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Original Article

Synthesis and secretion of a 38-kDA glycopolypeptide coincides with L6 myoblast fusion

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ABSTRACT Rat L6 myoblastic cell line fused rapidly after two day cultivation in a medium containing horse serum and insulin. We analyzed whether the induction of plasma membrane or secreted proteins occurred simultaneously with ongoing fusion. Thus the cells were metabolically labeled with [^{35}S]methionine followed by biotinylation of the cell surface proteins. Detergent-solubilized proteins derivatized with biotin were isolated with streptavidin-agarose and subjected to SDS polyacrylamide gel electrophoresis. This analysis did not show fusion-associated induction of any surface proteins. However, analysis of the microsomal fraction revealed a fusion-associated 38-kDa glycopolypeptide. This polypeptide appeared simultaneously with the formation of the multinucleated cells and then declined with decreasing fusion activity. Pulse-chase labeling experiments showed that the 38-kDa component was secreted into the medium. These results indicate that a secreted protein component is induced during the fusion of L6 myoblasts.

KEY WORDS: *muscle, myoblast, cell-cell fusion*

Introduction

Biological membrane fusions are spatially and temporally strictly regulated developmental processes. So far the best known fusion system is that of the enveloped viruses which utilize integral membrane proteins as fusogens (for a review, see White, 1990). Such fusogens are able to mediate the fusion between cells when expressed on the cell surface (White *et al.*, 1981). Fusions between intracellular vesicles and membranes apparently require a reusable system, and soluble protein components are involved (Beckers *et al.*, 1989; Weidman *et al.*, 1989; Clary *et al.*, 1990). Nevertheless, the mechanism of cellular fusion occurring during the formation of multinucleated cells, such as skeletal muscle cells, osteoclasts, syncytial trophoblasts and foreign body giant cells, has remained elusive.

During myogenesis, a family of differentiation genes are successively activated and their products induce the commitment into a muscle phenotype. Before the actual fusion, cell surface adhesion molecules anchor the cells together. Such adhesion molecules have been partially characterized (Knudsen, 1985; Knudsen *et al.*, 1990), but we have only a confused picture of the molecular mechanisms lying behind the fusion of the apposed plasma membranes. Thus, several cell surface proteins have been described that appear during myogenesis (Moss *et al.*, 1978; Pauw and David, 1979; Walsh and Phillips, 1981; Kaufman *et al.*, 1985). Such proteins could be a part of the fusion machinery but evidence for their involvement in fusion is lacking. It has been shown that the influx of calcium prior to fusion occurs (Entwistle *et al.*, 1988; Rapuano *et al.*, 1989). Furthermore, activation of protein kinase C (David *et al.*, 1990) or an increase in intracellular cyclic GMP level (Choi *et al.*, 1992) cause precocious fusion. Typically cell-cell fusions are dependent on metalloproteinase activity (Couch and Strittmatter, 1983; Cates *et al.*, 1984; Baldwin and Kayalar, 1986), which in the cases studied is located in the cytoplasm (Couch and Strittmatter, 1984). Changes in polypeptide phosphorylation have been reported to occur concomitantly with fusion (Sénéchal *et al.*, 1982). Lognonne and Wahrmann (1990) reported that inhibition of ecto-protein kinase activity blocks fusion. Such findings support a view that cell-cell fusions are under cytoplasmic and/or extracellular regulation. Plasma membrane protein fusogens such as exist with enveloped viruses very likely mediate the actual fusion. Such a cellular analogue of a viral fusion protein seems to be the sperm PH-30 protein (Blobel *et al.*, 1992).

Fusion models for exoplasmic fusion between cells, if analogous with the enveloped viral system or the intracellular fusion system, need protein components located in the plasma membrane and/or in the medium. In this study we have tried to see whether such cell surface or secreted proteins are induced in an L6 myogenic cell line which undergoes fusion. The most prominent change we found was

Abbreviations used in this paper: ConA, Concanavalin A; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, Dulbecco's phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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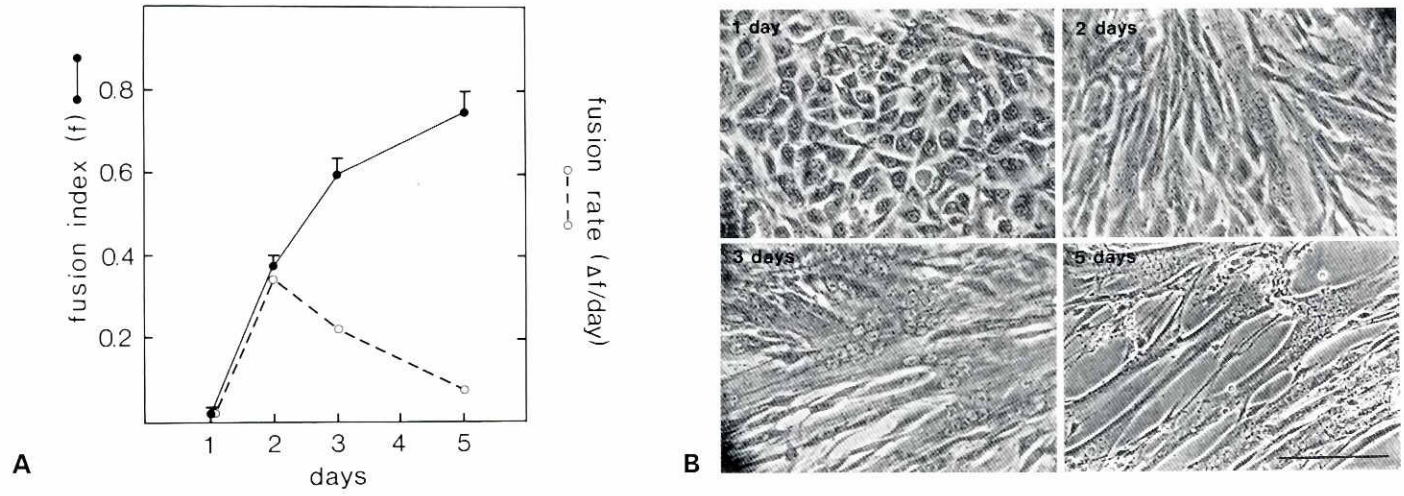

Fig. 1. Fusion of L6 myoblasts with horse serum and insulin. *The horse serum-insulin containing medium was given after two days growth in DMEM containing 10% FCS.* **(A)** *Fusion index (f; White et al., 1981) was determined at indicated days after administration of the fusion medium. Mean of three determinations and SD are shown. Fusion rate represents the difference in fusion index on successive days.* **(B)** *Shows phase contrast pictures of the L6 cells at the indicated times of cultivation in the fusion medium. Bar, 100 μm.*

the induction and secretion of a 38-kDa polypeptide. We did not detect changes in the plasma membrane polypeptide composition during the fusion of L6 myoblasts. Our findings are compatible with the view that the fusion machinery pre-exists in the plasma membrane and is regulated from inside and/or outside the cell.

Results

Fusion of the L6 myoblasts

The L6 myoblasts fused slowly with a rather constant fusion rate when grown in the presence of 1% horse serum. We observed a fusion index of about 80% after six-day induction with 1% horse serum. Addition of insulin induced the fusion to start more rapidly. Syncytia formation began to occur after one day from the administration of the induction medium and reached a maximal rate at two days (Fig. 1). At this time point, two to five nuclei were found in a cell and 35-50% (variation from experiment to experiment) of the nuclei were syncytial. The syncytial muscle cells fused further during the next few days but their fusion rate progressively declined. The fusions ceased at the level of 70-80%. Those cells that did not fuse became round and detached from the dish. This fraction was 10-20% of the initial cell number.

Insulin alone in DMEM resulted in a fusion rate of about 10% when measured three days after administration while insulin in DMEM containing 1% FCS caused a fusion rate of about 20%. No fusions occurred within five days when the L6 cells were grown in 10% FCS. After 3 days growth in 10% FCS the L6 cells lost their capability to fuse with the horse serum and insulin treatment.

Major cell surface proteins during the fusion

In exoplasmic fusion, cell surface proteins or proteins in the medium can be expected to mediate the fusion of adjacent plasma membranes. We analyzed whether new plasma membrane proteins were induced concomitantly with the fusion of myoblasts into multinucleated muscle cells. For this purpose, fusing and nonfusing L6 cells were metabolically labeled with [^{35}S]methionine, followed by 1 h chase (see Materials and Methods). Plasma membrane proteins were then biotinylated and purified by streptavidin-agarose affinity chromatography. SDS polyacrylamide gel electrophoresis and autoradiography of the material purified by streptavidin-agarose showed that the intensity of several polypeptides changed slightly upon fusion. An example is shown in Fig. 2. However, these changes were not reproducible in repeated experiments. Thus, we could not detect new, major plasma membrane polypeptides when the L6 cells fused.

A secreted polypeptide is induced concomitantly with fusion

Proteins destined for secretion are initially found within intracellular membrane compartments and vesicles. Thus the microsomal fraction prepared from cells labeled with [^{35}S]methionine (15 min) was analyzed. We found that certain polypeptides showed decreasing intensity when the L6 cells fused while a polypeptide of 38-kDa appeared in the microsomal fraction under the fusion stimulation (Fig. 3). We used sodium hydroxide washing (Mudd, 1974) to separate integral microsomal membrane proteins from the soluble and peripheric proteins. This procedure revealed that the components exhibiting decreasing intensity were integral membrane proteins (Fig. 3). Presumably they were not plasma membrane-associated since changes in the intensity of such polypeptides were not seen by the surface biotinylation (Fig. 2). The 38-kDa polypeptide that appeared during fusion was washed off the membranes at the high pH. Furthermore, phase separation with Triton X-114 (Bordier, 1981) resulted in the equilibration of the 38-kDa polypeptide in the water phase (not shown). This behavior suggests that it was not membrane associated but rather a soluble luminal component. The 38-kDa species bound to ConA agarose and eluted with α-methylmannoside, suggesting a glycoprotein nature. Fig. 4 shows [^{35}S]methionine-labeled polypeptides after the ConA-purification step in non-fusing and fusing cells.

To analyze whether the 38-kDa component was secreted, the L6 cells were pulsed with [^{35}S]methionine for 15 min and then chased for 2 h. We found that the amount of the 38-kDa polypeptide

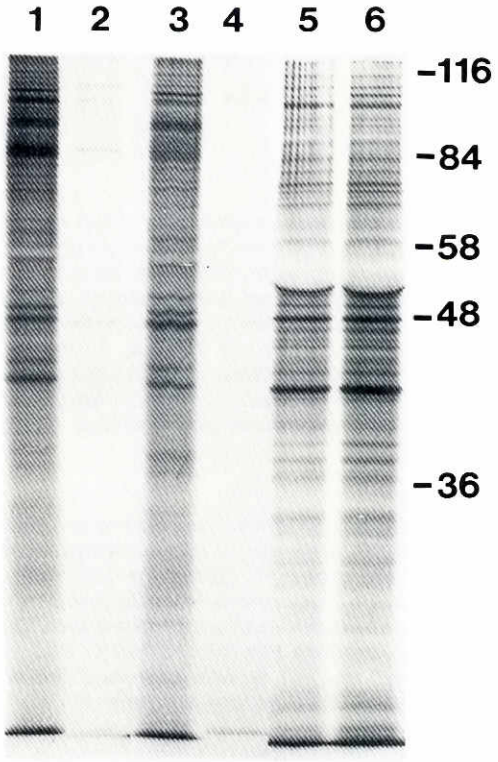

Fig. 2. Metabolically labeled surface polypeptides in nonfusing and fusing L6 myoblasts. *Cells were labeled with [^{35}S]methionine (15 min, followed by a 1 h chase) and surface proteins were biotinylated. Following isolation of the biotinylated proteins with streptavidine agarose, SDS polyacrylamide gel electrophoresis was performed. Autoradiography shows the labeled surface polypeptides in nonfusing cells* **(lane 1)** *and fusing cells* **(lane 3)**. **(Lanes 2 and 4)** *show similarly treated material from lysates of cells that were not biotinylated. Labeled polypeptides in postnuclear cell lysates of nonfusing* **(lane 5)** *and fusing cells* **(lanes 6)** *are shown.*

decreased in the microsomal fraction (not shown). It was found in the chase medium instead (Fig. 4). The intensity of the 38-kDa polypeptide increased 4- to 7-fold (assay to assay variation) during the first two days under fusion conditions, as determined by scintillation counting of excised bands (Fig. 4) after autoradiography. Fig. 5 shows a typical time course of the intensity of cell-associated 38-kDa band during fusion.

The 38-kDa polypeptide exhibited a short half life in the medium. Thus, after a 6 h chase, this band could no longer be discerned. Protein detection methods such as silver staining revealed a weak, horse serum- and insulin-dependent band at 38-kDa when microsomes were analyzed by SDS gel electrophoresis after the ConA affinity purification. Accordingly, the 38-kDa species was a minor component with a rapid turn-over. Creatine kinase, a differentiation indicator in muscle cell culture, appeared after the 38-kDa component reached its maximal synthesis level and after the fusion was practically completed (Fig. 5).

Discussion

Insulin has been found to potentiate the fusion of L6 cells together with transferrin and selenium (Pouliot *et al.*, 1990). In our study, the presence of insulin induced the fusions to start abruptly and a time point of maximal fusion rate was clearly observed (Fig. 1). Myoblast fusion induced by horse serum alone proceeded more linearly and is compatible with earlier findings (Wakelam, 1985). The fusions induced with horse serum and insulin started after a lag of about one day, leaving time for the induction of new protein synthesis. We did not, however, find detectable changes in the plasma membrane polypeptide composition during the period when the fusion reached a maximal rate. Thus, changes in major cell surface protein components did not occur when the fusion was stimulated.

High concentrations of viral fusogens are needed for the induction of cell-cell fusion (Doxsey *et al.*, 1985). This is obtained during viral infection (White *et al.*, 1981), in cells transfected with viral spike cDNA (Kondor-Koch *et al.*, 1983) or by implantation methods (Metsikkö *et al.*, 1986). If such fusogens mediate myoblast fusion, they must already exist on the cell surface before the fusion. Their fusogenic activity could be triggered by cytoplasmic or extracellular factors, e.g. kinases, phosphatases or proteases. Thus okadaic acid, a phosphatase inhibitor (Sardet *et al.*, 1991), was shown to

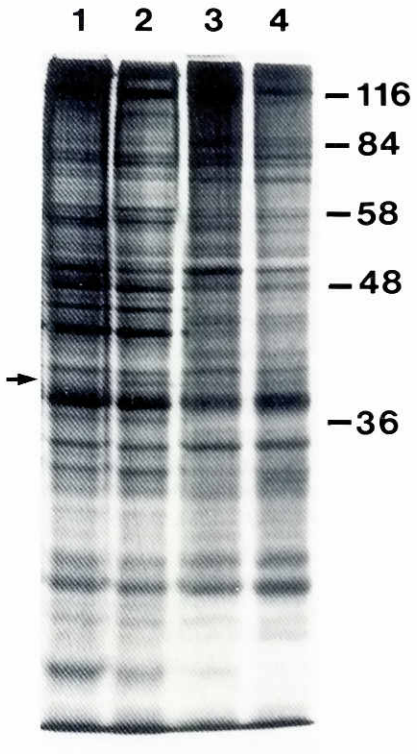

Fig. 3. Induction of microsomal polypeptides in fusing L6 cells. *Cells were labeled for 15 min with [^{35}S]methionine, and the microsomal fraction (see Materials and Methods) was analyzed by SDS gel electrophoresis.* **(Lane 1)** *shows the microsomal proteins synthesized in nonfusing cells that were grown for 4 days in 10% FCS.* **(Lane 2)** *shows the corresponding proteins in fusing cells that were grown for 2 days in 10% FCS followed by 2 days growth in 1% horse serum and insulin.* **(Lanes 3 and 4)** *show the microsomal polypeptides after alkaline washing (Mudd, 1974) in the nonfusing and fusing cells. The arrow marks a 38-kDa polypeptide that appears in the fusing cells.*

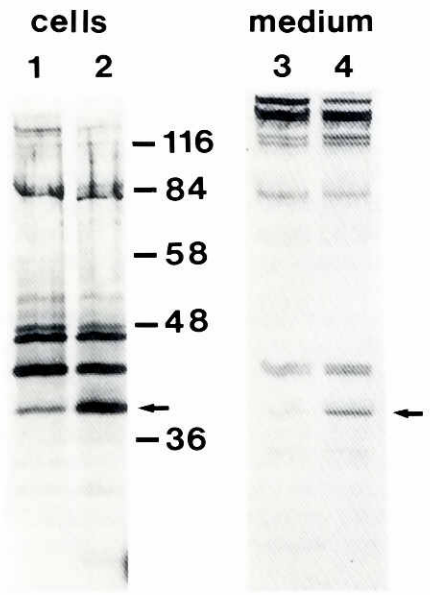

Fig. 4. Secretion of the inducible 38-kDa polypeptide. *L6 cells were pulse-labeled for 15 min with [^{35}S]methionine. Cells on one set of dishes were then solubilized and the nuclei were removed. Another set of dishes was chased for 2 h and the medium was recovered. The media and the postnuclear cell lysates were subjected to ConA agarose affinity chromatography and analyzed by SDS gel electrophoresis.* **(Lane 1)** *nonfusing cells;* **(lane 2)** *fusing cells;* **(lane 3)** *medium of nonfusing cells;* **(lane 4)** *medium of fusing cells. The arrows indicate the 38-kDa polypeptide. All the material released by α-methylmannoside was applied on the SDS gel.*

block chick myoblast fusion with a concomitant change in the phosphorylation stages of many proteins (Kim *et al.*, 1991). Chen and Lo (1991) have reported that myogenesis is dependent on the ecto-phosphorylation of a 112-kDa protein. Furthermore, Rosenberg *et al.* (1985) reported a reduction in the amount of a 105-kDa glycopolypeptide and the appearance of a 90-kDa polypeptide concomitant with fusion. Whether these changes triggered fusion remains open. Many of the changes in the cell surface polypeptide composition that have been reported to occur during spontaneous fusion (Moss *et al.*, 1978; Pauw and David, 1979; Walsh and Phillips, 1981) are probably associated with the differentiation of the myotubes rather than fusion itself.

We observed an induction of a ConA-binding 38-kDa polypeptide that was found in the medium, indicating secretion. Gel filtration studies suggested that this polypeptide was a part of a larger protein complex (data not shown). A 46-kDa ConA-binding polypeptide which resides in the endoplasmic reticulum is associated with muscle cell fusion (Nandan *et al.*, 1988). A monoclonal antibody against this polypeptide (kindly provided by B. Sanwal) did not recognize our 38-kDa polypeptide. It is thus plausible that these two species were not related to each other.

Comparison of Figs. 1 and 5 shows that the time course of the intensity of the 38-kDa polypeptide matches with the fusion rate. The finding that our 38-kDa polypeptide showed a maximum level of synthesis when the fusion rate was maximal suggests that it could have a role in the fusion process. Soluble fusion factors are known to exist in the fusion machinery of the intracellular vesicles (Weidman *et al.*, 1989). Further characterization of the 38-kD polypeptide is needed to determine whether it has a role in myoblast fusion and differentiation.

Materials and Methods

Cells and fusion

L6 myogenic cells originally isolated by Yaffe (1968) were obtained from The American Type Culture Collection. They were split at the density of 10^6 cells/dish (7 cm^2) and then grown for 2 days in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Gibco Laboratories). During this period a subconfluent monolayer of cells was obtained. To induce fusion, the medium was changed for DMEM (2 ml) containing 1% horse serum (Gibco Laboratories) and 0.4 U/ml of insulin (Lente MC, Novo Industri, Denmark) (fusion medium). Control dishes were grown further in the original DMEM containing 10% FCS.

Metabolic labeling

Metabolic labeling with [^{35}S]methionine (Amersham, Bucks, England) was performed in 5% CO_2-atmosphere in minimal essential medium without methionine (Gibco Laboratories). The cells were pulsed for 15 min (100 μCi/ml) followed by 1 or 2 h chase periods in DMEM containing 0.1% bovine serum albumin and 10x normal methionine concentration. Cells grown for 2 days in a medium containing 10% FCS and then for 2 days in the fusion medium were labeled. As a control, cells grown for 2 days or 4 days in a medium containing 10% FCS (nonfusing cells) were similarly labeled.

Preparation of crude microsomes

Cells were broken by hypotonic lysis. In brief, cell monolayers were washed with Dulbecco's phosphate buffered saline (PBS) followed by a rinse with 10 mM Tris buffer, pH 8.0. One ml of 10 mM Tris, pH 8.0, was then added and the monolayer was scraped with a cell scraper at 0°C. To break

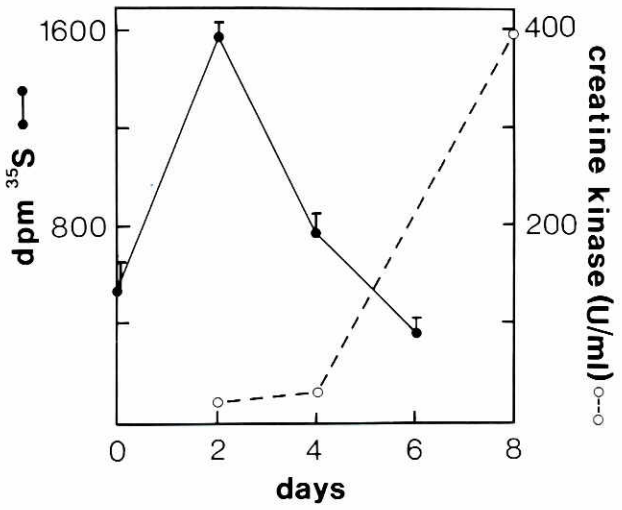

Fig. 5. Time dependency of the appearance of the 38-kDa polypeptide and creatine kinase in fusing L6 myoblasts. *[^{35}S]methionine-labeled microsomal proteins purified by ConA agarose were separated by SDS gel electrophoresis. Radioactivity in the gel bands was quantitated by excising the bands after autoradiography and by performing liquid scintillation counting. Means of duplicate determinations and the range are shown. Background radioactivity was subtracted. Determinations were at indicated days of growth in the fusion medium.*

the cells, the suspension was drawn ten times through a 0.6x25 syringe. Nuclei were spun down (5000 g, 5 min), and the microsomes were pelleted at 100000 g for 1.5 h at 4°C.

ConA agarose affinity chromatography

Cells or crude microsomes were solubilized in PBS containing 1% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride. Nuclei and insoluble material were removed by centrifugation (5000 g, 5 min, followed by 100000 g for 1.5 h) from the solubilized cells. Extracts were agitated with ConA agarose (Sigma Chemical Company, St. Louis, MO) for 1 h at 20°C, using 50 μl of packed gel for adsorption of proteins from solubilized cells grown on a 7 cm^2 petri dish. Chase media were centrifuged at 100000 g for 1.5 h, and then agitated with ConA agarose (50 μl packed gel/2 ml). The gel was washed with the solubilizing buffer five times for 5 min. Adsorbed proteins were eluted with 0.5 M α-methylmannoside in 50 mM Tris buffer, pH 7.4 (200 μl).

Surface protein biotinylation and isolation

Biotin labeling was as described (Lisanti *et al.*, 1988; Matter *et al.*, 1990) with some modifications. The cell monolayers were washed twice with PBS and incubated with sulfo-NHS biotin (Pierce, Oud-Beijerland, Netherlands) (0.5 mg/ml) for 30 min on ice. Excess label was then quenched with 50 mM ammonium chloride for 10 min at 0°C. The cells were solubilized with 50 mM Tris buffer, pH 8.0, containing 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.2% bovine serum albumin, and 0.5 mM phenylmethylsulfonyl fluoride (lysis buffer) (500 μl/dish). Nuclei were removed by low-speed centrifugation. The supernatant was centrifuged at 100000 g for 1 h at 4°C, and the soluble fraction was subjected to affinity chromatography with streptavidin agarose (Sigma Chemical Co.) as described by Hare and Lee (1989). Adsorbed proteins were solubilized by boiling for 3 min in SDS gel electrophoresis sample buffer containing 5 mM dithiothreitol. About 0.5% of the radioactivity incorporated into the cells was recovered.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Samples were alkylated with iodoacetamide. Prestained molecular weight standards (Sigma Chemical Co.) were used.

Creatine kinase

Creatine kinase activity was determined from cell lysates by using a commercial assay kit (Boehringer, Mannheim, Germany).

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