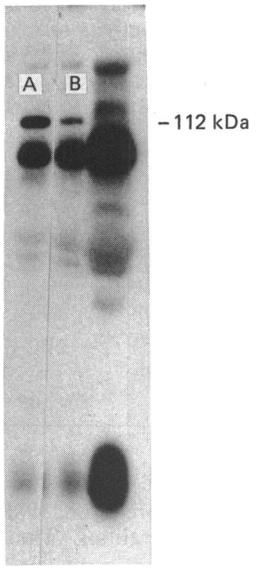

Fig. 2. Western-blot analysis of L6 and D1/S4 cells

Electrophoresis (70 μg of protein per lane) and transfer on to nitrocellulose paper were performed as described in the Materials and methods section. Nitrocellulose strips were incubated overnight with anti-PMV antibody (1 μg/ml) in 5%-SMP/TBS buffer. After washing, the strips were incubated for 4 h with 10^5 c.p.m. of ^{125}I-goat anti-rabbit IgG, washed, air-dried and exposed to X-ray film. Lane A, L6 cells; lane B, D1/S4 cells; other lane, horse serum proteins. Eight separate trials yielded virtually identical results.

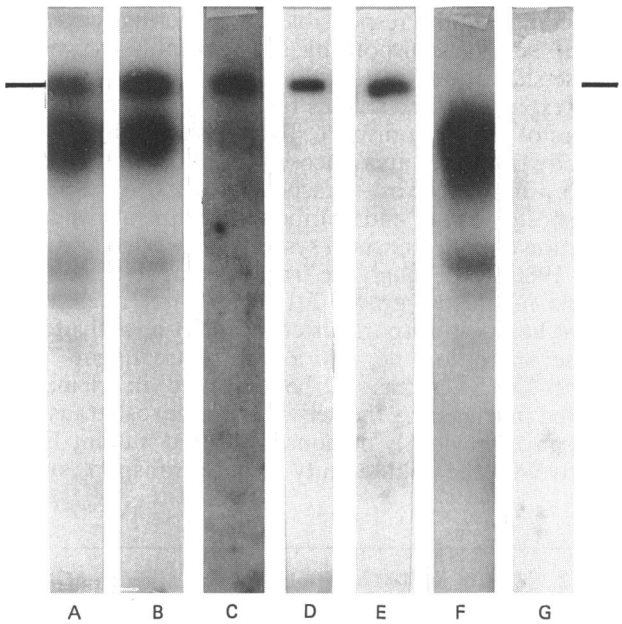

Fig. 3. Western-blot analysis with crude and affinity-purified anti-PMV antibodies

The conditions used were the same as those described for Fig. 2. Lane A, F72 cells probed with anti-PMV antibody; lane B, L6 cells probed with anti-PMV antibody; lane C, L6 cells probed with affinity-purified anti-PMV antibody; lane D, PMV from L6 cells probed with anti-PMV antibody; lane E, PMV from L6 cells probed with affinity-purified anti-PMV antibody; lane F, horse serum proteins probed with anti-PMV antibody; lane G, horse serum proteins probed with affinity-purified anti-PMV antibody. The horizontal line indicates the position of the 112 kDa protein. Four separate trials yielded virtually identical results.

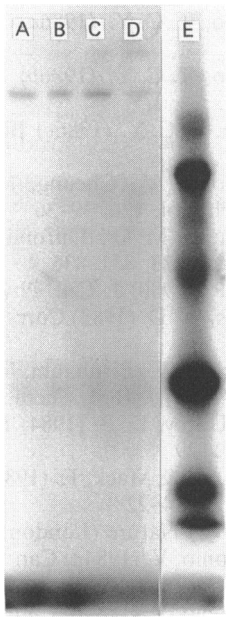

Fig. 4. Western-blot analysis of PMVs prepared from different types of cells

The conditions used were the same as those described for Fig. 2. Lane A, 70 μg of PMV from glucose-grown L6 cells; lane B, 70 μg of PMV from fructose-grown L6 cells; lane C, 100 μg of PMV from fructose-grown F72; lane D, 70 μg of PMV from glucose-grown D1/S4 cells; and lane E, molecular-size markers [phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa)]. Four separate trials yielded virtually identical results.

DISCUSSION

Considerable effort has been focused on the regulatory mechanism of hexose transport. Insulin, epidermal growth factor, phorbol esters, glucose starvation and viral transformation have all been shown to stimulate hexose transport in responsive cells (Salter *et al.*, 1982; Gliemann & Rees, 1983; Kalckar & Ullrey, 1984; Klip *et al.*, 1984; Simpson *et al.*, 1985; Van Putten & Krans, 1985; Haspel *et al.*, 1986). At the moment, the mechanism(s) involved in the regulation of hexose transport is not clearly established. It is possible that hexose transport may be modulated by more than one mechanism.

Most of the approaches used in studying the regulation of hexose transport in eukaryotic cells are based on the response of the cells to various reagents and to changes in the growth conditions. Although these physiological approaches have provided valuable information on the regulatory processes, it is at times difficult to demonstrate unequivocally the involvement of certain regulatory components. One approach to determine more precisely the regulatory mechanisms is through the use of mutants altered in response of their hexose-transport system to various changes. For example, a mutant defective in phosphoglucose isomerase has been used extensively to study the role of hexose metabolism in regulating hexose transport (Kalckar & Ullrey, 1984). Unfortunately, because of the lack of suitable mutants, such an approach has not been used extensively by other workers. The present paper reports the use of two hexose-transport regulatory mutants in examining the possible components involved in the regulation of hexose transport. Similar mutants have not been examined previously by other workers.

Studies on the hexose-transport system in undifferentiated L6 rat myoblasts reveal that substrate translocation is the rate-limiting step in the uptake of hexose analogues and that two hexose-transport systems are present in these cells (D'Amore & Lo, 1986*a*,*b*; D'Amore *et al.*, 1986*a*). We have previously demonstrated that hexose transport in L6 cells can be activated by anti-L6 antibody (Lo & Duronio, 1984*a*,*b*; D'Amore *et al.*, 1986*b*). This activation is independent of protein synthesis, but is dependent on antibody-mediated dimerization of cell-surface components (Lo & Duronio, 1986*a*,*b*). Figs. 2–4 indicate that the anti-L6 IgG binds to a 112 kDa plasma-membrane protein that is present in much diminished amounts in mutant D1/S4.

Kinetic analysis of the hexose-transport systems in D1/S4 cells reveals that the hexose-transport systems in this mutant cannot be activated by glucose starvation nor by antibody treatment. Thus this mutant may be defective in factor(s) normally responsible for triggering the antibody-mediated or glucose-starvation-mediated activation of hexose transport. Thus the possibility exists that this 112 kDa protein may actually be the factor responsible for triggering the activation of hexose transport. Since D1/S4 is also defective in myogenesis (Fig. 1), this 112 kDa protein may also play a role in myogenesis.

Studies with whole cells and PMVs show that glucose starvation results in increased hexose-transport capacity, with no change in affinity (D'Amore & Lo, 1986*a*,*b*; D'Amore *et al.*, 1986*b*). Table 2 shows that although the high-affinity hexose-transport system of mutant F72 cannot be activated by glucose starvation, its activity can be restored to that of the glucose-grown wild-type upon incubation with anti-L6 antibody for 35 min. Since the antibody-mediated activation is independent of protein synthesis (Lo & Duronio, 1984*a*,*b*), this suggests that the high-affinity hexose transporter is normally present in F72, except that it is not in a functional state. In support of this, cytochalasin B-binding studies have shown that this transporter is present in substantial quantities in this transport-defective mutant (S. Chen & T. C. Y. Lo, unpublished work). It is therefore conceivable that, in glucose-grown L6, the high-affinity transporter may be present in both active and inactive forms. Under physiological conditions, activation of the inactive form may probably be brought about by a positive regulator. Glucose starvation results in increased synthesis of this positive regulator. Our results suggest that F72 may be defective in this positive regulator; consequently the high-affinity transporter is present mainly in the inactive form, and glucose starvation has no effect on this inactive transporter. Data presented in Table 2 suggest that this inactive form can be activated by events resulting from the binding of antibody to the 112 kDa protein. The finding that antibody treatment of F72 cannot restore the transport activities to those of the antibody-treated glucose-grown L6 cells suggests that events resulting from the antibody treatment cannot

completely replace the functioning of the positive regulator.

It may be apparent from the present study that although treatment of cells or PMVs with specific antibodies may not have much physiological relevance, this approach does serve as a useful probe to examine the regulation of the hexose-transport system. Studies using the antibodies reveal that dimerization of the 112 kDa protein is essential for the activation process (Lo & Duronio, 1984*b*) and that mutant F72 is actually a hexose-transport regulatory mutant. It is interesting to note that the inactive transporter in this mutant can still be activated by antibody-mediated events. This, though, would be expected, since this mutant has amounts of the 112 kDa protein similar to those in the parental L6 cells. The present study also shows that mutants defective in this antibody-mediated activation can also serve as a powerful tool in defining the component involved in the regulation of hexose transport. Studies with these mutants suggest that the 112 kDa plasma-membrane protein may play an important role in triggering the activation of hexose transport by antibody treatment. It may be apparent from the above studies that transport regulatory mutants are valuable tools in dissecting the regulatory mechanism of hexose transport.

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