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On the Nature of Obligate Intracellular Symbiosis of Rickettsiae – *Rickettsia prowazekii* Cells Import Mitochondrial Porin

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Abstract—Mitochondrial porin was identified in *Rickettsia prowazekii* by Western blot analysis of whole cells and membrane fractions with monoclonal antibody against porin VDAC 1 of animal mitochondria. Using the BLAST server, no protein sequences homologous to mitochondrial porin were found among the rickettsial genomes. Rickettsiae also do not contain their own porin. The protein imported by rickettsiae is weakly extracted by nonionic detergents and, like porin in mitochondria, is insensitive to proteinase K in whole cells. Immunocytochemical analysis showed that it localizes to the outer membrane of the bacterial cells. These data support an earlier suggestion about import by rickettsiae of indispensable proteins from cytoplasm of the host cell as a molecular basis of obligate intracellular parasitism. They are also consistent with the hypothesis invoking a transfer of genes specifying surface proteins from the last common ancestor of rickettsiae and mitochondria to the host genome, and preservation by rickettsiae of the primitive ability to import these proteins.

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Key words: *Rickettsia prowazekii*, mitochondria, rickettsia-like endosymbiont, common ancestor, protein import, porin, nature of endocellular symbiosis

Rickettsiae are obligate endosymbionts of the order *Rickettsiales* classified within the α subdivision of Proteobacteria. All rickettsial species, except for *Rickettsia prowazekii*, are in effect endosymbionts of arthropods, but not parasites. *Rickettsia prowazekii*, an epidemic typhus agent, is pathogenic for humans and lice. The nature of obligate endocellular symbiosis of rickettsiae has remained enigmatic for a century [1].

In full agreement with the modern version of endosymbiont theory for the origin of organelles, numerous molecular data point to the order *Rickettsiales* as a taxonomic source of mitochondria [2-7]. The order *Rickettsiales* comprises a large group of bacteria, often called the family Rickettsiaceae, and a group of the so-called rickettsia-like endosymbionts (RLE) such as the most well-characterized paramecium endosymbionts *Holospora obtusa* and *Caedibacter caryophilus* [2, 5, 8]. Most detailed phylogenetic analyses of 16S rRNA and chaperonin Cpn60 show that RLE diverge after free-living α-Proteobacteria, but before Rickettsiaceae and mitochondria sister groups [2, 4, 5]. Based on these data, it was earlier suggested that the true rickettsiae, i.e., family Rickettsiaceae, and mitochondria have commonly derived from an already reduced RLE. According to this hypothesis, the last common ancestor of rickettsiae and mitochondria not only have lost redundant genes (e.g., those specifying biosynthetic pathways), but also transferred to the host genome some indispensable genes. It was further suggested that the endosymbiont possessed some ability to import proteins encoded by these transferred genes. The dependence on the import of essential proteins, inherited by rickettsiae from their last common

Abbreviations: Mab) monoclonal antibody; (M)OMP) (major) outer membrane protein; OM) outer membrane; PPIase) peptidyl-prolyl *cis/trans* isomerase; RLE) rickettsia-like endosymbiont.

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ancestor with mitochondria, was considered by the author of this hypothesis as a plausible basis of their obligate intracellular symbiosis [2, 4, 5, 9, 10]. Obviously, the genes for envelope proteins might have first successively passed to the eukaryotic genome from the last common ancestor of rickettsiae and mitochondria, given that such proteins might supposedly have been incorporated into outer membrane with the aid of a minimum number of factors [5].

In the present study, we carried out immunoblotting of the proteins of whole cells and membrane fractions of *R. prowazekii*, and immunoelectron microscopy of rickettsiae, grown in yolk sacs of chicken embryos, using monoclonal antibody (Mab) against mitochondrial porin of animals. It was first shown that the rickettsial cells incorporate in their membranes (i.e., import) mitochondrial porin. Thus, these data agree well with the above two hypotheses.

MATERIALS AND METHODS

Rickettsiae and mitochondria. *Rickettsia prowazekii* (strains Madrid E, EVir, and Breinl) were propagated in yolk sacs of chicken embryos and purified from material of yolk sacs as described previously [11, 12] using differential centrifugation and three consecutive centrifugations in Renografin density gradients. This purification procedure is known to efficiently eliminate the mitochondria [12]. Contamination of rickettsiae with organelles was checked by Western blot analysis with Mab to mitochondrial cytochrome *c*.

Because of a low mitochondrial content in yolk sacs, mitochondria were isolated from a tissue of chicken embryos using a modification of a published method [13] including two consecutive gradients of Percoll.

Total protein concentration in rickettsiae and mitochondria was determined using bicinchoninic acid [14] and adjusted to 5 mg/ml in 10 mM Tris-HCl, pH 7.5 (buffer T).

Antibodies. Mab to porin VDAC 1 of human mitochondria (catalog No. 529538) was from Calbiochem (USA). This antibody recognizes an externally exposed N-terminal epitope of 11 amino acid residues, which is conserved among animal porins [15]. Mab against mitochondrial cytochrome *c* was purchased from Pharmingen (Germany). The antibodies were diluted 1 : 3000 for Western blotting.

Fractionation of *R. prowazekii* cells. Membrane fractions of rickettsial cells were obtained as described previously by Emelyanov [16, 17]. *Rickettsia prowazekii* cells were suspended in a three-fold TES-buffer (Tris-EDTA-sucrose) at 4°C, and upon addition of chicken lysozyme to 0.2 mg/ml the suspension was slowly diluted by an addition of two volumes of cold bidistilled water (so-called controlled osmotic shock, during which the outer but not cytoplasmic membrane is broken). Unbroken cells and spheroplasts were pelleted by a low-speed centrifugation. The supernatant, containing isolated outer membranes (OM), was subjected to ultracentrifugation for 2 h at 160,000*g* (4°C). The spheroplasts were suspended in a small volume of TES and broken by addition of 20 volumes of cold water (cold osmotic shock). The supernatant after clarification of the suspension by low-speed centrifugation was subjected to high-speed centrifugation as above to obtain the fraction of total membranes (a mixture of cytoplasmic membranes and OM) [16].

In order to obtain rickettsial OM, an approach based on membrane blebbing was also used [12]. A suspension of rickettsial cells was incubated in hypotonic buffer (buffer T) for half-an-hour at 45°C on a shaker with subsequent pelleting of cells by centrifugation for 15 min at 14,000*g*. The supernatant (membrane vesicles) was ultracentrifuged for 2 h at 200,000*g* [12, 16]. The pellet was suspended in buffer T.

Treatment of *R. prowazekii* cells and total membranes with proteinase K and nonionic detergents. A suspension of *R. prowazekii* cells in buffer T was treated half-an-hour at room temperature with proteinase K at final concentration 0.1 mg/ml. After addition of 0.2 M phenylmethylsulfonyl fluoride (PMSF) to 2 mM, the cells were pelleted by centrifugation for 15 min at 14,000*g*, resuspended in a buffer followed by addition of PMSF to 2 mM, recentrifuged, and again resuspended in the above buffer. After addition of two-fold concentrated buffer for electrophoresis, the samples were immediately boiled for 5 min [16].

Whole *R. prowazekii* cells with protein concentration 3 mg/ml were treated with 1% (w/v) Triton X-100, octyl glucoside, or digitonin in buffer T at room temperature with occasional shaking. The cells were pelleted for 15 min at 14,000*g*, washed once with buffered detergent and once with buffer with recentrifugation, and resuspended in original buffer [16].

Total membranes with protein concentration 2 mg/ml were treated with 0.5% nonionic detergents as above, with recentrifugations for 2 h at 160,000*g*.

Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Mitochondrial and rickettsial proteins were separated by 10% SDS-PAGE by the Laemmli method [18]. Proteins were electrophoretically transferred onto PVDF membrane (Amersham, England) in a liquid chamber (Bio-Rad, USA) and stained with Ponceau S. Molecular mass markers and major *R. prowazekii* proteins [16] were marked with pencil. Immune reactions with Mab were detected using ECL (Amersham). Conjugates of antibodies to mouse IgG with peroxidase were from Bio-Rad.

Proteins were stained in the gels with silver nitrate by the method of Oakley et al. [19].

Immunoelectron microscopy. Yolk sacs infected with *R. prowazekii* were fixed by the Ito–Karnovsky method

[20] with an additional fixation using osmium tetroxide and infiltrated with LR White resin as described by Neuman et al. [21]. Thin sections were obtained using LKB 3 ultratome and labeled in a post-embedding mode [22]. The above-described Mab to mitoporin were used as a primary antibody, and anti-mouse antibody conjugated with 10-nm colloidal gold (Amersham Pharmacia Biotech, Sweden) served as secondary antibody.

After immunolabeling, thin sections were stained by the method of Reynolds [23] and viewed on a JEOL 100B electron microscope.

RESULTS

Based on phylogenetic data, it was earlier suggested that the true rickettsiae, i.e., the species of the family Rickettsiaceae, and mitochondria have originated from within a group of the rickettsiae-like endosymbionts, called RLE [2, 5]. It was also argued that some indispensable genes might have been transferred to the eukaryotic genome from this last common ancestor of rickettsiae and organelles. These might have been, first of all, the genes encoding surface proteins such as the oxygen sensor TspO (mitochondrial homolog—peripheral benzodiazepine receptor) or porin, incorporation of which into the envelope might have been assisted by a small number of factors [5].

Porin is a widespread and indispensable protein of the OM of Gram-negative bacteria and mitochondria. Although bacterial and mitochondrial porins are dissimilar at the level of the primary structure, both form β-structured trimers in the OM [24, 25]. Porin was so far not reported in rickettsiae [3]. Among major outer membrane proteins (MOMP) of *R. prowazekii*, each of the proteins with molecular mass of 31 and 30 kD, determined by SDS-PAGE, was earlier considered as a candidate for porin [16, 17]. The 30 kD protein was subsequently shown to be peptidyl-prolyl *cis/trans* isomerase (PPIase) of parvulin type [26, 27]. The 31-kD MOMP likely represents a C-terminal cleavable polypeptide of a precursor of surface protective antigen [1, 28]. Although this protein is rich in β-barrels, there is no structural similarity to porins.

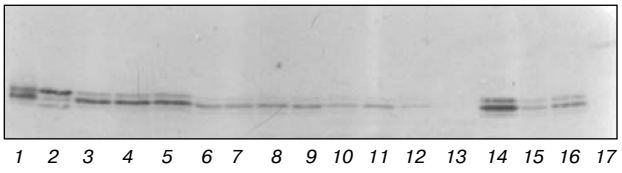

Fig. 1. Western-blot analysis of polypeptides of bacteria and mitochondria with monoclonal antibody against mitochondrial porin. The 10% polyacrylamide gel was loaded with total proteins of *R. prowazekii* strains Breinl (lanes *1* and *5*), Madrid E (*3*), EVir (*4*), mitochondria of hen embryos (*2*), and *E. coli* (*17*), and also with proteins of membrane vesicles (*12*), isolated OM (*13*), and total membranes of *R. prowazekii* strain Breinl (*14*). Rickettsiae were treated before electrophoresis by proteinase K (*8*) at 0.1 mg/ml and 1% detergents octyl glucoside (*6*, *7*), Triton X-100 (*9*, *10*), and digitonin (*11*) in the presence (*6*, *9*) and absence (*7*, *10*, *11*) of 5 mM $MgCl_2$. *15*, *16*) Detergent-insoluble material after treatment of *R. prowazekii* total membranes by 0.5% Triton X-100 in the absence and presence of magnesium ions, respectively.

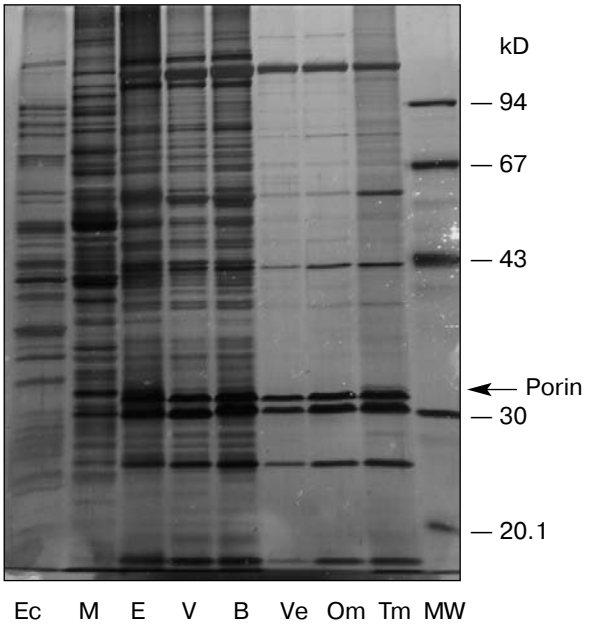

Fig. 2. Silver-stained proteins of rickettsiae and mitochondria. Proteins of mitochondria (M), *R. prowazekii* strains Breinl (B), Madrid E (E), EVir (V), *E. coli* (Ec), membrane vesicles (Ve), isolated OM (Om), total membranes (Tm), and markers (MW) were fractionated by SDS-PAGE. The position of mitochondrial porin is shown by an arrow. Molecular masses of markers in kD are on the right.

We used Mab raised against human porin in Western blot analysis of *R. prowazekii* and mitochondrial proteins. This Mab, called porin 31 hl, recognizes porins of animal mitochondria [15]. The results of immunoblotting are shown in Fig. 1. Strikingly, along with mitochondrial protein, a similar rickettsial protein appeared to be immunoreactive with the anti-porin antibody. However, this polypeptide is not encoded by a bacterial genome—a search of GenBank for homologous proteins using the BLAST server did not reveal any similarity with the finished and unfinished genomes of rickettsiae including *R. prowazekii* [3]. This previously uncharacterized minor *R. prowazekii* protein has molecular mass, determined by SDS-PAGE, ~32 kD. It is worth noting that porin is normally represented by three forms in both the mitochondria and rickettsiae, which are presumably the products of proteolytic degradation. Only these three polypeptides of bacteria and organelles reacted in Western blot with the monoclonal antibody. No immunoreactive proteins were

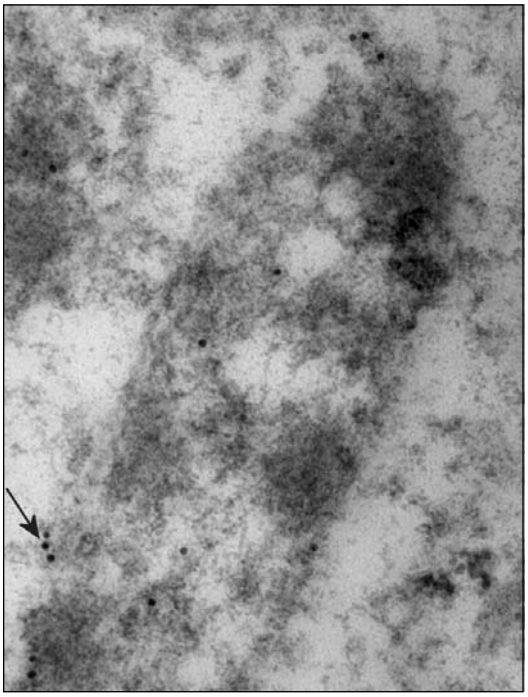

Fig. 3. Thin section of *R. prowazekii* cell in yolk sac tissue. Arrow indicates gold particle in the envelope of the bacterial cell.

found in *E. coli* (Fig. 1 and data not shown). The 32-kD protein of *R. prowazekii* is weakly sensitive to proteinase K in whole cells (like VDAC 1 in mitochondria [29]) and hardly extracted from cells and total membranes by nonionic detergents Triton X-100, octyl glucoside, and digitonin even in the absence of magnesium ions (Fig. 1), addition of which normally impedes solubilization of porins of Gram-negative bacteria [25].

It is curious in this regard, that porin is not detected in OM fractions obtained by two different methods. As seen from Fig. 2, these fractions are typified by the presence of integral OMPs such as the 31- and 30-kD proteins [12, 16, 17]. In agreement with this observation, porin is revealed on silver-stained gels in mitochondria, and in whole cells and total membranes of rickettsiae, but not in OM fractions (Fig. 2).

To further confirm a specific incorporation of mitoporin in the rickettsial OM, we carried out immunocytochemical experiments using electron microscopy and Mab to porin. As seen in Fig. 3, gold particles conjugated with antibody to mouse immunoglobulins localize for the most part on a surface of *R. prowazekii* grown in yolk sacs. Indistinct appearance of the cell wall may be accounted for by partial autolysis of the bacteria in the hen embryo that died from rickettsial infection. Control *in situ* experiments, omitting the primary antibody, did not specifically label any cellular structures (not shown). These observations were made on several samples for electron microscopy.

DISCUSSION

In a full agreement with the universally accepted theory of organelle origin, first clearly formulated by the Russian scientist Constantin Merezhkowsky just a century ago [30], mitochondria might have endosymbiotically originated from within the bacterial order *Rickettsiales* [2-5]. There is little doubt that, during long-term symbiotic relationship with primitively amitochondriate host, many redundant bacterial genes have been lost, and still others passed to the eukaryotic genome. A protein import system might have concomitantly evolved, making it possible to recover back the proteins encoded by nuclear genes [31]. Modern mitochondria possess a very complex import apparatus. It consists of OM (TOM and TOB/SAM) and inner membrane (TIM) complexes, as well as a battery of cytosolic and mitochondrial factors such as chaperones and matrix PPIase of a cyclophilin class [32]. In the framework of the modern endosymbiont theory, the origin of such a complex system would be difficult to explain, especially given that the homologs of the mitochondrial import machinery have actually been not found among bacteria. It is quite clear that a multicomponent system of mitochondrial protein import could not have arisen suddenly, at once. From an evolutionary perspective, it is also clear that this system could not have originated through an acquisition of the constituents one-by-one. It follows almost inevitably from this consideration, that some primitive system of protein import must have arisen in a bacterial precursor of the organelle. The availability of such system would have made possible a functional transfer of endosymbiont genes to the host cell genome. This would have allowed the host to take partial control over the invader—a prerequisite of the origin of the eukaryotic organelle. It is easy to suggest that the genes for surface proteins of endosymbiotic bacterium must have first passed to the host genome, as their insertion into the outer membrane might have been assisted by a small number of subunits [5, 6].

Based on the results of phylogenetic analysis of 16S rRNA and Cpn60, a hypothesis was earlier put forth that mitochondria and the species of the family Rickettsiaceae have derived from an already strongly reduced α-proteobacterial endosymbiont. In line with the above consideration, some essential genes might have been transferred to the host cell genome from this common ancestor. It has supposedly been just the latter to possess some imperfect system of import of protein encoded by nuclear-transferred genes. Transfer of essential genes and recovery by endosymbiont of the proteins might have been first step towards "domestication" of a bacterium and its conversion into an organelle [5, 6]. In a sense, protein import must have arisen *prior to* (sophisticated) import systems. Primitive import machinery could have been naturally preserved in rickettsiae from their common evolutionary ancestor with mitochondria. By an analogy with mito-

chondria, chaperonin Cpn60 and PPIase—membrane-bound proteins in *R. prowazekii*—might have been the components of a primitive import system [4, 17].

In the present study, we have shown that *R. prowazekii* cells import mitochondrial porin. This cannot be explained by a contamination of the rickettsial samples by mitochondria, as total rickettsial proteins do not react in immunoblot with Mab against mitochondrial cytochrome *c* (data not shown). In view that porin is detected not only in mitochondria but also in other cell compartments including plasma membrane [15], one might have supposed that *R. prowazekii* cells are contaminated by host membranes. However, to reconcile this explanation with the absence of porin in isolated OM (Figs. 1 and 2), one would have to make an unrealistic assumption that only those envelope fragments are separated from rickettsial cells during OM isolation procedures used, which do not contain "stuck" host membranes. Mechanistic contamination of bacterial samples with host membranes also should be excluded logically, in view of the methodology of the rickettsial OM isolation (see "Materials and Methods").

Since porin is not present in OM fractions of rickettsiae, a justified assumption would be that it localizes to adhesion zones (Bayer's zones), i.e., contact sites of inner and outer membranes of Gram-negative bacteria [25], which probably remain associated with rickettsial cells during OM isolation by the methods used. Otherwise, the absence of envelope protein in isolated OM is difficult to explain. If mitochondrial porin is indeed localized in bacterial adhesion zones, an intriguing assumption arises that they may be an evolutionary prototype of mitochondrial megachannels, which presumably contain porin and benzodiazepine receptor [33]. The latter has earlier been considered theoretically as a candidate for mitochondrial protein imported by rickettsiae [5]. Interestingly, insertion of porin and other β-barrel proteins into mitochondrial OM is mediated by a membrane complex TOB (SAM), a key component of which—Tob55, or Sam50—is the only evolutionarily conserved membrane component of mitochondrial import machinery. As could be anticipated, phylogenetic analysis of Tob55 and its bacterial homolog Omp85 showed the closest relationship of mitochondria with α-proteobacteria [29, 32, 34].

These data are fully consistent with the above-described hypotheses. Furthermore, they clearly point to a nature obligate endosymbiosis of rickettsiae. As the protein import is very complex process involving cytoplasmic factors, rickettsiae can multiply only inside a living, functioning cell. Unlike many pathogens secreting exotoxin to kill target cells, phospholipase activity of rickettsiae manifests only at close contact of endosymbiont with host cell [1], facilitating its penetration. Slow growth of rickettsiae in cytosol seems to be due to competition with mitochondria for imported proteins [2, 4, 5, 9, 10].

An alternative view is that, irrespective of an origin of the gene encoding porin, rickettsiae possessed porin once in their evolutionary history. In this case, its loss might have occurred after acquisition by rickettsiae of an ability to import mitochondrial porin encoded by the nuclear gene. From an evolutionary standpoint, however, this scenario is biologically meaningless. More probably, the gene coding for porin has been transferred to the host genome just at the origin of rickettsiae and mitochondria from rickettsiae-like endosymbiont. It is easy to suggest that after divergence of rickettsiae and mitochondria the first ones must have preserved a primitive capability to import some proteins, which are a subset of those imported by mitochondria via the complex import system that has emerged in the course of their individual evolution [5].

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