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Association of glycolytic enzymes with the cytoplasmic side of the plasma membrane of glioma cells

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A latex phagocytosis technique was used to prepare relatively pure plasma membranes with inside-out orientation. This method was adapted through a number of modifications in order to evaluate the association of glycolytic enzymes with the cytoplasmic side of the plasma membrane of C6 glial cells. As phosphorylation is strictly coupled with transport in these cells, glycolytic enzymes, especially hexokinase, could metabolize glucose in close vicinity to its transporter. Of the enzymes tested, hexokinase is present in considerable quantities on these membranes (nearly 40% of homogenate specific activity), followed by D-glyceraldehyde-3-phosphate dehydrogenase (10%), pyruvate kinase (8%), and 3-phosphoglycerate kinase (1%). Except for hexokinase, the enzyme pattern presented here is different from that published for other membrane preparations.

Introduction

Previous studies have established that transport of glucose is rate-limiting for its utilisation in C6 glioma cells, indicating a 'membrane-controlled' (limited) type, with a close coupling between glucose transport and phosphorylation [1,2]. Early publications reporting that glycolytic enzymes are associated with the cytoplasmic side of plasma membranes [3] were thus of particular interest. Apart from their binding to internal membranes, like mitochondrial or sarcoplasmic reticulum membranes, a few glycolytic enzymes were reported to occur on the cytoplasmic membrane surface of human erythrocytes [4,5]. Plasma membranes from other cell types, however, suffer from inadequate characterisation, especially when considering the sides that are exposed. Further disadvantages are the contamination with other cellular membranes, the formation of vesicles containing solubilised cytoplasmic proteins, and the association with cytoskeletal components.

The phagocytosis procedure with polystyrene particles, described by Charalampous in 1977 [6], and modified thoroughly in our laboratory [7,8], provides a new approach to this problem. The established phagocytic activity of glial cells in culture [9] enables us to obtain a sufficient amount of relatively pure inside-out plasma membranes. Much attention has been paid on ways of minimising the portion of non-internalized beads which are lacking in plasma membranes, but avidly bind proteins, and therefore influence the outcome of the enzyme pattern. Out of the six glycolytic enzymes tested, D-glyceraldehyde-3-phosphate dehydrogenase, hexokinase, 3-phosphoglycerate kinase and pyruvate kinase were found to be associated with the cytoplasmic surface of C6 glioma cells.

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Materials and Methods

The C6 glioma cell line was obtained from the American Type Culture Collection, Rockville, MD, and cultured as described previously [1]. At the confluent stage, the monolayers were used to prepare inside-out plasma membranes, according to the general published procedure [7,8]. In order to reconcile sufficient phagocytosis with minimal lysosomal fusion and almost complete removal of non-internalized beads, the monolayers were first incubated with polystyrene particles (diameter 1.1 μm, final concentration $1 \cdot 10^8$) for 1 h (receiving sufficient membrane yield). They were then washed three times with 0.9% NaCl/0.1 mM EDTA (removing non-internalised beads), reincubated with fresh media for 2 h (to phagocytose beads still attached), and rinsed again three times with NaCl/EDTA. By use of trypsin/EDTA (0.05%/0.02%) for 10 min at 37 °C, cells were detached from the flasks and beads still adhering were removed from the cell surface. The following preparative steps were taken at a temperature of 0 °C. Cells were transferred to precooled siliconised glass tubes and after repeated (4–7) washings and centrifugation steps (each at $500 \times g$ for 10 min), were homogenised in 33% sucrose.

To study the enzyme pattern of non-internalised beads which had been in contact with cell proteins and internal membranes, polystyrene beads were added to an extra cell homogenate and subjected to the following isolation procedure. The cell homogenates of both preparations were centrifuged in a three step (10%/20%/30%) sucrose gradient (SW 28 rotor, $100\,000 \times g$ for 70 min) and the particle fractions (called 'nude' and 'membrane' beads) were collected from the 10%/20% interphase. The particle suspensions were pelleted ($165\,000 \times g$ for 35 min), resuspended, counted by a Coulter Counter, adjusted to a standard concentration of $1 \cdot 10^{10}$ particles/ml and finally stored in liquid nitrogen overnight in order to measure the enzyme activities the next day.

The specific activities (U/mg protein) of the glycolytic enzymes aldolase (EC 4.1.2.13), D-glyceraldehyde-3-phosphate dehydrogenase (EC1.2.1.12), hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), 3-phosphoglycerate kinase (EC 2.7.2.3) and pyruvate kinase (EC 2.7.1.40) as well as the marker enzymes 5′-nucleotidase (EC 3.1.3.5) (plasma membrane) and succinate-cytochrome-*c* reductase (mitochondria) were determined according to the procedures of Bergmeyer [10] and Tisdale [11], with slight modifications.

All results are presented as means ± S.D. from three separate experimental sets, covering the whole procedure from cell culture up to enzyme analysis.

Substrates and enzymes were obtained from Boehringer, Mannheim, F.R.G.

Results

Specific activity of glycolytic enzymes of the cell homogenate

The determination of specific activities of glycolytic and marker enzymes in the homogenate was carried out to establish the ratio of membrane to homogenate enzyme activity. This finally allows us to estimate the percentage of specific activity associated with the cytoplasmic membrane side. The order of specific enzyme activities (Fig. 1) was 3-phosphoglycerate kinase > pyruvate kinase > D-glyceraldehyde-3-phosphate dehydrogenase > phosphofructokinase > aldolase > hexokinase. The

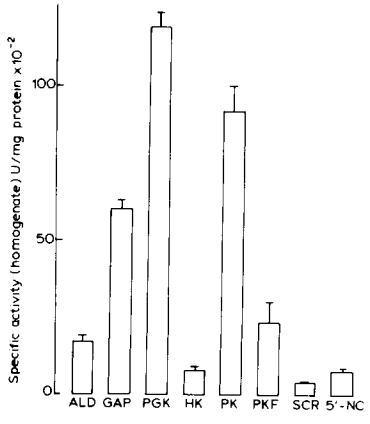

Fig. 1. Specific activities of different glycolytic enzymes and marker enzymes, measured in the homogenate of C6 glioma cells. ALD, aldolase; GAP, D-glyceraldehyde-3-phosphate dehydrogenase; PGK, 3-phosphoglycerate kinase; HK, hexokinase; PK, pyruvate kinase; PFK, phosphofructokinase; SCR, succinate-cytochrome-*c* reductase, 5′-NC, 5′-nucleotidase.

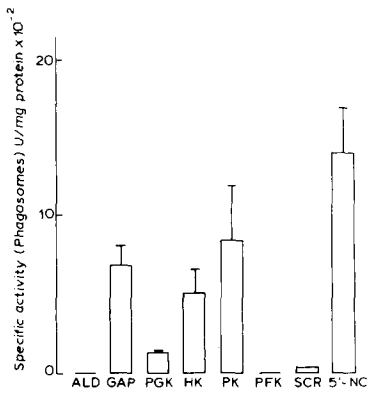

Fig. 2. Specific activities of different glycolytic enzymes and marker enzymes, measured at the inside-out-orientated plasma membranes of C6 glioma cells. Abbreviations as in Fig. 1 legend.

hexokinase is listed at the end, being 14-times less active in the homogenate than the 3-phosphoglycerate kinase. Compared to the above glycolytic enzymes, the marker enzymes 5′-nucleotidase and succinate-cytochrome-*c* reductase exhibit the lowest activity of all enzymes measured.

Glycolytic enzymes associated with the cytoplasmic surface

These data document that aldolase and phosphofructokinase were not detectable at the cytoplasmic side of the plasma membrane, thus differing from results being published by others on red cell membranes or F-actin in muscle (see Discussion). Although in the given order pyruvate kinase > D-glyceraldehyde-3-phosphate dehydrogenase > hexokinase > 3-phosphoglycerate kinase, hexokinase was listed after D-glyceraldehyde-3-phosphate dehydrogenase, the difference between pyruvate kinase, with the highest activity, and hexokinase, is quite small ($8.4 \cdot 10^{-2}$ vs. $5.1 \cdot 10^{-2}$ U/mg protein), compared to the homogenate. As expected for a plasma membrane marker enzyme, the 5′-nucleotidase exhibited the highest activity of all the enzymes listed.

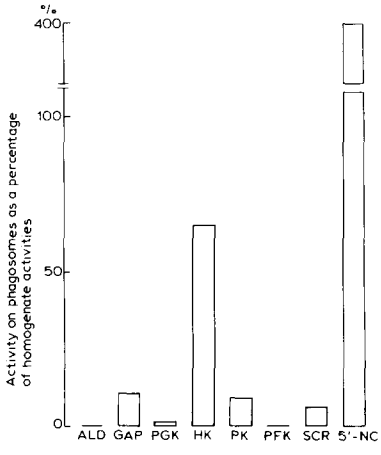

Fig. 3. Specific activities of different glycolytic enzymes and marker enzymes on phagosome membranes, shown as percentage of the homogenate specific activities. Abbreviations as in Fig. 1 legend.

Taking into account the ratio of membrane to homogenate specific activity of all the glycolytic enzymes (Fig. 3), the hexokinase is the enzyme which (on the basis of homogenate specific activity) has, by 65%, the highest degree of association with the cytoplasmic surface of the plasma membranes of C6 glioma cells. Furthermore, this pattern clearly documented that those enzymes with the highest homogenate activity, like D-glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase, are listed far behind the hexokinase, with 11 and 9%, respectively.

Evaluation of contaminating factors

In order to calculate the influence contamination might have on the results, original latex beads were added to the cell homogenate and after collecting them by the same procedure as described for internalised beads, were measured to obtain the enzyme activity. Aldolase and phosphofructokinase showed no affinity for the polystyrene surface, as no activity was detectable, whereas the remaining glycolytic enzymes were found in the following order of activity: pyruvate kinase > D-glyceraldehyde-3-phosphate dehydrogenase > hexokinase > 3-phosphoglycerate kinase. From the two marker enzymes, 5′-nucleotidase was bound to a small extent only to 'nude' compared to 'membrane' beads ($0.7 \cdot 10^{-2}$ vs. $14 \cdot 10^{-2}$ U/mg protein). In contrast, latex particles bind succinate-cytochrome-*c* reductase much better (i.e., 39% of homogenate activity). This means that the very low activity of succinate-cytochrome-*c* reductase found associated with plasma membranes pointed to a minute contamination either by homogenate enzymes adsorbed to 'nude' particles or

by mitochondrial fractions sticking to membrane-surrounded beads.

Enzyme pattern after correction for contamination

To avoid misinterpretation of the raw data, it seemed reasonable to consider the main location of hexokinase activity within the cell. It is known essentially from Wilson's laboratory that the predominant part of hexokinase is on the outer mitochondrial membrane [12]. Succinate-cytochrome-*c* reductase activity as a marker for mitochondrial contamination was found to be extremely low in our membrane preparation, thus excluding a major degree of hexokinase from mitochondria. This was confirmed by electron microscopy, which did not detect mitochondrial structures with these membranes (pictures not shown). Taking into account the succinate-cytochrome-*c* reductase ratio of 'membrane' to 'nude' beads (i.e., 15%), and considering that hexokinase activity on 'nude' beads was 1.5-times higher than membrane activity, the particular part of hexokinase associated with the cytoplasmic surface still remained remarkably high and amounted to 42% of specific homogenate activity ($15\% \times 1.5 = 23\%$, $65\% - 23\% = 42\%$). The following comparison shows that, even after correction for contamination, the three glycolytic enzymes that are associated with the plasma membrane of C6 glioma are hexokinase, 42%, D-glyceraldehyde-3-phosphate dehydrogenase, 10%, pyruvate kinase, 8%, and 3-phosphoglycerate kinase, 1% (percentage of specific homogenate activity).

Discussion

Taking advantage of previous findings that C6 glioma cells and primary astrocytes are able to phagocytose exogenous material [9,13], plasma membranes with inside-out orientation could be obtained by phagocytosis of polystyrene beads [7,8]. Previous studies [6,7], complemented by our data, indicate that contamination of this preparation with proteins of internal membranes is rather low. The fusion of phagosomes with lysosomes was minimised by shortening the incubation period to 1 h which, on the other hand, has proved to be long enough to receive an adequate membrane yield. During the subsequent gradient centrifugation, cytoplasmic components and loosely attached membrane fractions are released from the 'membrane' beads, while moving upwards through the 20% sucrose layer, whereas other cell constituents remained pelleted on the bottom of the centrifugation tubes. A further advantage is that these particles can easily be sedimented with a Beckman minifuge (i.e., $8000 \times g$), and that the membrane surface area can be determined exactly.

Studying the occurrence of glycolytic enzymes, especially of hexokinase, on the cytoplasmic surface of plasma membrane, one requires a rough estimation of the extent to which internal membranes like mitochondria can influence the results. Therefore, after contact with cell homogenate, beads without membranes were measured for enzyme activities, since soluble proteins stick to polystyrene with different affinities. Within our membrane preparations, such contamination by 'nude' beads has been reduced as far as possible by a further phagocytosis period after removal of the particle suspension, the use of trypsin to release beads still attached to the cell surface, and the many rinsing and centrifugation steps. In order to calculate contamination still remaining, we measured the mitochondrial marker enzyme succinate-cytochrome-*c* reductase. This enzyme seems to be especially useful, as it binds to polystyrene with high affinity, thus being a sensitive marker for contaminations by 'nude' particles and mitochondria membrane fractions, respectively. Therefore, the degree of contamination was estimated on the basis of the distribution of succinate-cytochrome-*c* reductase between 'nude' and 'membrane' beads.

Taking into account the fact that the succinate-cytochrome-*c* reductase activity of 'membrane' beads is $0.2 \cdot 10^{-2}$ U/mg protein, and $1.3 \cdot 10^{-2}$ U/mg protein on 'nude' particles, one can calculate that if succinate-cytochrome-*c* reductase exclusively stems from the latter, its part in the whole preparation amounts to 15% at the most. Considering this, about 40% of the specific hexokinase activity measured in the homogenate is still associated with the cytoplasmic surface. In comparison to hexokinase, only a small amount of D-glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and 3-phosphoglycerate kinase was found associated with the plasma membrane

cytoplasmic side. Surprisingly, the second key glycolytic enzyme phosphofructokinase and also aldolase were not detectable in this membrane preparation. From the literature, it is known that these enzymes are both bound to F-actin or actomyosin [14,15], and also to band 3 of the erythrocyte membrane [4,5]. In our preparation, however, no actin was detected by electrophoresis in SDS polyacrylamide gels (data not shown). It is assumed that actin filaments, which are part of a cytoskeletal weave-like structure, are located just beneath the plasma membrane and usually bind phosphofructokinase and aldolase. In contrast to conventional preparation techniques, those structures were not found in phagocytosed membranes. In addition, it cannot be excluded that the surface membrane becomes partly cleared of special proteins before being internalised [16]. This could explain the different enzyme pattern obtained with these membranes compared to others like red cell plasma membrane [4] and heart muscle sarcoplasmic reticulum [17], respectively.

Hexokinase, as pointed out, is bound in considerable quantities to the cytoplasmic side of the plasma membrane, thus confirming previous reports of other authors on cells like erythrocytes [3], hepatoma [18], intestinal mucosa [19–21] and Ehrlich ascites carcinoma [22]. In the case of glial cells, the C6 glioma line belongs to a membrane-regulated [23] cell type characterised by a close coupling between glucose transport and phosphorylation [1,2]. The association of the first glycolytic key enzyme with the cytoplasmic surface, which causes an immediate phosphorylation in the vicinity of the glucose transporter, is the one basic requirement of the observed strict coupling between transport and phosphorylation in C6 glioma cells. There are suggestions that hexokinase may thus be an integral part of the glucose transmembrane transporter [24]. This was recently discussed by other authors in relation to transport-associated phosphorylation in adipocytes [25].

Acknowledgements

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