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Inhibition of the Mitochondrial Calcium Uniporter by Antibodies against a 40-kDa glycoprotein[T]

Nils-Erik L. Saris,[1] Tatjana V. Sirota,[2] Ismo Virtanen,[3] Kaija Niva,[1] Timo Penttilä,[1] Ludmila P. Dolgachova,[4] and Galina D. Mironova[2]

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Polyclonal rabbit antibodies against a Ca^{2+}-binding mitochondrial glycoprotein were found to inhibit the uniporter-mediated transport of Ca^{2+} in mitoplasts prepared from rat liver mitochondria. Spermine, a modulator of the uniporter, decreased the inhibition. This glycoprotein of M_r 40,000, isolated from beef heart mitochondria and earlier shown to form Ca^{2+}-conducting channels in black-lipid membranes, thus is a good candidate for being a component of the uniporter. Antibody-IgG was found to specifically bind to mitochondria in human fibroblasts.

KEY WORDS: Calcium transport; fibroblast; glycoprotein; heart; mitochondria; spermine; uniporter.

INTRODUCTION

The molecular species involved in the uptake of Ca^{2+} by animal mitochondria have not been unequivocably identified in spite of the fact that this transport process was discovered more than 30 years ago. Several laboratories have endeavored to elucidate the mechanism and to purify components of the transport system; see reviews by Saris and Åkerman (1980), Carafoli and Sottocasa (1984), and Gunter and Pfeiffer (1990). One likely candidate is a glycoprotein isolated from ox liver mitochondria in extracts obtained by hypotonic treatment (Lehninger, 1971, Sottocasa *et al.*, 1972), especially since antibodies against the protein specifically inhibited the uptake of Ca^{2+} by the ruthenium red-sensitive mechanism, the so-called uniporter (Panfili *et al.*, 1976; Sottocasa *et al.*, 1981). With improved purification, the protein—called calvectin—no longer contained sugar moieties (Panfili *et al.*, 1983). It was probably a dimer with an M_r for the monomer of about 14,000. Unfortunately, the antibodies are no longer available. Another candidate protein is a small hydrophobic protein—calciphorin—with ionophoretic properties (Jeng and Shamoo, 1980a), isolated by Jeng and Shamoo (1980b) and Ambudkar *et al.* (1984) from beef heart and rat liver mitochondria, respectively. Since attempts to reconstitute Ca^{2+} transport in artificial membrane systems with calvectin were unsuccessful (Prestipino *et al.*, 1976), it has been speculated that calvectin and calciphorin together might bring about Ca^{2+} transport (Carafoli and Sottocasa, 1984). Recently, a ruthenium red-sensitive Ca^{2+} uptake by proteoliposomes containing cytochrome oxidase and mitochondrial proteins with an $M_r > 35$ kDa was reported (Zazueta *et al.*, 1991). Ca^{2+} uptake by cytochrome oxidase vesicles has been reported before (Rosier and Gunter, 1980). Mironova *et al.* (1982) have isolated a Ca^{2+}-binding (Sirota *et al.*, 1987) mitochondrial glycoprotein by ethanol extraction. This protein shows ruthenium red-sensitive Ca^{2+}-channel properties in a black-lipid membrane system in the presence of the high concentrations of Ca^{2+} (5–10 mM) that have to

[1]Department of Medical Chemistry, University of Helsinki, SF-00170 Helsinki, Finland.
[2]Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Russia.
[3]Department of Anatomy, University of Helsinki, Helsinki, Finland.
[4]Institute of Theoretical and Experimental Biophysics, Academy of Sciences of Russia. Present address: Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Russia.

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be used in this system. The protein was reported to consist of a glycoprotein part and a small putative channel peptide that may be dissociated from it (Mironova and Utesheve, 1989). In the present communication it is shown that antibodies raised against this protein preparation inhibited the uniporter in a mitoplast preparation at micromolar concentrations of Ca^{2+}, at least under some conditions. The glycoprotein preparation thus is likely to contain a component of the calcium uniporter. This paves the way for a renewed attempt to characterize this transport system on the molecular level. Interest in this kind of protein has also been augmented by the recent finding of Igbavboa and Pfeiffer (1991) that an intermembrane component is involved in the regulation of the activity of the uniporter.

MATERIALS AND METHODS

Rat liver mitochondria were prepared by a conventional procedure and mitoplasts by digitonin treatment of mitochondria (Schnaitman and Greenawalt, 1968). The glycoprotein was prepared as described by Mironova *et al.* (1982). Antiserum against the glycoprotein was produced in rabbits by intramuscular injection in the hind leg of 0.5 mg glycoprotein mixed with Freund's complete adjuvant. After a week 0.2 mg antigen plus Freund's complete adjuvant was injected into the popliteal lymphoglandula of the hind leg. This procedure was repeated 3 weeks later. After 2 weeks 0.1 mg was given intravenously. The IgG fraction of antiserum giving a precipitate by the Ouchterlony immuno-diffusion test was obtained by diluting the antiserum 1 : 1 by 155 mM NaCl, 10 mM phosphate, pH 7.4 (PBS), then absorbing the IgG to a Prosep™-A column (Protein A affinity absorbent, Bioprocessing Ltd., England). The column was washed with PBS and the IgG eluted with 0.1 M glycine-HCl, pH 3.0. The A_{280} of the collected fractions were measured and the IgG-containing fractions pooled and dialyzed against PBS and then the incubation medium before concentration by ultrafiltration. Dr E. Panfili, University of Trieste, Italy, kindly provided a crude and a purified sample of calvectin. Changes in the activity of Ca^{2+} in the medium were reported by a calcium-sensitive electrode (Radiometer A/S, Copenhagen, Denmark) coupled to a pH meter (Saris and Allshire, 1989). Ca^{2+} transport was also followed with the radioisotope method (^{45}Ca was obtained from The Radiochemical Centre, Amersham, U.K.), counting

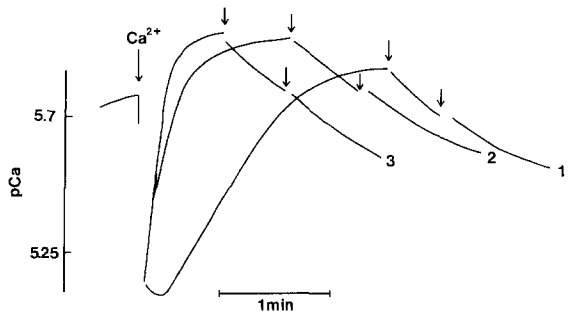

Fig. 1. Inhibition of the uniporter but not ruthenium red-insensitive Ca^{2+} fluxes by antiglycoprotein antibodies. Mitoplasts, 0.82 mg protein, were incubated with 0.54 mg antibody-containing IgG for 1 h at 0°C in a volume of 115 μl. For the measurement of Ca^{2+} transport by the calcium-sensitive electrode technique, the mitoplasts were then transferred to the incubation medium with a final volume of 2.5 ml. The medium contained 200 mM sucrose, 20 mM KCl, 10 mM Hepes, pH 7.4, 1 mM KH_2PO_4, 2 mM succinate, and 6 μM rotenone. Where indicated, 5 μM Ca^{2+} was added, and when its uptake was completed, 1 μM ruthenium red was added (first arrow), followed by the addition of 10 mM Na^+ (last arrow). In trace 1, Ab-containing IgG was used during the preincubation, trace in 2, control IgG, and in trace 3, medium alone.

the radioactivity of mitochondria retained on 0.45 μm pore-size filters. The content of divalent cations of the reagents was reduced by passing through a CHELEX column. For indirect immunocytochemistry, cultured human fetal fibroblasts were seeded on small coverslips. The cells were obtained from a local source and were cultured in RPMI1640 medium (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (Gibco) and antibiotics. Then subconfluent cultures were fixed with methanol, cooled to −20°C, and washed with PBS. Then the rabbit antiserum was applied in different dilutions on the specimens. An intense reaction was obtained with dilutions up to 1:3200–1:6400. After rabbit antiserum the coverslips were washed and reacted with fluorescein isothiocyanate-coupled sheep anti-rabbit antiserum (1:150, Organon Teknika, Cappel Laboratories, West Chester, Pennsylvania). After washing, the coverslips were mounted and examined in a light Aristoplan microscope with oil immersion optics.

RESULTS

Inhibition of the Mitochondrial Calcium Uniporter by Antiglycoprotein Antibodies

Figure 1 shows a clear inhibition of Ca^{2+} uptake by mitoplasts that had been incubated with IgG con-

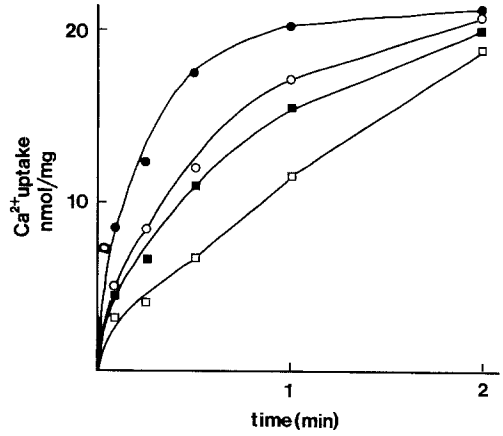

Fig. 2. Inhibition of mitoplast Ca^{2+} uptake by varying amounts of glycoprotein antibodies. Mitoplasts, 0.57 mg protein, were incubated for 1 h at 0°C with 0.14 (open circles), 0.28 (filled squares), and 0.57 mg (open squares) IgG purified from antiserum against the mitochondrial glycoprotein. In the control (filled circles) incubation was with medium alone. The medium was as in Fig. 1. The experiment were started by addition of 5 μM Ca^{2+} labeled with ^{45}Ca, and at the points of 400-μl samples were removed for Millipore filtration and counting.

taining glycoprotein antibodies in comparison with mitoplasts incubated with plain medium or control IgG not containing antibodies. The inhibition in this experiment using a calcium-sensitive electrode was about 80%. The uptake trace in the presence of control IgG deviates slightly in its upper part from that of control mitoplasts incubated in medium alone. This could be due to slight inhibition of uptake by IgG as reported by Panfili *et al.* (1976). There was no inhibition of ruthenium red-insensitive efflux of Ca^{2+}.

In Fig. 2 the amount of antibody-containing IgG was varied. The highest amount of IgG used inhibited Ca^{2+} uptake by about 65%.

The Effect of Spermine on the Inhibition of the Uniporter by Glycoprotein Antibodies

Spermine is a potent stimulator of the uniporter (Nicchitta and Williamson, 1984; Allshire and Saris, 1986; Kröner, 1988) at low concentrations of Ca^{2+} while it is inhibitory at higher. Since the mitochondrial glycoprotein could be involved in the regulation of the uniporter activity (Igbavboa and Pfeiffer, 1991), we studied the effect of the antibodies on the activation of the uniporter by spermine. In Fig. 3A it is seen that there was a clear inhibition of uptake of the small amounts of Ca^{2+} present endogenously by the antibodies in the absence of spermine. In the presence of spermine, the inhibition by the antibodies was largely overcome. Uptake of an added aliquot of 5 μM Ca^{2+} is shown in Panel B. In the control the mitoplasts were able to lower the setpoint for Ca^{2+} to a level below the initial level (pCa is above the level before the addition), presumably as a result of the stimulation of the uniporter activity by Ca^{2+} itself (Saris and Kröner, 1990). This stimulation is not seen in the presence of antibodies. In the presence of 0.5 mM spermine, the uptake of added Ca^{2+} was clearly inhibited and no inhibition by antibodies is discernible. At this level of Ca^{2+} load, spermine inhibited the uptake and no further inhibition was obtained by the antibody treatment.

The Specificity of the Antiglycoprotein Antibodies

In an Ouchterlony immunodiffusion test a single sharp precipitation line was formed by the antibodies [corresponding to 10 μl antiserum and the purified glycoprotein (4 μg)] while no precipitation occurred (data not shown) with the purified or the crude preparation of hypotonically extracted protein (25 μg) described by Sottocasa *et al.* (1972). The antibodies have been reported to be species and organ-unspecific (Dolgachova and Scarga, 1984).

In cultured human fibroblasts the rabbit antiserum against the mitochondrial glycoprotein gave a bright cytoplasmic reactivity (Fig. 4). The immunoreaction was confined solely to wormlike fibrillar structures both close to the nucleus but also in the peripheral part of the cells. This pattern is typical for mitochondria as reported earlier (Johnson *et al.*, 1980; Amchenkova *et al.*, 1988).

DISCUSSION

The Nature of the Proteins Involved in Uniporter-Mediated Mitochondrial Ca^{2+} Transport

Mironova *et al.* (1982) have shown that the 40-kDa glycoprotein isolated from beef heart mitochondria is able to form ruthenium red-sensitive Ca^{2+} channels in a black-lipid-membrane system in the presence of 5–10 mM Ca^{2+}. The channel-forming part of it was a component with a low molecular weight. It probably is a peptide since proteinase treatment inhibits its Ca^{2+}-transporting activity. The finding that antibodies raised against this protein preparation inhibit the uniporter activity of mitoplasts at micromolar concentrations of Ca^{2+} (Figs. 1–3) demonstrates that we are dealing with a protein involved in the uniporter activity.

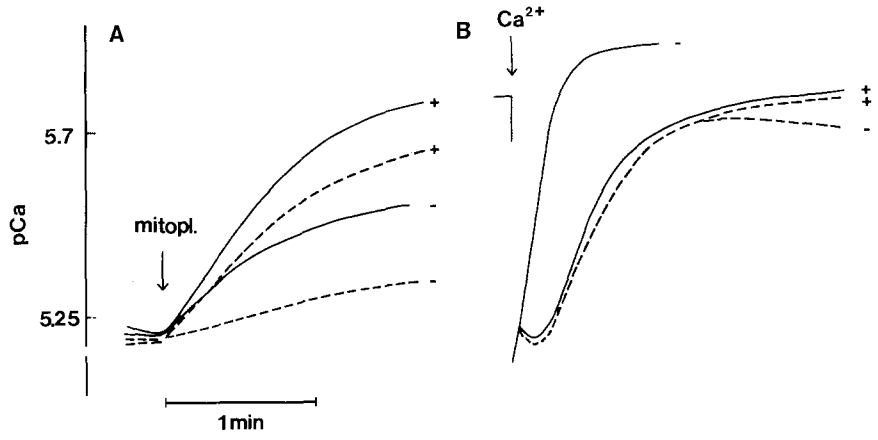

Fig. 3. Inhibition of spermine-stimulated Ca^{2+} uptake by glycoprotein antibodies. Experimental conditions were as in Fig. 1, but the Ca^{2+} uptake was reported by a Ca-sensitive electrode. Mitoplasts, 0.95 mg, were incubated with antibody-containing IgG, 0.54 mg (broken lines), or 0.54 mg control IgG (continuous lines), and used in experiments at a mitoplast concentration of 0.38 mg protein/ml. +, 0.5 mM spermine present; −, without spermine. In A, the experiment was started by addition of the mitoplast preparation. After the completion of uptake, an addition of 5 μM Ca^{2+} was made in B.

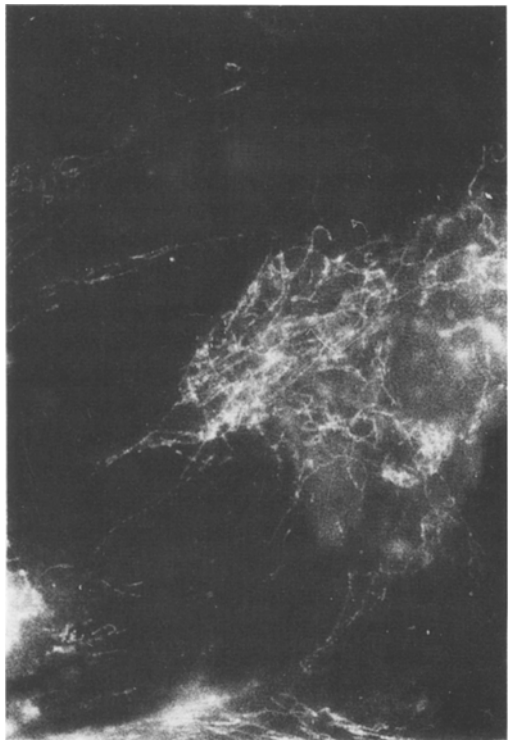

Fig. 4. The localization of the glycoprotein in cultured human fibroblasts. The immunocytofixation of the glycoprotein antibodies and their visualization was carried out as described in the Materials and Methods section. The threadlike structures are mitochondria.

Glycoproteins have been implicated as components of the uniporter in mitochondria isolated from various mammalian tissues (see reviews by Saris and Åkerman 1980; Carafoli and Sottocasa, 1984). The M_r of the glycoprotein from different sources varied. This could at least partly be due to the presence of varying amounts of lipids and carbohydrate in the preparations. Panfili *et al.* (1976) purified a protein from ox liver mitochondria which they first believed to be a glycoprotein but finally obtained a preparation that was devoid of sugar (Panfili *et al.*, 1983). Since antibodies raised against this protein inhibited the uniporter, and the Ca^{2+}-binding properties of the protein were identical to those of their previous glycoprotein, these authors concluded that the sugars were contaminants, possibly glycolipids, removable by acetone extraction. Acetone did not remove the sugar moieties from the presently studied protein (data not shown) that thus is a genuine glycoprotein. It is of interest that the mitochondrial outer and inner membranes contain distinct systems for the synthesis of N-linked glycoconjugates via the dolicholpyrophosphate pathway (Levrat *et al.*, 1989). The inner membrane, in addition, is able to transfer sugar moieties from sugar nucleotides directly to endogenous protein acceptors (Levrat *et al.*, 1990). Like the "glycoprotein" of Panfili *et al.* (1976), our glycoprotein (Mironova *et al.*, 1982) contains a large proportion of acidic amino acids, but there are clear differences in the reported amino acid composition of these proteins

(Carafoli and Sottocasa, 1974). The inability of the antibodies raised against our glycoprotein to precipitate calvectin also indicates that we are dealing with separate proteins. Still, the presence in our antiserum of nonprecipitating antibodies that could react with calvectin cannot be excluded.

It is of interest that Mironova *et al.* (1982) found a small peptide to be formed from the glycoprotein and that this peptide was the channel-forming component. It is tempting to speculate that it could be the equivalent of calciphorin—a small hydrophobic mitochondrial polypeptide with an M_r of 3000—reported to have ionophoretic properties in being able to transfer Ca^{2+} to an organic phase (Jeng and Shamoo, 1980a,b; Ambudkar *et al.*, 1984). However, a phospholipid preparation from rat liver mitochondria has been reported to behave as calciphorin, which casts doubt upon calciphorin as a component of the uniporter (Sokolove and Brenza, 1983). It was then shown by the laboratory of Shamoo that delipidated calciphorin still exhibited high-affinity Ca^{2+}-binding, it was binding the Ca^{2+} analog Eu^{3+} tightly and that this complex exhibited fluorescence distinct from those of phospholipid-Eu^{3+} complexes (Herrmann *et al.*, 1984). Our peptide, however, is a channel-forming one, which would be appropriate for the uniporter that has features compatible with a channel mechanism (Saris and Åkerman, 1980), especially a fast-gated pore (Gunter and Pfeiffer, 1990).

Is the Glycoprotein Involved with the Regulation of the Uniporter Activity?

It is well known that the uniporter activity can be modulated by both divalent cations and polyamines; see reviews by Saris and Åkerman (1980), Carafoli and Sottocasa (1984), and Gunter and Pfeiffer (1990). The finding that a mitochondrial intermembrane component is involved in the regulation of the uniporter (Igbavboa and Pfeiffer, 1991) is of interest in this context. The antibodies inhibit the uniporter under some but not all conditions; see Fig. 3. Thus, modulators, like spermine, seem to change the extent of inhibition. The antibodies may conceivably interact with a regulatory component.

CONCLUSION

The inhibition of the mitochondrial uniporter by antibodies against a mitochondrial glycoprotein or protein complex together with the earlier finding that this complex or a peptide derived from it creates ruthenium red-sensitive Ca^{2+} channels in black-lipid membranes indicates that these proteins indeed are components of or regulate the uniporter. An effort can now be made to characterize the molecular species involved in greater detail and to endeavor to elucidate their molecular biology.

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