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Extramitochondrial Porin: Facts and Hypotheses[1]

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Mitochondrial porin, or VDAC, is a pore-forming protein abundant in the outer mitochondrial membrane. Several publications have reported extramitochondrial localizations as well, but the evidence was considered insufficient by many, and the presence of porin in nonmitochondrial cellular compartments has remained in doubt for a long time. We have now obtained new data indicating that the plasma membrane of hematopoietic cells contains porin, probably located mostly in caveolae or caveolae-like domains. Porin was purified from the plasma membrane of intact cells by a procedure utilizing the membrane-impermeable labeling reagent NH-SS-biotin and streptavidin affinity chromatography, and shown to have the same properties as mitochondrial porin. A channel with properties similar to that of isolated VDAC was observed by patch-clamping intact cells. This review discusses the evidence supporting extramitochondrial localization, the putative identification of the plasma membrane porin with the "maxi" chloride channel, the hypothetical mechanisms of sorting porin to various cellular membrane structures, and its possible functions.

KEY WORDS: Porin; VDAC; caveolae, plasmalemma, cholesterol transport, maxi chloride channel, patch clamp, biotin

INTRODUCTION

Eukaryotic porin was originally described as a characteristic channel activity in planar bilayer membranes containing mitochondrial outer membrane fractions or the isolated protein itself (Freitag *et al.*, 1982; Colombini, 1979). In planar bilayer experiments, the channel exhibits a high-conductance anion-selective fully open state and multiple, often cation-selective substates adopted upon application of voltages > approx. 20 mV (Colombini, 1994; Benz, 1994). Because of these properties, the pore is also referred to as voltage-dependent anion-selective channel (VDAC). Gene knockout experiments performed in *S. cerevisiae* indicated that at least two isoforms of porin were encoded by eukaryotic genomes (Dihanich *et al.*, 1987, 1989). It is now clear that closely related sequences [human (Blachly-Dyson *et al.*, 1993); murine (Sampson *et al.*, 1997); plant (Fischer *et al.*, 1994)] or more distantly related porin isoforms [yeast (Blachly-Dyson *et al.*, 1997)] exist in the same organism. Nonetheless, most of the biochemical results obtained in many laboratories pertain only to the single porin isoform, which was reproducibly and reliably purified from tissues and cells, called "porin31HL," "VDAC1," or "porin isoform 1" by the various authors. In this paper we will only deal with data related to this polypeptide, referring to it simply as "porin."

The porin is the most abundant protein in the outer membrane, where it represents 5% of total protein (rat liver mitochondria; Linden *et al.*, 1984). The mito-

[1] Key to abbreviations: NH-SS-biotin, sulfosuccinimidyl-2-[biotin-amido]-ethyl-1,3-dithiopropionate; VDAC, voltage-dependent anion channel.

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chondrial outer membrane has been thought for a long time to be completely permeable to low-molecular weight ions and metabolites. Accordingly, porin was considered a simple molecular sieve. Apparently unrelated reports drew renewed attention to this protein. As a component of the peripheral benzodiazepine receptor (McEnery *et al.*, 1992), porin has been proposed to participate in the transport of cholesterol (Papadopoulos *et al.*, 1997) and heme precursors (Rebeiz *et al.*, 1996). It regulates the ATP supply to hexokinase (Adams *et al.*, 1991; Brdiczka and Wallimann, 1994). Porin might be involved in the mitochondrial events in apoptosis (Kroemer *et al.*, 1997) and it might be able to mediate nucleic acid translocation (Szabo *et al.*, 1998).

The presence of porin in extramitochondrial locations was reported for the first time by Thinnes and co-workers (Thinnes *et al.*, 1989; Kayser *et al.*, 1989). Despite subsequent confirmatory reports of its presence in the plasma membrane (Konig *et al.*, 1991; Cole *et al.*, 1992; Puchelle *et al.*, 1993; Lisanti *et al.*, 1994a; Dermietzel *et al.*, 1994; Jakob *et al.*, 1995), the issue remained debated. These studies relied mostly, although not exclusively, on immunocytochemical techniques. Skeptics suspected the presence of artifacts, because of unspecific binding of antibodies or to the redistribution of porin during the fractionation of cellular membranes (Yu *et al.*, 1995).

We have now obtained (Bathori *et al.*, 1999) evidence that porin is present in caveolae and caveolae-like domains. Caveolae are specialized, cholesterol and sphyngolipid-rich plasma membrane regions also enriched in a number of GPI-anchored and integral membrane proteins, believed to be involved in signal transduction (reviews: Anderson, 1993; Lisanti *et al.*, 1994b; Okamoto *et al.*, 1998), transcytosis, potocytosis (*e.g.*, Anderson *et al.*, 1992; Parton *et al.*, 1994; Henley *et al.*, 1998; Oh *et al.*, 1998), and cholesterol traffic (Smart *et al.*, 1996; Uittenbogard *et al.*, 1998). Caveolae-like domains, or lipid "rafts," are domains similar to caveolae, lacking caveolin-1, which do not form the characteristic membrane invaginations of caveolae.

The presence of porin in the plasma membrane adds new questions to the porin reevaluation process. What machinery governs the targeting of the molecule to different membrane compartments? How can the strong permeability barrier of the plasma membrane be maintained in the presence of porin? What is the function of the porin in caveolae? This review is meant to give a short overview of the relevant facts concerning the extramitochondrial location of the porin. We would also like to present a collection of emerging questions and suggest some answers.

LOCALIZATION OF PORIN IN PLASMA MEMBRANES

The presence of porin in the plasma membrane of eukaryotic cells was suggested to Thinnes and coworkers by its copurification with human transplantation antigens (Thinnes *et al.*, 1989; Thinnes, 1992). The earliest immunocytochemical data supporting this hypothesis (Thinnes *et al.*, 1989) utilized antibodies raised against porin 31HL (purified from a crude membrane preparation from human lymphocytes), applied to an Epstein Barr virus-transformed lymphocyte line to generate secondary immunofluorescence images. Subsequent confirmatory studies (Konig *et al.*, 1991; Cole *et al.*, 1992) used normal human lymphocytes as well as a series of lymphocytic cell lines (including CEM cells), a panel of eight monoclonal antibodies raised against the 19 N-terminal amino acids of porin 31HL [whose sequence had meanwhile been determined (Kayser *et al.*, 1989; Jurgens *et al.*, 1991)] (Babel *et al.*, 1991), and flow cytometry as well as EM immunogold labeling and immunofluorescence techniques. The images illustrating these papers show a striking punctuate distribution pattern on the lymphocyte surface, which is hard to reconcile with aspecific binding of the antibodies and is compatible instead with a localization in subdomains of the plasma membrane. The gold-particle labeling EM studies confirmed that porin was localized preferentially in subdomains of the cell membrane (Cole *et al.*, 1992). The punctuate distribution of the immunolabeling may also appear as artifact of polymerization of the bivalent antibodies. However the risk of this artifact is low, since the polymerization is normally prevented by the presence of 1% BSA, which was present in the incubation medium of lymphocytes during the immunogold labeling. While relatively high concentrations needed to be applied for some of the antibodies (cf. Konig *et al.*, 1991, Table 3), their specificity had been tested in immunoblots (Babel *et al.*, 1991), and, indeed, they subsequently proved capable of discriminating between isoforms, namely HVDAC1 (*i.e.*, porin 31HL) and HVDAC2 (Winkelbach *et al.*, 1994). The experiments with monoclonal Abs thus led to the conclusion that the N-terminal of plasma membrane porin was exposed on the cell surface. In 1993, Puchelle *et al.*, following up on the previous reports that porin could

also be detected on the surface of epithelial cells (Konig *et al.*, 1991; Thinnes, 1992), published a study of freshly dissociated human surface respiratory epithelium cells, coming to the conclusion that porin was present also in their apical plasma membrane. Furthermore, these authors reported a colocalization of porin and the cystic fibrosis transmembrane conductance regulator (CFTR), and observed a high incidence of porin-associated gold labeling in subapical vesicles. These vesicles might well have been endosomes, since a recent study (Reymann *et al.*, 1998) has reported extensive labeling of endosomal vesicles in rat renal cortex by an antiporin monoclonal antibody. The hypothesis that plasma membrane porin might correspond to the so-called "maxi chloride channel" (see discussion below) was formulated immediately (Thinnes *et al.*, 1989; Jurgens *et al.*, 1991; Thinnes, 1992). A channel with characteristics very similar to those of isolated mt porin had been observed by patch-clamping, among other cell types, astrocytes. Dermietzel *et al.* (1994) cloned a protein (called BRVDAC), nearly identical to porin from bovine brain. The starting material for the initial protein isolation was a detergent-resistent membrane fraction shown to contain only trace amounts of mitochondrial F_1-ATPase (β subunit). The authors generated a monoclonal (mAb1) antibody against the purified protein, and used this antibody as well as one of the mAbs raised versus the N-terminal sequence of porin31HL (mAb2) for *in situ* immunolocalization of BRVDAC. The cell membrane of astrocytes was labeled by both; in addition, mAb1 recognized the neurons and Purkinje cells of some brain regions. Most interestingly, mAb2 (at 2.5 μg/ml) rapidly blocked the activity of the porin-like anion channel in patch-clamp experiments, while mAb1 and polyclonal antibodies raised versus an internal sequence of BRVDAC did not. These coupled immunotopological and electrophysiological approaches provided strong evidence for the presence of VDAC in the plasma membrane of nervous tissue cells, which had already been suggested by the copurification of a protein with 70% identity to porin (possibly cross-reacting with mAb1) with the $GABA_A$ receptor (Bureau *et al.*, 1992).

In a finding reminescent of Dermietzel *et al.*'s isolation of porin from a detergent-resistent membrane preparation, Lisanti *et al.* (1994a) reported the presence of a protein with sequence homology to porin in caveolae-enriched fractions isolated from lung tissue by a procedure utilizing Triton X-100.

The reliability of all these evidence—as well as of data suggesting a nonmitochondrial subcellular localization of porin (see below)—was questioned by Yu *et al.* (1995), who investigated the subcellular distribution of epitope-tagged porins. They introduced in the HVDAC1, HVDAC2 and HVDAC2′ cDNAs tag sequences encoding known epitopes at the extreme C-terminus of each protein. Transfection of cultured cells by the modified porins cDNAs provided a convenient way to study the intracellular targeting of each porin isoform. Experiments with antibodies against the tagged proteins showed that all three isoforms were exclusively located in fractions or subcellular regions that contained mitochondrial marker proteins. The authors concluded that the data supporting a nonmitochondrial localization of VDAC were probably artifacts, due perhaps to unspecific immunoreactions and redistribution of porin among subcellular fraction in procedures involving the use of detergents.

On the opposite side in the debate, Jakob *et al.* (1995) labeled intact cells with the membrane impermeable reagent NH-SS-biotin. Because of the membrane impermeability of the label, it was assumed that NH-SS-biotin reacted only with protein segments exposed to the external surface of the plasma membrane. In two-dimensional polyacrylamide gel electrophoresis chromatograms of the biotinylated cell proteins, blotting with antiporin antibody and with streptavidin-produced decorated spots in overlapping positions, strongly suggesting that at least a fraction of the porin molecules had been labeled by the biotinylating reagent and, therefore, originated from the plasma membrane.

Our own work addressed the question of whether the porin is really present in caveolae (and, therefore, in the plasma membrane). The approach we followed was to purify and characterize electrophysiologically the putative plasma membrane porin from isolated caveolae. Usually the starting material for the isolation of caveolae or caveolaelike domains is the total tissue homogenate or cells, including mitochondria. Caveolae are separated on the basis of Triton insolubility of the caveolar domain. During the solubilization, an obvious risk of contamination, i.e., nonspecific association of porin to caveolar proteins, should be considered (Yu *et al.*, 1995). In order to separate the plasma membrane porin from the eventual mitochondrial contamination, we started from neoplastic hematopoietic CEM cells labeled with NH-SS-biotin and developed a purification procedure in which the classical isolation method (De Pinto *et al.*, 1987) was combined with

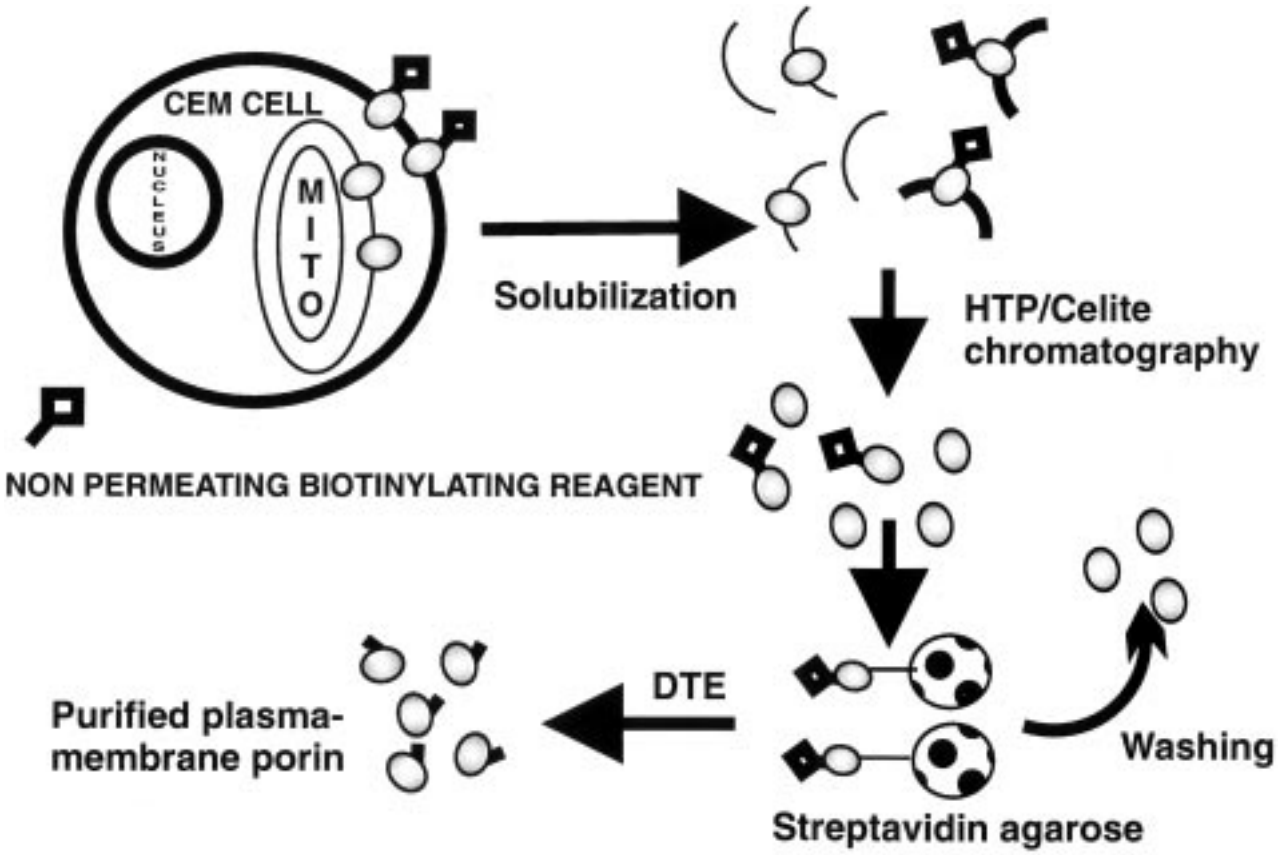

Fig. 1. Scheme of plasma membrane porin purification procedure. CEM cells were incubated with NHS-SS biotin (0.5 mg/ml). After 30 min at 4°C, the reaction was stopped. Cells were disrupted and subjected to the caveolaelike purification procedure (Bathori *et al.*, 1999). Such a purified material was solubilized by 10% Triton X-100, centrifuged, and the supernatant loaded onto a hydroxyapatite:celite 2:1 dry column. The fractions containing the pass-through were incubated for 4 h with an equal volume of swollen immobilized streptavidin–agarose. After two washing steps, the streptavidin–agarose was incubated with 0.1 M dithioerythreitol (DTE) for 30 min, to release any protein (i.e. porin) labeled by NHS-SS and centrifuged. The released protein were recovered in the supernatant.

an affinity purification step on streptavidin–agarose (Bathori *et al.*, 1999). In control experiments, we demonstrated that NH-SS-biotin reacts exclusively with proteins exposed on the outer surface of the cell membrane. The basic steps of the procedure are schematically presented in Fig. 1. In the final step, treatment with dithioerythrietol removed the biotin label. The procedure resulted in a single protein band on SDS-PAGE, which was identified with porin on the basis of MW, reactivity with polyclonal antibodies developed against mitochondrial porin or monoclonal antibodies against a synthetic peptide reproducing the porin N-terminal, and typical electrophysiological activity displayed in planar bilayers after reconstitution (Fig. 2).

Typical porin activity also was found in bilayers reconstituted with isolated caveolar or caveolar raft membrane fraction from rat brain caveolas and neoplastic hematopoetic CEM cells. Western blots demonstrated the presence of porin in preparations of caveolae from brain, lung, and heart, and in caveolar rafts of normal hemopoetic cell from human peripheral blood (except granulocytes) and of neoplastic counterparts (CEM, UT-7, HL-60). In all these preparations, a mitochondrial marker protein, namely the phosphate carrier, could not be detected in Western blots. The absence of porin in the rafts originating from granulocytes is remarkable: it points to a finely differentiated expression of the porin, and, furthermore, constitutes

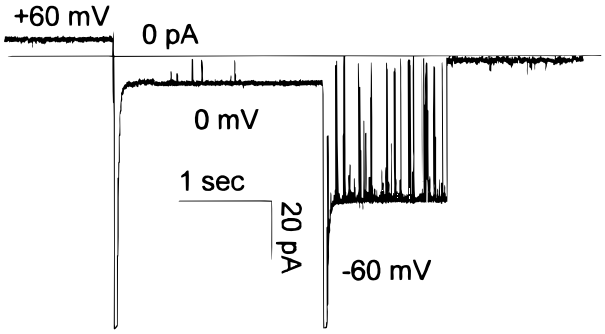

Fig. 2. Detection of the isolated plasmamembrane porin. Sample current trace from an experiment in 650 (*cis*)/100 (*trans*) m*M* KCl. One plasma membrane porin channel, isolated by the procedure described in the text (see also legend of Fig. 1), was incorporated into a phosphatidylethanolamine planar bilayer. The channel, which occupied a low-conductance state at $V_{cis}-60$ mV, is opened to an anion-selective 780 pS state upon switching the voltage to zero (central part of the trace), and closed to a very low conductance level soon after −60 mV application. Notice the brief closures to a nonconducting state.

a validation of our method. Porin purified from rat brain caveolas also exhibited the characteristic electrophysiological activity.

PORINLIKE CHANNEL ACTIVITY IN THE PLASMA MEMBRANE DETECTED BY PATCH CLAMP

The electrophysiological properties of purified VDAC inserted into planar bilayers are well established (see Introduction). Strikingly, isolated porin-like activity has been difficult to demonstrate in patch-clamp studies of mitochondria (Kinnally *et al.*, 1987; Tedeschi *et al.*, 1987; Tedeschi *et al.*, 1989; Moran *et al.* 1992; Mirzabekov *et al.*, 1993; Sorgato and Moran, 1993; [but see Wunder and Colombini (1991), who, however, utilized proteoliposomes containing total mitochondrial membranes and not the organelles themselves). Porinlike channels (often referred to as "maxi chloride channels") were instead documented in the plasma membrane as early as 1983 (Blatz *et al.*, 1983), and have been extensively characterized (*e.g.*, Schwarze and Kolb, 1984; Kolb *et al.*, 1985; Gogelein, 1988; Nobile and Galietta, 1988; Schlichter *et al.*, 1990; Schwiebert *et al.*, 1990, Light *et al.*, 1990; Groschner and Kukovetz, 1992; Pahapill and Schlichter, 1992; Sun *et al.*, 1992; Bajnath *et al.*, 1993; Brown *et al.*, 1993; Dermietzel *et al.*, 1994; Hurnak *et al.*, 1995; Mitchell *et al.*, 1997b; O'Donnell *et al.*, 1998). Typically these channels are not active in the cell-attached mode and can only be observed in excised patches, *i.e.*, they are strictly regulated by soluble cell components. There is clear evidence of regulation of the maxi chloride channel by protein kinase C (PKC) (Saigusa and Kokubun, 1988) and G proteins (Schwiebert *et al.*, 1990; Mitchell *et al.*, 1997a). Interestingly, PKC, G proteins, and porin colocalize in caveolae (Lisanti *et al.*, 1994a; Parolini *et al.*, 1996; Mineo *et al.*, 1998; Bathori *et al.*, 1999), and porin contains evolutionary conserved PKC phosphorylation sites in a region of the protein supposed to form an exposed loop (Sampson *et al.*, 1997). According to some reports, maxi chloride channels also appear to require Ca^{2+} to operate, and/or display temperature sensitivity. As mentioned, the hypothesis was formulated early on that they might correspond to porin, which also appears to be regulated by soluble cellular compounds (Holden and Colombini, 1988, 1993; Liu and Colombini, 1992; Zizi *et al.*, 1994; Lee *et al.*, 1996; Heiden *et al.*, 1996), and this hypothesis was supported by the results of Dermietzel *et al.* (1994). It was, therefore, of interest to establish whether a pore with properties similar to isolated porin could be detected in intact CEM cells, from which we isolated plasma membrane porin (see above). Porin-like activity could never be observed when recording in the cell-attached mode. On the other hand, activity similar to, but not identical with, that of reconstituted porin was detected in about 50% of experiments with excised inside-out patches. This behavior, and the properties of the channel, correspond to those of the maxi chloride channel. One point of difference with isolated porin was that this channel appeared to gate mostly between completely closed and open states (although substates could be detected), while isolated porin often adopts a variety of substates, and seldom closes completely. Furthermore, the CEM plasma membrane channel exhibited fast kinetics. We have, however, recently reported (Bathori *et al.*, 1998) that isolated mitochondrial VDAC can also close completely for brief periods, and it does often display fast gating kinetics. Isolated plasma membrane porin behaves similarly.

It must be also considered, that the inhibitory or regulatory effects might be more pronounced in natural than in artificial membranes. The properties of porin may well be modified by isolation/reconstitution, in particular, by the use of detergents. A previous study has demonstrated, by means of a hexokinase assay on

intact mitochondria, that trace amounts of Triton may diminish the sensitivity of porin to Konig's polyanion and presumably against other modifiers (Bathori *et al.*, 1995). In agreement with this suggestion, Shoshan-Barmatz *et al.* (1996) and Shafir *et al.* (1998) found that, if porin (from both SR and mitochondria) was isolated by means of the detergent nonidet P-40, it became strongly sensitive, in bilayer experiments, to *N, N′*-dicyclohexylcarbodiimide (DCCD) [which also labeled it in both mitochondria (De Pinto *et al.*, 1985) and SR].

PORIN IN NONMITOCHONDRIAL ORGANELLES

A few reports have placed porin in cellular compartments other than mitochondria or the plasma membrane, namely, the nuclear envelope (Thinnes, 1992; Junankar *et al.* 1995), the sarcoplasmic reticulum (Lewis *et al.*, 1994; Junankar *et al.*, 1995; Shoshan Barmatz *et al.*, 1996; Shafir *et al.*, 1998), and, recently, endosomes (Reymann *et al.*, 1998).

The first observations of a high-conductance anion channel of the sarcoplasmic reticulum were performed on "sarcoballs" (fused membrane hemispheres of sarcoplasmic reticulum developed in skinned muscle fibers after appropriate treatment) (Hals *et al.*, 1989). The channel displayed a bell-shape symmetrical voltage dependence. However, some other properties did not match the classical porin behavior. The sarcoball anion channel had a lower slope conductance than the porin and closed completely at voltages higher than $+/-20$ mV. Its conductance exhibited nonlinear salt concentration dependence whereas the porin conductance increased linearly with the ionic concentration of the media. Presumably contamination problems were also present. Sarcoballs develop on the surface of the skinned fibers in areas where the vesicles of sarcoplasmic reticulum, T-tubules, and mitochondria are clustered, and some portion of the sarcoball membrane certainly does not originate from the sarcoplasmic reticulum (Lewis *et al.*, 1992). Unfortunately, both contaminating components may add porin to the sarcoball membrane (the presence of porin in T-tubules was reported by Jurgens *et al.*, 1991). However, Lewis *et al.* (1994) found porin-specific immunolabeling of sarcoplasmic reticulum in preparations from *Bufo marinus*. In a subsequent study (Junankar *et al.*, 1995), immunogold labeling of skeletal and cardiac muscle sections with antiporin monoclonal antibody decorated all three structures (mitochondria, sarcoplasmic reticulum, and T-tubules). The relative densities of gold particles in the mitochondrial outer membrane, sarcoplasmic reticulum, and plasmalemma were in the ratio 7:3:1. Fractionation of the muscle cell membranes by discontinuous sucrose density gradient centrifugation produced highly purified sarcoplasmic reticulum vesicles ($<0.75\%$ contamination by mitochondrial membranes). In Western blots of the purified fraction the antiporin antibody detected a 31 kDa band, corresponding to the expected porin MW. Further support for a sarcoplasmic localization of porin was presented by Shoshan-Barmatz *et al.* (1996) and Shafir *et al.* (1998). These reports are particularly relevant, since the porin was not only detected by immunogold electron microscopy and immunoblot in different SR preparations, but was also isolated, partially sequenced, and characterized in planar bilayers. Importantly, the starting material for these authors' isolation procedure was the subcellular membrane fraction (sarcoplasmic vesicles) and not total cellular membrane. These sarcoplasmic vesicles were rich in porin, but contained only 1% (specific activity in comparison to mitochondria) of a mitochondrial outer membrane marker activity (NADH:cyt *c* oxido reductase).

TARGETING THE PORIN TO VARIOUS COMPARTMENTS

In cells, the targeting of freshly synthesized proteins to the various subcellular compartments is governed by specific segments of the proteins, the so-called targeting sequences or import signal sequences (von Heinje, 1990). Proteins without specific signals seem to be carried automatically to the cell surface via the consecutive pathway (Burgess *et al.*, 1987). The targeting of porin to various compartments is not yet understood. A VDAC mitochondrial targeting sequence has not been identified, although its insertion into the mitochondrial outer membrane depends on the receptor MOM19 (Harkness *et al.*, 1994). Porin insertion into artificial membranes has been reported to be a self-catalytic event (Xu and Colombini, 1996).

The first attempt to explain the phenomenon of a differentiated targeting of porin polypeptides to topologically distinct cellular districts was based on the existence of multiple porin genes. In mouse, three functioning genes for porins have been reported (Sampson *et al.*, 1997) and a similar situation is now also known in man (Rahmani *et al.*, 1998, Messina *et*

al., 1998). A differentiated utilization of the various gene products is an obvious hypothesis for the presence of the porin multigene family. In plants, both amyloplasts and mitochondria have been shown to contain porins with very similar functional and structural features (Fischer *et al.*, 1994). Plasma membrane porin however appears to be the same isoform (VDAC1) routinely isolated from mitochondria.

There are very few examples of proteins targeted either to mitochondria or to another cellular district. Larsson *et al.* (1996) discovered a first example: a single mouse gene encoding protein isoforms targeted to the mitochondria or the nucleus. The gene for the mitochondrial transcription factor A can be alternatively transcribed in testis to give an isoform lacking the mitochondrial targeting presequence. This isoform is localized in the nucleus. More recently, by sequencing the only gene for porin in *Drosophila melanogaster*, we discovered the existence of alternate transcripts differing only in the 5′UTR region added to the mature mRNAs (Oliva *et al.*, 1998). The porin gene in the fly shows conserved structural features common to the more evolved mammal genes. In addition, it is able to yield two alternative mRNAs, differing exclusively in the sequence preceding the start coding codon (Fig. 3). Neither of these alternative exons is able to confer to the protein any polypeptide presequence, but, as already mentioned, porin insertion into mitochondrial membranes does not require any presequence. The alternative exons just add different 5′UTRs to the coding sequence. The presence of such an alternative splicing of type I was discovered by chance, analyzing several clones derived by 5′RACE experiments: we stress that the identity of the clone was deduced only because we had previously sequenced the whole intronic regions of the fly gene (Oliva *et al.*, 1998). The alternative mRNA is much rarer than the previously discovered mRNA encoding porin, cloned in two different laboratories (Messina *et al.*, 1996, Ryerse *et al.*, 1997): we found only one clone in ten of the alternate transcript following 5′RACE experiments. We do not know if a similar situation is present also in mammals, but this hypothesis cannot be ruled out. 5′RACE experiments onto mammalian mRNA were never addressed to the identification of alternative transcripts. Such an evidence could easily be considered just an artifact, also because the sequence of the mammalian gene introns was not determined. On the other hand, we noticed that the gene for the porin isoform 1 (VDAC1) both in mouse and in man has its largest intron (about 14 kbp) between exons 1 and 2 (Sampson *et al.*, 1997). This feature is analogous to what we found in the *Drosophila* gene, where introns are very small, with the exception of the intron between the exons IA and II, exactly where we discovered the alternative exon IB (Oliva *et al.*, 1998); such an intron is thus also a candidate for the localization of an alternate exon in mammals.

Since the existence of two alternative mRNAs for the same protein is the only hint we actually have to distinguish differently localized proteins, otherwise identical, we are working on the hypothesis that a differentiated targeting could be somehow related to such a difference. This hypothesis does not contradict the results presented by Yu *et al.* (1995) on the epitope-tagged porin subcellular localization (Yu *et al.*, 1995); their results, indeed, do not rule out a differentiated targeting of transcripts containing alternative 5′ untranslated sequences, since they used a single species of HVDAC1 mRNA.

UTR regions of mRNA have been frequently invoked as responsible for mRNA-specific intracellular localization. mRNA localization (reviewed in St Johnston, 1995) is an efficient tool for the cell to exert a local translational control; in turn, this is useful to prevent the expression of the protein it encodes in the wrong regions of the cell. Examples of such a strategy were discovered by analyzing the development of fertilized eggs in *Drosophila* and *Xenopus*, where mRNA localization is one of the most important ways for the generation of protein gradients and the determination of cell fate (St Johnston, 1995; Curtis *et al.*, 1995).

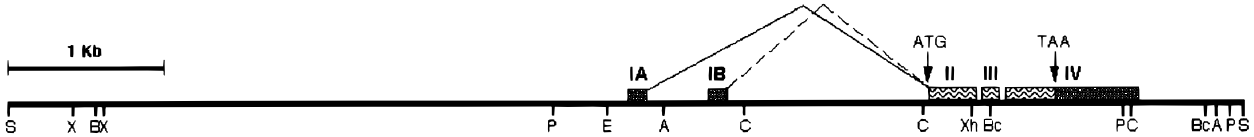

Fig. 3. Structural organization of the *Drosophila melanogaster* porin gene. Exons are shown as boxes above the line and numbered IA, IB, II, III, and IV. Shadowed boxes are the noncoding regions, while coding region boxes are filled with wave patterns. IA and IB are the two alternative leader exons. Letters below the line point to restriction sites. ATG and TAA are the positions of the starting and stop codons, respectively.

mRNA localization and its translational control are thus considered two intimately linked biological processes (St Johnston, 1995; Curtis *et al.*, 1995).

How is such a mechanism implemented? Responsibility for mRNA localization is considered to lie with *cis*-acting sequences in the mRNA molecules and *trans*-acting elements. Many examples of *cis*-acting sequences localized in 3′UTR have been reported. Much less information is available about *trans*-acting elements, most likely proteins able to interact with the mRNAs. Only recently some information appeared in the literature concerning the 5′UTR sequences. The 5′UTR sequence can be involved in this process essentially in two ways: because of independently sensitive *cis*-acting elements or through interactions of the 5′UTR region with the 3′UTR region.

The primary sequence of the 5′UTR can affect the rate of translation. Stable stem-loop structures anywhere in the 5′UTR can block ribosome scanning. The sequence of 5′UTR thus establishes the intrinsic rate of initiation of an mRNA. An example of this mechanism is the regulation of mammalian ferritin mRNA translation by iron: the iron-response element, a 5′UTR localized stem/loop structure, can be bound by the iron regulatory protein, an mRNA binding protein, in an iron-sensitive manner (Gray and Hentze, 1994). Thus proteins that stabilize structures in the 5′UTR can be potent inhibitors of translation.

Interactions between the 5′UTR and 3′UTR regions of the same mRNA have been shown to exist. There is evidence of physical interactions between these two regions. In fact, electron micrographs of cells actively synthesizing secreted peptide hormones show that the great majority of membrane-bound polysomes are circular (Christensen *et al.*, 1987). Very recently, a functional interaction between the 5′ and 3′ ends of *oskar* mRNA, a factor involved in the development of *Drosophila melanogaster*, has also been demonstrated (Gunkel *et al.*, 1998). When *oskar* mRNA reaches the posterior pole of the *Drosophila* oocyte, its translation is derepressed by an active process that requires a specific element in the 5′UTR of the mRNA. Its functional interaction with the previously identified repressor region in the *oskar* 3′UTR is required for the localized *oskar* mRNA translation.

In conclusion, even if the study of these processes is still in its infancy (St Johnston, 1995) we cannot rule out that the presence of different sequences in the 5′UTR of mRNAs encoding the same polypeptide can be involved in a differentiated mRNA localization and, in turn, in a differentiated targeting of the protein product.

A second hypothesis in the plasma membrane targeting of porin does rely on its affinity for cholesterol. One could think that porin is present in caveolae and caveolaelike domains simply because these are stations on the way to its final destination(s). Porin binds preferentially to cholesterol (De Pinto *et al.*, 1989; Popp *et al.*, 1995). Caveolae are especially rich in cholesterol, and play an important role in cholesterol traffic. Newly synthesized cholesterol forms a complex with caveolin, heat-shock protein 56, cyclophilin 40, cyclophilin A, and some other unidentified proteins (Uittenbogaard *et al.*, 1998). The complex travels to the caveolae and the freshly synthesized cholesterol first appears in caveolae. Thus, an alternative working hypothesis may be that porin mRNA(s) are translated in the cytosol and, randomly, some molecules are transported to the caveolar region by means of the caveolin–cholesterol complexes. Porin is then relocated to subcellular compartments via the intracellular cholesterol transport routes. This could explain the presence of porin in various cellular membranes, but contradicts the results presented by Yu *et al.* (1995). Consistent with this hypothesis, a sterol repressing element (SRE-1) is present in the promoter region both of VDAC1 and VDAC2 genes (Sampson *et al.*, 1997). Sterol-repressing elements regulate the synthesis of proteins that are involved in cholesterol traffic. Porin was reported to be part of the peripheral benzodiazepine receptor, which appears to be involved in the transport pathway for cholesterol between the outer and inner mitochondrial membranes (McEnery *et al.*, 1992; Papadopoulos *et al.*, 1997).

POSSIBLE ROLES OF VDAC IN EXTRAMITOCHONDRIAL LOCATIONS

Only speculations are possible on this topic at this time. An obligate preliminary assumption is that VDAC's function is, in any case, that of forming regulated, wide pores, which may function in the transport of ions, metabolites, and, perhaps also, macromolecules. One well-established (e.g. Rostovtseva and Colombini, 1996, 1997) role of VDAC is as an ATP diffusion pathway, so that it is logical to propose this is (one of) its task(s) also in nonmitochondrial locations (*e.g.*, Shoshan-Barmatz *et al.*, 1996). Thinnes (Thinnes *et al.*, 1990; Thinnes, 1992; Puchelle *et al.*, 1993; Winkelbach *et al.*, 1994; Reymann *et al.*, 1995; Thinnes

and Reymann, 1997) has proposed that VDAC may function as a CFTR-associated chloride release channel, *i.e.*, as a component of the outwardly rectifying depolarization-induced channel (ORDIC) complex disregulated in cystic fibrosis. This hypothesis finds some support in data indicating a colocalization of VDAC and CFTR (Puchęlle *et al.*, 1993) and in the ability of both VDAC (Florke *et al.*, 1994; Thinnes *et al.*, 1994), and ORDIC to bind ATP and DIDS. However, in the patch-clamp recordings of Solc and Wine (1992), the ORDIC displayed small (25–50 pS) conductances. According to this hypothesis, an important role of VDAC would be in chloride secretion from epithelial cells. This notion is not supported by the phenotype of a patient with porin deficiency in muscles (Huizing *et al.*, 1994). Unfortunately, too little information is still available about the molecular biology of such a rare pathology to draw any firm conclusion about its physiopathological meaning. The caveolar localization of porin supported by our recent data is more compatible with a role in the uptake of solutes via potocytosis (Lisanti *et al.*, 1994b). In this process, the material to be internalized is first enclosed in caveolae-derived vesicles, which remain close to the plasmalemma, then released into the cytoplasm via an unknown pathway, presumably a regulated channel (Hooper, 1992; Anderson *et al.*, 1992).

CONCLUSIONS

Sufficient data have now accumulated to make the presence of porin in extramitochondrial cellular compartments more than an hypothesis. Some difficulties, however, remain. One weak point is that most of the immunotopological evidence was obtained with the same set of anti-VDAC antibodies. The studies involving biotinylation of cell surfaces provide, however, independent evidence. In addition, the results of Yu *et al.* (1995) must be accounted for. We have presented above an hypothesis that might explain why porin localized exclusively in mitochondria in these authors' experiments. Much exciting work remains to be performed to clarify the porin addressing mechanism(s) and its functions in the cell.

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