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Isolation of functional pure mitochondria by superparamagnetic microbeads

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ABSTRACT

Isolation of mitochondria by current methods relies mainly on their physicochemical properties. Here we describe an alternative approach to obtain functional mitochondria from human cells in a fast, reproducible, and standardized procedure. The new approach is based on superparamagnetic microbeads conjugated to anti-TOM22 antibody. The bead conjugates label the cytoplasmic part of the human mitochondrial membrane protein TOM22 and, thus, allow for a gentle isolation of mitochondria in a high gradient magnetic field. By comparing the MACS (magnetic cell separation) approach with mitochondria isolation methods using differential centrifugation and ultracentrifugation we demonstrate that the MACS approach provides the highest yield of isolated mitochondria. The quality, enrichment, and purity of mitochondria isolated with this protocol are comparable to mitochondria obtained using the ultracentrifuge method, and a typical separation procedure takes only approximately 1 to 2 h from initial cell homogenization. Mitochondria isolated with the new approach are sufficient for protein import, blue native gel electrophoresis, and other mitochondrial assays.

Mitochondria play a central role in many cellular functions, including bioenergetics, apoptosis, and the metabolism of lipids, iron, nucleotides, and amino acids. As mounting evidence has implicated mitochondria as key participants in degenerative diseases [1,2] and aging [3], mitochondria have become the subject of intense study in numerous fields, including biomedical research, drug discovery, and proteomics.

Because experimental conditions can be precisely controlled in isolated mitochondria compared with intact cells or tissues, isolated organelles provide a unique tool to investigate not only mechanisms of apoptosis, reactive oxygen species (ROS)[2] production, and biogenetics but also mitochondrial DNA (mtDNA), mitochondrial RNA (mtRNA), and mitochondrial protein synthesis. Among various approaches for mitochondria purification, differential centrifugation (DC) is the most frequently used protocol due to its quick and inexpensive nature. This method, developed during the 1950s [4] and modified in many ways [5–8], provides a mitochondrial fraction in which integrity and functionality of the organelle are maintained. Other purification protocols that also result in highly pure mitochondrial fractions, such as preparation of an isopycnic gradient followed by ultracentrifugation (UC) [9,10], are time-consuming and expensive and require access to an ultracentrifuge.

Here we describe a fast and easy method to obtain pure functional mitochondria with high yield from human cultured cells. The new method is based on magnetic sorting, which was originally developed for the separation of cells [11] but has also been successfully used to purify cell compartments such as Golgi vesicles [12], endosomes [13], lysosomes [14], nuclei [15], and plasma membranes [16]. Here we compared the new approach, MACS (magnetic cell separation), with the DC and UC methods by testing the organelles' performance using different assays for purity and function.

Materials and methods

Mitochondria isolation with superparamagnetic microbeads

Anti-TOM22 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were developed for the magnetic isolation of mitochon-

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[2] *Abbreviations used:* ROS, reactive oxygen species; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; DC, differential centrifugation; UC, ultracentrifugation; MACS, magnetic cell separation; TOM22, 22-kDa translocase of outer mitochondrial membrane; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; IgG1, immunoglobulin G1; APC, allophycocyanin; FACS, fluorescence-activated cell sorter; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TFAM, mitochondrial transcription factor A; RCR, respiratory control ratio; COXI, cytochrome *c* oxidase subunit I; BN–PAGE, blue native gel electrophoresis.

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dria from human cells. Microbeads are a colloidal suspension of extremely small (50 nm diameter) superparamagnetic particles. Conjugated to a monoclonal anti-TOM22 antibody, the microbeads bind specifically to the 22-kDa translocase of outer mitochondrial membrane (TOM22) on the surface of human mitochondria and, thereby, magnetically label the organelles. The labeled mitochondria can then be efficiently isolated in the magnetic field of a MACS Separator.

Different human cell lines were harvested at approximately 90% confluency and washed twice with phosphate-buffered saline (PBS). Cells (1×10^7) were lysed in 1 ml of ice-cold PBS, including Complete Protease Inhibitor Cocktail Tablets (Roche, Germany), by shearing through a needle approximately 20 times. With respect to different cell sizes, different needle diameters were used for cell disruption: 26G for 293 HEK cells, 29G for HeLa cells, and 27G for osteosarcoma cells. Glass homogenizers were employed for large numbers of cells. After cell disruption, an aliquot of the lysate was examined for trypan blue exclusion to ensure that 80% of the cells were lysed. For magnetic labeling, the crude cell lysate was incubated with 25 µl of anti-TOM22 MicroBeads for 15 to 60 min at 4 °C. Subsequently, the suspension was loaded onto a preequilibrated MACS Column (Miltenyi Biotec), which was placed in the magnetic field of a MACS Separator (Miltenyi Biotec). Columns were washed three times with 3 ml of PEB buffer (PBS [pH 7.2], 2 mM ethylenediaminetetraacetic acid [EDTA], and 0.5% bovine serum albumin [BSA]). After removing the column from the magnetic field, retained mitochondria were eluted with 5 ml of PEB buffer. Following centrifugation at approximately 13,000*g* for 1 min, the mitochondrial pellet was washed twice with 0.32 M sucrose, 1 mM EDTA, and 10 mM Tris–HCl and was finally resuspended in the appropriate incubation buffer.

Staining and FACS analysis of isolated mitochondria

Isolated mitochondria from approximately 1×10^5 cells were resuspended in 100 µl of cold PEB buffer prefiltered through a 0.22-µm membrane. Mitochondria were incubated with a primary antibody (e.g., anti-TOM22 monoclonal mouse antibody or anti-TOM22–biotin, Miltenyi Biotec) for 10 min at 4 °C. Washing was performed by the addition of 1 ml of cold PEB buffer to the mitochondria and subsequent centrifugation for 2 min at 13,000*g* at 4 °C. The supernatant was aspirated except for the last 25 µl (no pellet was visible due to the low amount of starting material). Mitochondria were resuspended, and the volume was adjusted to 100 µl with cold PEB buffer. Then mitochondria were incubated in the dark with the secondary antibody (e.g., rat anti-mouse immunoglobulin G1 (IgG1)–allophycocyanin (APC) or anti-biotin–APC, Miltenyi Biotec) for 10 min at 4 °C. After three washing and centrifugation steps, mitochondria were resuspended in 1 ml of cold PEB buffer and fluorescence measurement was performed on a fluorescence-activated cell sorter (FACS) (FACSCalibur, Becton–Dickinson, Heidelberg, Germany). Data were analyzed using CellQuest software (Becton–Dickinson).

Mitochondria isolated from stably transfected 293 HEK cells expressing the enhanced green fluorescent protein (EGFP) fused to a mitochondrial targeting sequence were directly analyzed using a FACS.

Mitochondria isolation using DC and UC

Mitochondria were purified by DC, as described in detail previously [7]. Purification of mitochondria using a Percoll/Metrizamide gradient and UC was described in detail previously [10]. Because Metrizamide was not available, Nycodenz (Serva, Heidelberg, Germany) was used to prepare a Percoll/Nycodenz gradient.

Antibodies used in Western blot analysis

Mouse monoclonal antibodies used were from the following: against 70 kDa subunit of complex II (Molecular Probes, Eugene, OR, USA), against core 2 subunit of complex III (Molecular Probes), against subunit I of complex IV (Molecular Probes), against β-actin (Sigma, Taufkirchen, Germany), against cytochrome *c* (BD Biosciences, San Jose, CA, USA), against 22 kDa translocase of outer mitochondrial membrane (Miltenyi Biotec), against KDEL (Abcam, Cambridge, UK), against Rab4 (BD Biosciences), against lamin A/C (BD Biosciences), and against Golgin-97 (Molecular Probes). A rabbit antiserum was used against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling, Danvers, MA, USA), and a rabbit antiserum had previously been raised against recombinant human mitochondrial transcription factor A (TFAM) [17]. The following secondary antibodies were used: peroxidase-conjugated goat anti-mouse IgG (Perbio Science, Bonn, Germany) and peroxidase conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, Cambridgeshire, UK).

Results

Mitochondria isolation using the MACS approach

To test whether mitochondria could be successfully isolated with the MACS protocol, we generated a 293-reporter HEK cell line persistently expressing EGFP fused to a mitochondrial targeting sequence derived from the precursor of subunit VIII of human cytochrome *c* oxidase. Import of EGFP into, and fluorescence labeling of mitochondria in the stably transfected 293 HEK reporter cell line was verified by fluorescence microscopy (data not shown). An aliquot of the crude reporter cell lysate before mitochondria separation, the flow-through and washes, and eluted mitochondria were analyzed with a FACS. Mitochondria could be detected as green fluorescence in the crude reporter cell lysate (Fig. 1A). In the flow-through and wash fractions, a marginal EGFP signal was detected (Fig. 1B), whereas the mitochondrial fraction exhibited a strong EGFP signal overlapping with the original fraction (Fig. 1C and D). These data strongly indicate that mitochondria can be efficiently isolated by the MACS approach.

To find out the advantages and disadvantages of the new approach, we compared the MACS protocol with the DC and UC methods, focusing on relevant parameters of mitochondria preparations such as yield, quality, and purity of the isolated organelles.

Yield of isolated mitochondrial fractions

The amount of mitochondria isolated from 293 HEK cells using the MACS protocol was twofold higher than that using DC (see Fig. S1A in supplementary material). In comparison with the UC method, the MACS approach yielded four times more mitochondria from osteosarcoma cells (see Fig. S1B). These results clearly indicate that the highest yield in two different cell types was achieved with the MACS protocol.

Quality of mitochondrial preparations

Well-coupled mitochondria are a prerequisite to achieving reliable reproducible results in nearly all functional assays. Coupling of isolated mitochondria reflecting the quality of the preparations was measured using an oxygen electrode. Respiratory control ratios (RCRs) higher than 3 are well accepted to indicate tightly coupled mitochondria [18]. All three methods provided tightly coupled mitochondria (see Fig. S1C in supplementary material). However, mitochondria isolated with the MACS protocol were found to be