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RIBONUCLEOTIDE REDUCTASE: EVIDENCE FOR SPECIFIC ASSOCIATION WITH HeLa CELL MITOCHONDRIA

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Mammalian mitochondria contain pools of deoxyribonucleoside 5'-triphosphates that behave differently from the much larger whole-cell pools. To investigate the origins of these pools, we analyzed HeLa cell mitochondria for ribonucleotide reductase activity. Three findings suggest specific association of a reductase with mitochondria: (1) enzyme activity in extracts of washed mitochondria, (2) stimulation of that activity by dATP at levels inhibitory to the major cellular activity, and (3) association of immunoreactive material with washed and fractionated mitochondria.

What are the capacities of mitochondria for synthesis of the deoxyribonucleotide precursors to mitochondrial DNA? Earlier studies from this laboratory (1,2) showed that HeLa cell mitochondria contain pools of deoxyribonucleoside triphosphates (dNTPs) representing up to ten percent of the total intracellular dNTP pools, even though mitochondrial DNA represents but one percent of the total DNA in these cells. In various ways these pools behaved differently from the whole-cell pools, suggesting that they are synthesized by a distinct pathway, perhaps within the mitochondrion itself. Mammalian mitochondria contain a

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number of deoxyribonucleoside kinases (3), indicating that salvage routes to dNTPs are localized within these organelles. A growing body of evidence implicates mitochondrial DNA metabolism as a target for cytotoxic effects of antiviral nucleoside analogs (3). Therefore, further information about both salvage and *de novo* synthetic routes to dNTPs in mitochondria is desirable. One of us (4) had obtained evidence for the existence of thymidylate synthase in mammalian mitochondria. Accordingly, we decided to analyze mitochondria for the first enzyme of *de novo* dNTP synthesis—ribonucleoside diphosphate (rNDP) reductase.

MATERIALS AND METHODS

Mitochondria for enzyme assay were isolated from suspension cultures of HeLa S3 cells. Cells were centrifuged and resuspended in about six cell volumes of hypotonic buffer (10 mM Tris-HCl, pH 6.7, 10 mM KCl, and 0.15 mM $MgCl_2$). After five minutes' incubation on ice, the cells were lysed in a Dounce homogenizer and the suspension centrifuged at low speed to remove unbroken cells. Mitochondria were pelleted by 15 minutes of centrifugation at 27,000 x g and resuspended in 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 0.15 mM $MgCl_2$. Following a repetition of this differential centrifugation procedure, the mitochondria were washed by three successive suspensions in, and centrifugation from, a buffer containing 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 3 mM EDTA. This procedure was monitored by following activities of citrate synthase and cytochrome c oxidase.

Mitochondria for immunoassay were prepared from monolayer cultures grown in MEM medium supplemented with glutamine, penicillin/streptomycin, and 5% fetal calf serum. When cultures had attained 75% confluency, they were treated with trypsin and replated, at about 60% confluency. Twenty four hours later plates were aspirated and washed with calcium- and magnesium-free phosphate-buffered saline (PBS). Cells were removed either by trypsin treatment or by scraping with a rubber policeman, centrifuged, resuspended in three cell volumes of PBS, and stored frozen. After thawing, cells were centrifuged and suspended in one cell volume of 10 mM HEPES-NaOH, pH 7.4, 0.25 M sucrose, 1 mM EGTA, and 0.5% bovine serum albumin. Lysis was accomplished with 8 strokes in a Teflon homogenizer, and mitochondria were purified essentially as described above.

Ribonucleotide reductase was assayed with CDP as the substrate, as previously described (5). Assays were conducted both on crude extracts and on ammonium sulfate precipitates obtained after adjusting extracts to 45% of saturation.

Immunological experiments used the AD203 monoclonal antibody to the R1 (large) subunit of mouse rNDP reductase (6). This was purchased from

InRo Biomedtek. Polyclonal antiserum to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was a gift from Dr. Ann Brodie of this department.

Sucrose gradients were prepared discontinuously, by layering into Beckman SW41 rotor centrifuge tubes solutions of 1 mM EDTA, 0.1% bovine serum albumin, 10 mM HEPES, pH 7.4 containing, respectively, 2.0, 1.6, 1.3, and 1.0 M sucrose. Gradients were frozen and then thawed overnight at 4° C. Mitochondria were layered on, and centrifugation was carried out for 2 hours at 80,000 x g and at 5° C. Fractions (0.5 ml) were collected; diluted with two volumes each of 1 mM EDTA and 10 mM HEPES, pH 7.4; centrifuged for 20 minutes at 16,000 x g to pellet mitochondria; and the washing procedure repeated once more before immunassay and citrate synthase activity assay of each fraction.

RESULTS AND DISCUSSION

Our first experiments involved simply the preparation and assay of crude, cell-free extracts of HeLa cells and of washed mitochondria. As shown in Figure 1A, mitochondrial extracts had about three percent of the rNDP reductase activity seen in whole-cell extracts, on a per-cell basis. While the activity seen in mitochondria could represent contamination of the mitochondria with cytosol, the mitochondrial activity has a property distinctly different from that assayed in whole-cell extracts. As shown in Figure 1B, the allosteric inhibitor, dATP, showed significant *stimulation* of the mitochondrial activity, at concentrations yielding nearly complete inhibition of the activity in whole-cell extracts. This observation was reproducible. We attempted to study this difference in extracts from which endogenous nucleotides had been removed, by prior ammonium sulfate fractionation of the extracts. Recoveries of the mitochondrial activity were low in this experiment, about one percent of the activity of corresponding whole-cell preparations (data not shown). This was comparable to the level of lactate dehydrogenase that we assayed in the same preparations, as an index of cytoplasmic contamination. However, the mitochondrial reductase activity, while very low, was still stimulated by dATP (data not shown).

Results of the above experiments, therefore, were equivocal; lactate dehydrogenase assays suggested that the mitochondrial activity represented cytosolic contamination, while dATP stimulation of the mitochondrial activity suggested the presence of a novel ribonucleotide reductase in mitochondria. Therefore, we turned to a more extensive series of experiments involving mitochondria washed and fractionated

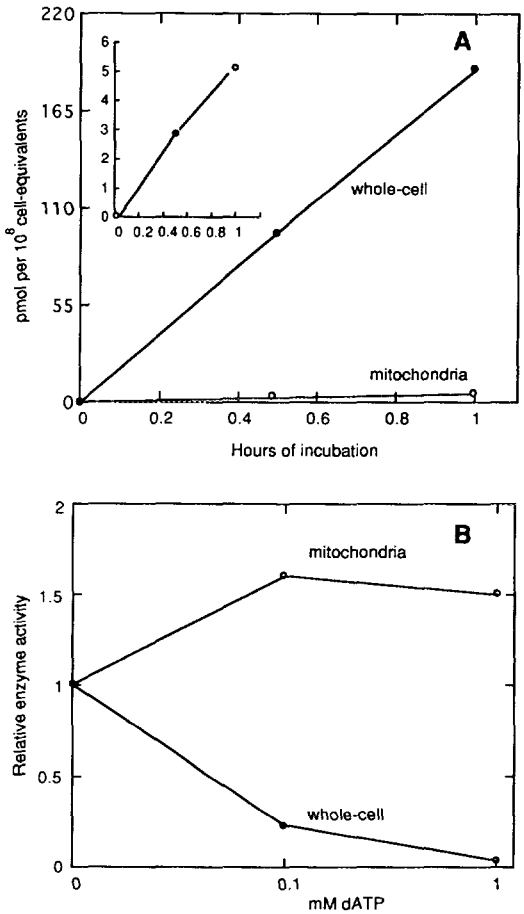

Figure 1. CDP reductase activity in HeLa cell mitochondria. A, time course of reactions in crude extracts of whole cells of washed mitochondria. Data for the mitochondrial extract are replotted in the inset. B, effect of dATP on enzyme activity in crude extracts.

in various ways. These experiments could have been difficult, because of the laborious nature of the enzyme assay at the low activities seen. However, we were aided by our finding that mitochondrial preparations contained material immunoreactive with a monoclonal antibody shown elsewhere (6) to be highly specific for the R1 subunit of mammalian rNDP reductase. Therefore, we used immunoblotting to detect and partially quantitate R1 in our preparations. Parallel immunoblotting experiments were done with a polyclonal antiserum against glyceraldehyde-3-phosphate dehydrogenase. G3PDH is a cytosolic protein that binds nonspecifically to membranes and organelles (7), so it was used as a control for non-specific association with mitochondria and other membranous structures.

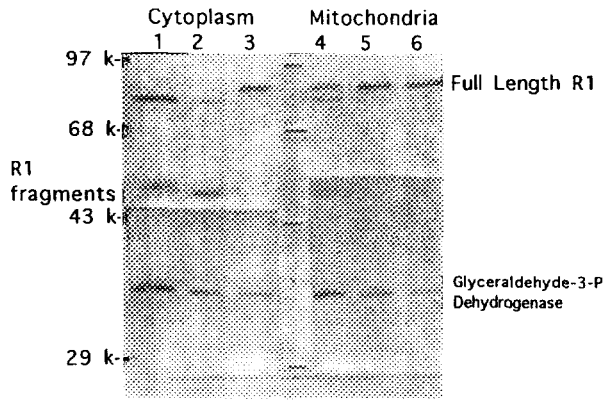

Figure 2. Detection of rNDP reductase R1 protein in mitochondria by immunoblotting. A 7.5% SDS polyacrylamide gel was used to resolve proteins in cytoplasmic (lanes 1–3) or mitochondrial (lanes 4–6) extracts. Molecular weight marker proteins were resolved in the lane between 3 and 4. After electrophoresis and transfer of the proteins to nitrocellulose, the blot was probed with AD203 anti-R1 monoclonal antibody. Following this, the bottom part of the filter, which was blank, was cut out and reprobed with polyclonal antiserum against glyceraldehyde-3-phosphate dehydrogenase. The band representing G3PDH is identified on the figure. All other bands represent protein species immunostained by the AD203 anti-R1 antibody. Lanes 1 and 4, extracts from cells detached by trypsin. Lanes 2, 3, 5, and 6, similar to 1 and 4, respectively, but cells were detached from plates by mechanical scraping instead of by trypsin treatment. In this experiment a cell homogenate was divided into two aliquots. To one was added the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). To the other was added 0.1 mg/ml of trypsin. After 15 minutes of incubation further proteolysis was inhibited by addition of PMSF. Each homogenate was fractionated, and cytoplasmic and mitochondrial proteins were analyzed as shown. Lane 2, cytoplasmic proteins from trypsin-treated homogenate; 3, cytoplasmic proteins from PMSF-treated homogenate; 5, mitochondrial proteins from trypsin-treated homogenate; 6, mitochondrial proteins from PMSF-treated homogenate.

Analysis of cytosolic and mitochondrial extracts suggested the presence in mitochondria of two immunoreactive polypeptides—one near the molecular weight of the major cytosolic R1 polypeptide and one of slightly higher molecular weight (Figure 2, lanes 1 and 4). However, we found the lower-molecular-weight species to represent an artefact of the trypsin treatment used to release cells from the culture dishes. When cells were detached instead by mechanical scraping, all of the immunoreactive material seen, both in cytosol and in mitochondria, was the higher-molecular-weight species (lanes 3 and 6). This finding suggested that the presence of two immunoreactive species in mitochondria from trypsinized cells represented (1) a cytosolic

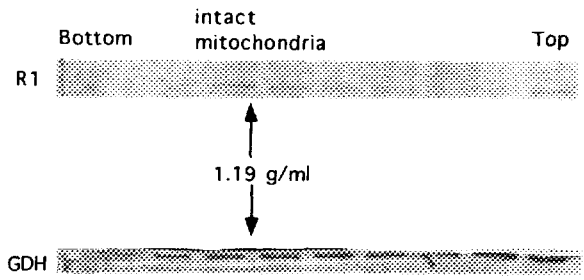

Figure 3. Specific association of R1-immunoreactive material with HeLa cell mitochondria. A crude mitochondrial preparation was subjected to sucrose gradient centrifugation. Fractions enriched in mitochondria were identified by refractometry (density of mitochondria is 1.19 g/ml) and by citrate synthase assay of each fraction (data not shown). Each fraction was analyzed by immunoblotting for R1 and for G3PDH, as shown.

contaminant, which was accessible to the trypsin and was partially degraded during or after detachment; and (2) an "intramitochondrial" form of the enzyme, protected from trypsin proteolysis by its specific association with mitochondria. This supposition was tested by treating a HeLa cell homogenate from scrape-detached cells with trypsin *in vitro*, followed by fractionation and separate analysis of cytosolic and mitochondrial proteins. As shown in lanes 2 and 5, this treatment converted all of the cytosolic R1 protein to the lower-molecular-weight species, while the immunoreactive protein in mitochondria was completely protected.

As is apparent from Figure 2, mitochondrial extracts, whether from trypsin-treated or scrape-detached cells, contained significant contamination, as judged by the presence of G3PDH-immunoreactive material (although scraped cells showed much less of this contamination than did trypsin-treated cells). It became important to determine whether the RNR-immunoreactive material was more specifically associated with mitochondria than was G3PDH. To this end, we further purified mitochondria by sucrose gradient centrifugation. The presence of mitochondria in this region of our gradients was confirmed by citrate synthase assays (data not shown). As shown in Figure 3, R1-reactive material was found both at the top of the gradient (presumably representing cytosolic contamination) and in those fractions containing intact mitochondria. By contrast, G3PDH-reactive material was distributed throughout the gradient. By this criterion, therefore, ribonucleotide reductase is more closely associated with mitochondria than is the control protein, glyceraldehyde-3-phosphate dehydrogenase.

Experiments comparable to those depicted in Figures 2 and 3 have been repeated many times. In general, our recoveries of mitochondria were quite low. However, semiquantitative analysis of immunoblots indicates, as does Figure 2, that the proportion of R1-reactive material in mitochondrial protein is comparable to that in cytosol. The observation that this material is protected from degradation by external proteases suggests that the enzyme is, in fact, specifically associated with mitochondria. Since the preliminary analysis shown in Figure 1 suggests that the mitochondrion-associated enzyme has distinctive feedback control properties, and since our earlier studies (1,2) showed that dNTP pools in mitochondria are regulated differently from whole-cell pools, analysis of the enzyme in a system allowing greater recovery of mitochondria now becomes an important goal.

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