Amino Acids (2012) 42:711–723
DOI 10.1007/s00726-011-0987-y

MINIREVIEW ARTICLE

Recent advances in the molecular biology of metazoan polyamine transport

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Received: 28 March 2011 / Accepted: 2 June 2011 / Published online: 4 August 2011

Abstract Very limited molecular knowledge exists about the identity and protein components of the ubiquitous polyamine transporters found in animal cells. However, a number of reports have been published over the last 5 years on potential candidates for metazoan polyamine permeases. We review the available evidence on these putative polyamine permeases, as well as establish a useful «identikit picture» of the *general* polyamine transport system, based on its properties as found in a wide spectrum of mammalian cells. Any molecular candidate encoding a putative «general» polyamine permease should fit that provided portrait. The current models proposed for the mechanism of polyamine internalization in mammalian cells are also briefly reviewed.

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Keywords Polyamines · Membrane transport · Permeases · DNA cloning · Vesicular transport

Introduction

The persisting gaps in our molecular knowledge of mammalian polyamine transport has serious adverse effects on the progress of the whole polyamine field. Although xenobiotic, radiolabeled, or fluorescent polyamines can be used to identify the sites of endogenous polyamine accumulation, there is currently no tool for accurately measuring the actual molecular changes in the tissue expression and activity of polyamine transporter entities under physiological or pathological conditions. Even the well-recognized increase in polyamine uptake activity that generally accompanies polyamine depletion upon ornithine decarboxylase (ODC)[1] inactivation by 2-difluoromethylornithine (DFMO) remains an empirical, albeit consistent observation (Byers and Pegg 1990; Lessard et al. 1995; Seiler 2003; Seiler and Dezeure 1990; Seiler et al. 1996), which has yet to be supported by a molecular explanation.

Indeed, despite much encouraging progress made in prokaryotes and, more recently, in yeast and trypanosomatids, the very question of the identity of the plasma membrane polyamine permeases (PMPPs) in metazoans is still the subject of intensive investigation by various teams.

[1] The abbreviations used are: PMPP, plasma membrane polyamine permeases; PTS, polyamine transport system; ODC, ornithine decarboxylase; DFMO, 2-difluoromethylornithine; CHO cells, Chinese hamster ovary cells; OAZ, ornithine decarboxylase antizyme; AZIN, antizyme inhibitor; SSAT, spermidine/spermine N^1-acetyltransferase; PSVs, polyamine-sequestering vesicles; RME, receptor-mediated endocytosis; HMA, 5-(*N,N*-hexamethylene)amiloride; Spd-MANT, *N*-spermidine-[N^4-(3-aminopropyl)] anthranylamide.

As of today, the sole "hard" genetic evidence for the molecular identity of these general PMPPs in mammalian species remains the cytogenetic localization of the locus responsible for the complete deficiency in diamine and polyamine uptake activity found in Chinese hamster ovary (CHO) cell transport mutants (CHO-MG cells). That locus maps to the *Z* hamster chromosome close to the highly syntenic region of the *IDH2* gene (Adair and Siciliano 1985). In addition, there is solid genetic evidence that mammalian polyamine transporters are encoded by one or more gene(s), as clearly shown by Byers et al. (Byers et al. 1989) with the restoration of polyamine import activity in CHO-MG cells upon transfection of total human genomic DNA. The last 25 years have been the scene of a spectacular increase in the number and structural diversity of permeases driving the transport of virtually any conceivable type of substrate. Permease DNA sequences have first been identified by standard cDNA library screening, and then by similarity cloning starting from a "founding member" of a given permease family, or, with the availability of complete genome information for most species of interest, by *in silico* identification of new members of a permease family and screening for substrates of interest. But despite the rapid progress and comprehensive understanding of the molecular biology of membrane transport of substrates as closely related as amino acids and neurotransmitter amines, the molecular description of mammalian polyamine transport is at best embryonic as of early 2011. However, there are some recent encouraging signs pointing to actual putative candidate permeases, as well as to the molecular and subcellular processes governing the polyamine internalization process.

The objectives of the present mini-review are multipronged. We first intend to briefly review the biochemical properties reported for mammalian polyamine transport since, to be validated, candidate permeases must account for a number of well-defined physicochemical characteristics and substrate requirements. Second, we will review the rather controversial models that have been proposed during the last decade to describe the process of polyamine internalization at the cellular level, including the role of endocytosis and vesicular transport. Finally, we will summarize the recent advances in the cloning and identification of polyamine permeases in metazoans, and especially mammals. One must be aware that given the current state of knowledge of the molecular players in the polyamine transport equation and fluxes, divergent conceptual models for the actual process at the subcellular level have been generalized from various cell systems with different intrinsic characteristics and idiosyncratic experimental designs. Not surprisingly, such models have generated a blend of interest and conflicting views due to the near absence of molecular information on the actual components of metazoan "*polyamine transport systems*" (PTSs). The latter denomination, which is still widely used in the field, eloquently denotes the rather blurred conceptualization of the nature of the permeases and molecular components supporting that ubiquitous biochemical activity.

Mammalian polyamine transport systems: an "identikit picture"

An identikit picture

Once a putative polyamine permease has been proposed or identified, it must be tested against a relatively well-defined set of properties that characterize mammalian transport activities reported in virtually all tissue types. Despite a few discrepancies that can most often be accounted on the experimental setup used, these ubiquitous mammalian polyamine importers, which we will call the general PTS, can be empirically described with a relatively concise list of specific properties. For coverage of the various physiological studies of mammalian polyamine transport in specific cell types or tissues, we refer the reader to Nikolaus Seiler's extensive reviews on the subject (Seiler and Dezeure 1990; Seiler et al. 1996; Seiler 2003).

A number of excellent reports on the topic have indeed been published in the 1980s and early 1990s using different species and cell types that greatly contributed to establish the foundations of polyamine influx as a specific, protein-mediated activity belonging to one of more class(es) or type(s), according to the study considered (reviewed in (Seiler and Dezeure 1990; Seiler et al. 1996; Igarashi and Kashiwagi 2010). However, the majority of these studies addressed only a subset of all properties that have since been found to characterize mammalian polyamine transport. In order to obtain a portrait as complete as possible of the physicochemical and biological properties of one representative human PTS, we published an extensive analysis of the characteristics and regulation of polyamine uptake in the estrogen-dependent ZR-75-1 human breast cancer cell line (Blais et al. 1996; Lessard et al. 1995; Poulin et al. 1995b). The latter cells offer the major advantages of exhibiting a very active PTS (in the order of 1–2 nmol spermidine (or putrescine) min^{-1} mg^{-1} DNA or $\sim$10 pmol min^{-1} 10^{-6} cells) and a polyamine pool almost completely constituted of spermine, with minimal ODC antizyme (OAZ; vide infra) activity under basal conditions. This series of investigations not only provided key information to refine the "identikit" portrait of the general mammalian PTS, but also a detailed list of physicochemical characteristics that could be cross-referenced with previous and later studies in other mammalian cell types.

Table 1 Characteristics of the "general" polyamine transport system (PTS) of mammalian cells. For details and references, refer to the main text

Parameter	Property	Comment
Na^+	→Yes (Put) → ±(Spd) → No (Spm) → Absent (all substrates)	Inhibition by high monovalent cation concentrations (e.g., choline > $Li^+ \gg Na^+$) often misinterpreted as "Na^+ dependence"
$\Delta\Psi_{pm}$	Strong dependence on electronegative $\Delta\Psi_{pm}$ (all substates)	Could provide a fraction of the electrochemical potential required for polyamine import
Divalent cations	Strong dependence on at least one divalent cation in the series $Mn^{2+} > Ca^{2+} > Mg^{2+} \gg Co^{2+}$ (all substates)	Significance unknown; property seen for some channels
pH dependence	→ Put transport: optimum (7.2–8.2), strong inhibition at slightly acidic (pH 7.2–6.0) → Spd transport: optimum (6.8–7.7), less sensitive to pH < 6.8	Might reflect titratable residues important for Put binding and/or translocation
Osmolality	Inversely related to Put (and to a lesser degree, Spd) transport activity	Possible role in maintaining Gibbs-Donnan equilibrium with osmolytes with lower thermodynamic activity (Put)
Chemical characterization	→ Cys residue(s)	Required for binding and/or substrate translocation
	→ Asp, Glu residues	Idem; evidence for –COOH groups at or near the binding site
Substrate binding site	Specific, competitive binding to *5-substituted amiloride derivatives*	Interaction with NHE (antiporters or ENAC channels excluded; reminiscent of some channels (mechanosensitive, viroporins)

Put putrescine, *Spd* spermidine, *Spm* spermine

Biochemistry of PTSs

Once the properties of the general PTS have been compiled from a sizable array of cell types and critically compared, a rather specific description of its characteristics can be made (Table 1). First and foremost, the mammalian PTS is saturable, strongly energy dependent, and displays a high affinity for its substrates (generally ranging from 10^{-7} to 10^{-5} M, depending on the polyamine species and cell type), which obviously points to the central role of an active transporter in the process (in addition to the reviews mentioned above, see early articles for extensive characterizations: (Pohjanpelto 1976; Feige and Chambaz 1985; Kano and Oka 1976; Gawel-Thompson and Greene 1988; Gordonsmith et al. 1983; Rinehart and Chen 1984; Byers and Pegg 1989). While many early reports claimed that the transport of putrescine and, to a lesser degree, spermidine was Na^+ dependent, i.e., used the inward Na^+ gradient as the driving force for the cotransport of the polyamine, analyses were in all cases done by isosmotic replacement of NaCl with other chloride salts (most often LiCl or choline chloride) to manipulate only the cationic species (Rinehart and Chen 1984; Feige and Chambaz 1985; Gawel-Thompson and Greene 1988; Nicolet et al. 1990). However, by comparing the effect of substituting NaCl with isosmotically equivalent amounts of a non-electrolyte such as sucrose or D-mannitol versus LiCl or choline chloride, the near suppression of putrescine or spermidine uptake observed upon replacement of Na^+ with Li^+ or cholinium$^+$ was shown to result in fact from an inhibition of polyamine transport by the high concentrations of the monocationic substitutes (Kumagai and Johnson 1988; McCormack and Johnson 1989; Osborne and Seidel 1990; Poulin et al. 1995b; Gordonsmith et al. 1983). In fact, complete replacement of NaCl with a non-electrolyte even increased apparent polyamine uptake activity (by up to ~40%), suggesting that Na^+ itself acts as an inhibitor of transporter activity (Poulin et al. 1995b). In fact, when re-assessing the reports claiming Na^+ dependence for various PTSs, it becomes quite clear that polyamine transporters are *not* Na^+-cotransporters.

Instead, there is ample evidence that polyamine entry is strongly dependent on the plasma membrane potential ($\Delta\Psi_{pm}$) (Kakinuma et al. 1988; Poulin et al. 1998, 1995b; Dot et al. 2000). Such a steep dependence might obviously be attributed to the contribution of an ion channel in the uptake mechanism, despite the fact that polyamine internalization is a high-affinity, saturable mechanism. On the other hand, *membrane potential dependence* is also a characteristic feature of cation transporters such as

members of the SLC7 family that includes major lysine/arginine/ornithine permeases (e.g., CAT1 = SLC7A1) (Kavanaugh 1993), and some members of the organic cation/anion/zwitterion transporter family (SLC22) such as OCT-1 (SLC22A1) and -3 (SLC22A3, the extraneuronal monoamine transporter) (Busch et al. 1996; Koepsell et al. 2007).

Key biochemical information on polyamine permeases has been derived from various other approaches. An important factor that was found to be strictly required for active putrescine and spermidine transport is the presence of at least one *exofacial divalent cation* in the relative order of efficiency $Mn^{2+} > Ca^{2+} > Mg^{2+} \gg Co^{2+}$ (Table 1) (Pohjanpelto 1976; Minchin and Martin 1997; Poulin et al. 1995b). Chelation experiments with either EDTA or EGTA indeed supported the fact that either Mn^{2+} or Mg^{2+} are likely bound to the exofacial portion of the native permease or of a tightly associated component, and are functionally essential for polyamine transport (Poulin et al. 1995b). The need for a chelation step to remove the tightly bound exofacial divalent cations might explain the variable results reported by other groups when appraising Ca^{2+} requirements for polyamine import (e.g., see (Groblewski et al. 1992); (Brachet and Tome 1992).

Furthermore, putrescine transport is much more sensitive than spermidine uptake activity to slightly *acidic pH* (pH 6.0–7.0; 70% decrease at pH 6.0) and exhibits a pH optimum (7.2–8.2) that is shifted toward more alkaline values (Brachet et al. 1995; Poulin et al. 1995b). These differences in the pH optimum profile for putrescine and spermidine uptake may support the existence of distinct permease types for the transport of diamines and polyamines. The specific requirement of a steep outward pH gradient for putrescine uptake might indicate the presence of a titratable amino acid residue (e.g., His) with a pK_a in the vicinity of physiological pH and involved in putrescine binding to the permease and/or its translocation. Another physicochemical parameter that exerts a strong effect on polyamine transport is *osmolality*. The major upregulation in polyamine biosynthesis observed from bacteria to mammalian cells shortly after a decrease in medium osmolality has been well documented (Munro et al. 1972, 1975; Perry and Oka 1980; Poulin and Pegg 1990; Mitchell et al. 1998). In addition to, and perhaps related to, the latter effect of hypoosmotic stress is a rapid, several-fold increase in the rate of putrescine transport, and to a lesser degree, spermidine transport in both bacterial and mammalian cells (Munro et al. 1972; Perry and Oka 1980; Käpyaho and Jänne 1982; Poulin et al. 1991, 1995b; Mitchell et al. 1998). The short-term (<15 min) nature of the latter response is consistent with a post-translational mechanism and is reminiscent of the behavior of several K^+ and Cl^- channels as well as inorganic cation: Cl^- permeases during regulatory volume decrease (Wehner 2006; Delpire and Gagnon 2008).

A number of studies have also established that polyamine permeases or essential components closely associated with these transporters have essential thiol and carboxyl side groups, as demonstrated by the complete inhibition of polyamine import activity upon treatment with *N*-ethylmaleimide (Rinehart and Chen 1984; Seiler et al. 1996; Seiler and Dezeure 1990) and 1,3-dicyclohexylcarbodi-imide (Torossian et al. 1996), respectively. Interestingly, selective modification of essential carboxyl groups with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide accessible to the solvent phase abolished spermidine uptake activity in CHO cells, a reaction which was protected by either putrescine, spermidine, or spermine in the low μM range (Torossian et al. 1996). Such substrate-mediated protection of permease activity against chemical modification is consistent with the presence of essential Glu or Asp residues either at the substrate binding site or in regions that become occluded upon substrate binding and internalization. Another key aspect of the polyamine binding site of the putative general permease(s) in mammalian cells is the ability to bind a subset of amiloride derivatives, especially 5-(*N,N*-hexamethylene)amiloride (HMA), with K_i values in the micromolar range for putrescine transport inhibition (Poulin et al. 1998). This property of a series of amiloride derivatives, and especially HMA, to inhibit putrescine with high potency can be useful not only for confirming the identity of putative permeases, but is remarkably similar to the ability of HMA to dock to certain cationic channels (e.g., viroporins, mechanosensitive channels) (Wilson et al. 2006; Wu and Davis 2001).

Molecular evidence for heterogeneity among polyamine permease subtypes has recently been obtained (cf. "Taking baby steps: the riddle of the identity of polyamine permeases"). A number of reports have provided evidence for at least two classes of polyamine permeases, often on the basis of the apparent "Na^+ dependence" discussed above (De Smedt et al. 1989; Rannels et al. 1989; Nicolet et al. 1990; Parys et al. 1990; Seiler et al. 1996; Seiler and Dezeure 1990). Since the latter property more likely corresponds to the differential degree of transport inhibition by high concentrations of monovalent cations, the mammalian PTSs can be divided into (a) putrescine or *diamine-preferential* transporters that are more monocation-sensitive and (b) spermidine/spermine- or true *polyamine-preferential* transporters that are less sensitive to monovalent cations (Seiler and Dezeure 1990). Such distinctions are closely related to the substrate selected for measurement, as some reports have claimed that spermidine uptake presents characteristics intermediate between diamine- and polyamine-preferential transporters (Byers et al. 1987). In fact, evidence has been shown in support of two PTS subtypes

that can be regulated in a distinct fashion, with cell transformation being associated with the specific induction of a high-affinity, putrescine-preferential subtype that is absent in non-tumoral pancreatic acinar cells (Nicolet et al. 1991, 1990). This inducible putrescine import system provides a clear example of an inducible PTS that is not expressed in differentiated, mature cells in at least certain tissues.

Substrate specificity

While the respective range of substrates that are specifically recognized by individual PTS subtypes is somewhat difficult to determine exactly, the general PTS considered as a whole has relatively well-defined requirements for substrate recognition. In fact, despite having no molecular identity yet assigned to it, the PTS has been the target of several extensive series of structure–activity relationship analyses with a staggering number of polyamine analogs and conjugates covering virtually all variations of the primary spermidine and spermine backbones (Gordonsmith et al. 1983; Bergeron et al. 1989; Li et al. 1997; Seiler 2003; Gardner et al. 2004; Delcros et al. 2006; Phanstiel et al. 2007; Casero and Woster 2009). Although a thorough discussion of this topic goes beyond the scope of this review, one can summarize the substrate requirements of the PTS as follows: (a) to be endowed with high affinity, a substrate must bear two cationic amine (primary to tertiary, more rarely quaternary) centers separated by a distance equivalent to $\geq$4 methylene groups (additional cationic centers increase the affinity and can be separated by propylene chains); (b) the presence of a carboxyl group or a negatively charged group *abolishes* recognition by the PTS; (c) various aliphatic or aromatic side chains can be added to the terminal primary amino groups via acylation or sulfonylation with loss of one charge but no loss of affinity, or even a net gain thereof (Burns et al. 2001); and (d) dimerization of two potential "good" substrates increases the affinity of the resulting polyamine dimer in a synergistic manner. Structural features important for inducing substrate translation or import are less clear, although there is some evidence that the presence of a carbonyl group prevents substrate internalization by the mammalian PTS while preserving binding affinity to a good extent (Huber et al. 1996; Graminski et al. 2002; Covassin et al. 2003).

Despite the requirements listed above, polyamine transport activity in vertebrates has sometimes been referred to as displaying a relatively loose specificity, because paraquat and methylglyoxal bis(guanylhydrazone) are well-recognized substrates of the PTS despite being very remote chemically from the common aliphatic polyamines (Seiler and Dezeure 1990; Seiler 2003). The latter examples further emphasize the major importance of an electrostatic interaction between the cationic centers of a divalent or trivalent PTS substrate and spatially constrained negatively charged residues of the permease, with a contribution of hydrophobic interactions with the spacers between the charged amines. Interestingly, various reports, including an elegant structure–activity relationship study comparing the affinity of hexahydropyrimidines, representing a folded configuration of spermidine in a "locked" state, have brought support for the hypothesis that the high-affinity substrates spermidine and spermine may well interact as *cyclical conformers* with the polyamine permeases (Bergeron and Seligsohn 1986; Lessard et al. 1995). Such conformers, arising from internal folding of the molecule unto itself via hydrogen bonding, would account for the well-known smooth continuum of substrate affinity for the aliphatic diamine series $^{+}H_3N(CH_2)_nNH_3^{+}$ ($2 < n < 9$). This series exhibits two peaks of maximal affinity at $n = 4$ (putrescine) and 7–8, with the particular distinction that diaminoheptane and diaminooctane are excellent competitors of spermidine transport, unlike putrescine (Porter and Bergeron 1983; Bergeron and Seligsohn 1986). Thus, by analogy with the prokaryotic PotABCD ABC permease that can use both putrescine and spermidine as substrates via interaction with partially overlapping residues in the substrate binding site of PotD (Kashiwagi et al. 1996), a single polyamine permease type might in fact be responsible for the uptake of both diamines and polyamines in mammalian cells (Feige and Chambaz 1985; Gawel-Thompson and Greene 1988; De Smedt et al. 1989). This hypothesis would also be consistent with the fact that *all* known mammalian cell mutants deficient in spermidine or spermine transport are also deficient in putrescine transport (Seiler 2003), and transfection of human genomic DNA invariably restores transport activity for all three substrates (Byers et al. 1989). Thus, the question regarding the actual subtype heterogeneity of the general PTS will await cloning of the actual polyamine permeases.

Regulation of the mammalian PTS

Also part of the "identikit picture" that any candidate general polyamine permease must match is a number of well-defined regulatory aspects. One of the major reasons that keep attracting the attention of polyamine biologists to polyamine transport is the frequently dramatic increase of this activity upon *oncogenic cell transformation*, which parallels the sustained induction of ODC activity (Roy et al. 2008; Redgate et al. 1997). Classic experiments using injection of radioactive polyamines to tumor-bearing animals have provided striking evidence in support of that general observation (Chaney et al. 1983; Volkow et al. 1983; Redgate et al. 1997). The increase in polyamine

transport activity found in cancer cells is not entirely unexpected as a number of cytokines and growth factors, many of them acting as promoters of cell cycle progression, are known to upregulate polyamine uptake activity (Feige and Chambaz 1985; Gawel-Thompson and Greene 1989; Rinehart and Chen 1984; Seiler and Dezeure 1990; Lessard et al. 1995; Blais et al. 1996). Not surprisingly, the same mitogenic growth factors also induce ODC expression. Conversely, growth-inhibitory cytokines are often potent suppressors of polyamine transport (Lessard et al. 1995; Blais et al. 1996).

The frequent coordination of polyamine transport activity with ODC induction bears considerable significance, as both processes are potently inhibited by ODC antizymes (OAZs). OAZs have been found from fungi to vertebrates, and all three OAZ genes (OAZ1, -2 and -3) found in mammalian species repress both ODC activity and polyamine transport, although OAZ3 does not induce ODC degradation (Coffino 2001; Kahana 2009). OAZs are unique in eukaryotic genomes in that the active, long isoforms of each OAZ type are translated only via a +1 translational frameshift that is induced with increases in free polyamine levels. The mechanism underlying the inhibition of polyamine transport by OAZs has not yet been established, and whether OAZs directly interact with polyamine permeases is still a matter of speculation. Interestingly, OAZ1 has been reported to consistently bind sorting nexin-5 (SNX-5) in yeast double-hybrid assays (R.A. Casero Jr., unpublished results), although the significance of that interaction is not yet clear. Nevertheless, the known roles of sorting nexin-5 in vesicular trafficking phenomena such as macropinocytosis and retromer complex (Wang et al. 2010; Wassmer et al. 2009) formation are intriguing in view of the body of evidence that connects polyamine transport to vesicular trafficking and transport.

The effectiveness of a simultaneous feedback inhibition of both polyamine biosynthesis and accumulation via polyamine-inducible translational frameshift provides an elegant and self-regulated system that can maintain the polyamine pool within optimal limits with a very short response delay. An additional level of complexity to polyamine transport control has been added upon the discovery and characterization of antizyme inhibitors (AZIN)-1 and -2, which are catalytically inactive ODC relatives whose expression is tightly coupled with that of ODC and the OAZs, and have a high affinity for OAZs. AZINs are thus activators of polyamine transport and ODC activities (Kahana 2009). Interestingly, AZIN2, which is preferentially expressed in the testis like OAZ3, localizes to the *trans*-Golgi network (where vesicular polyamine internalization has also been reported) and has been associated with vesicular membrane trafficking (Kahana 2009).

Like ODC, OAZ and AZIN proteins have short half-lives, which is critical for rapid adjustments of polyamine levels to changes in intra- or extracellular conditions. Thus, inhibition of protein synthesis in cells incubated with polyamines prevents OAZ induction, resulting in unabated polyamine accumulation and cell death (Mitchell et al. 1994; Poulin et al. 1995a). In addition to the short-term, virtually complete shutdown of polyamine import activity brought about by OAZ induction, there is evidence for a time-dependent, "trans-inhibition" of polyamine uptake activity that does not require de novo protein synthesis (Parys et al. 1990; Lessard et al. 1995). The molecular significance of this second, stable inhibitor of polyamine transport is still unknown, although our recent insertion mutagenesis experiments have identified one likely candidate (unpublished observations). Finally, a last backup system that limits the accumulation of polyamines bearing a 3-aminopropyl moiety is the induction of spermidine/spermine N^1-acetyltransferase (SSAT), albeit its latency is somewhat longer than in the case of OAZs (Poulin et al. 1993; Lessard et al. 1995).

As mentioned in the "Introduction", DFMO and other inhibitors of ODC biosynthesis upregulate polyamine transport activity, often severalfold, through an unknown mechanism that depends on the depletion of the free polyamine pool (Poulin et al. 1993). Indirect lines of evidence tend to suggest that the increase in PTS activity observed upon polyamine depletion is consistent with an increased abundance of the putative polyamine permeases rather than a lower abundance of OAZs (Seiler and Dezeure 1990; Seiler et al. 1996; Seiler 2003; Lessard et al. 1995). These observations raise the distinct possibility that the intracellular polyamine pool might negatively regulate either the expression or activity of polyamine permeases independently from OAZs. In view of the important role of vesicular trafficking in the regulation of the mammalian PTS (vide infra), polyamine depletion might well increase plasma membrane exposure of polyamine permeases via reduction of the constitutive internalization or turnover of plasma membrane polyamine permeases.

Polyamines accumulate in intracellular vesicles: where are the permeases?

When evidence is scarce, theories flourish. In the recent years, polyamine biologists have used inferences based on a paucity of observations to describe a number of microscopic observations, with rather confusing and mixed results. We will critically review the main models, keeping in mind that the worth of any such model is the accuracy with which it predicts the likelihood of information added *later* to the model.

Vesicular polyamine accumulation

Histochemical evidence from various sources has shown the presence of high concentrations of polyamines in vesicular structures such as neuroendocrine or mast cell secretory granules (Garcia-Faroldi et al. 2010). Moreover, kinetic evidence has shown that not only neurotransmitter amines, but also natural polyamines are actively transported into synaptic vesicles (Masuko et al. 2003). To obtain a more dynamic picture of how polyamines internalized by the mammalian PTS are handled intracellularly, a number of polyamine analogs bearing fluorophores have been synthesized and characterized. Most such fluorescent polyamines have been designed so as to preserve structural features essential for their high affinity and specificity of interaction with the PTS. Alternatively, polyamine internalization has been monitored by immunofluorescence using a well-characterized monoclonal antibody specific for spermine. The pioneering work of Aziz et al. (1998); Cullis et al. (1999) provided the first clear demonstration that fluorescent polyamines concentrate in small membrane-bound intracellular vesicles, so-called polyamine-sequestering vesicles (PSVs), in a wide variety of cell types. This behavior has been observed in polyamine conjugates as widely different as N^1-(fluoresceinyl)-spermine (Aziz et al. 1998) and *N*-spermidine-[N^4-(3-aminopropyl)] anthranylamide (Spd-MANT) (Cullis et al. 1999; Soulet et al. 2002), which display kinetic properties undistinguishable from those of the parent polyamine, but widely different affinities for the PTS. Thus, in addition to a saturable, energy-dependent uptake that is competitively inhibited by spermidine, the fluorescent polyamine analogs also induce a rapid feedback inhibition of transport consistent with OAZ induction.

However, biological interpretation of polyamine accumulation into PSVs has led to rather conflicting models and views. Our early attempts at identifying the nature of these PSVs using Spd-MANT or a highly fluorescent probe, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-*N*'-{*S*-[spermidine-(N^4-ethyl)]thioacetylethylenediamine} (Soulet et al. 2002) showed that only a minor fraction of these structures belonged to the clathrin-coated-dependent endosome recycling pathway that follows receptor-mediated endocytosis (RME), including small early endosomes as well as larger sorting endosomes. However, a more extensive analysis using CHO cell mutants defective at specific stages of early and late endocytosis, cell compartment-specific fluorescent markers, and various schedules of treatment with protonophores and the V-ATPase inhibitor, bafilomycin A_1, provided a consistent mechanistic explanation for PSV labeling (Soulet et al. 2004). The proposed model (Fig. 1a) predicted the existence of at least two classes of polyamine permeases that differ by their cellular localization, namely (i) classical PMPPs and (ii) vesicular H^+-coupled polyamine antiporters (VPAs). VPAs are novel entities that use the steep inward electrochemical proton gradient sustained by the activity of V-ATPases for importing free polyamines from the cytosol into PSVs by a proton:polyamine exchange mechanism. We clearly demonstrated that PSVs are in fact identical to more acidic vesicles of the late endocytosis compartment, i.e., multivesicular bodies, late endosomes, and lysosomes. Thus, the evidence supporting the model pointed to a *sequential* pathway of internalization that involves the initial import of polyamines into the cytosol, followed by their rapid sequestration in PSVs via VPAs.

A key aspect of our analysis is that vesicular polyamine accumulation acts in fact as the *major driving force* for intracellular polyamine accumulation by creating a "sink" effect, i.e., by reducing the thermodynamic activity of cytosolic polyamines, thus favoring import by PMPPs due to the steep inward polyamine gradient thus created (Soulet et al. 2004). In fact, the rapid and extensive sequestering of cytosolic polyamines via VPA activity might keep the *free* polyamine pool to values sufficient for PMPPs to mediate transport passively through diffusion via facilitators or ion channels. As a result, inhibition of VPA activity strongly depresses overall polyamine transport, which provides a novel level of regulation of polyamine transport, with obvious implications for the potential mechanism(s) of negative feedback regulation by OAZs and other factors. Another interesting aspect of this model for the intracellular organization of mammalian polyamine transport is its striking overall similarity with neurotransmitter recapture in the nervous system. Thus, according to our model, PSVs would play a role somewhat analogous to that of synaptic vesicles by sequestering not only newly internalized, but also de novo synthesized polyamines within membrane-bound compartments. The latter vesicles thus isolate the major bulk of the polyamine pool from the cytosol, remove the end products of polyamine biosynthetic pathways and polyamine import, and reduce the actual free polyamine pool to levels compatible with most cellular functions, including continuous polyamine biosynthesis itself. A further corollary that sets our model apart from the others is that PSVs exist independently of polyamine transport since they consist of vesicles of the late endocytic compartment.

Using rather different approaches, M. Belting and E. Gerner independently ended up with somewhat similar models for the significance of PSV formation. Belting and his colleagues (Belting et al. 1996) based their analysis on the premise that the heparan sulfate groups of glycosaminoglycans such as glypican-1 bind spermine, but neither spermidine nor putrescine, with high affinity. By acting as spermine receptors, glypican-1 can bind spermine with

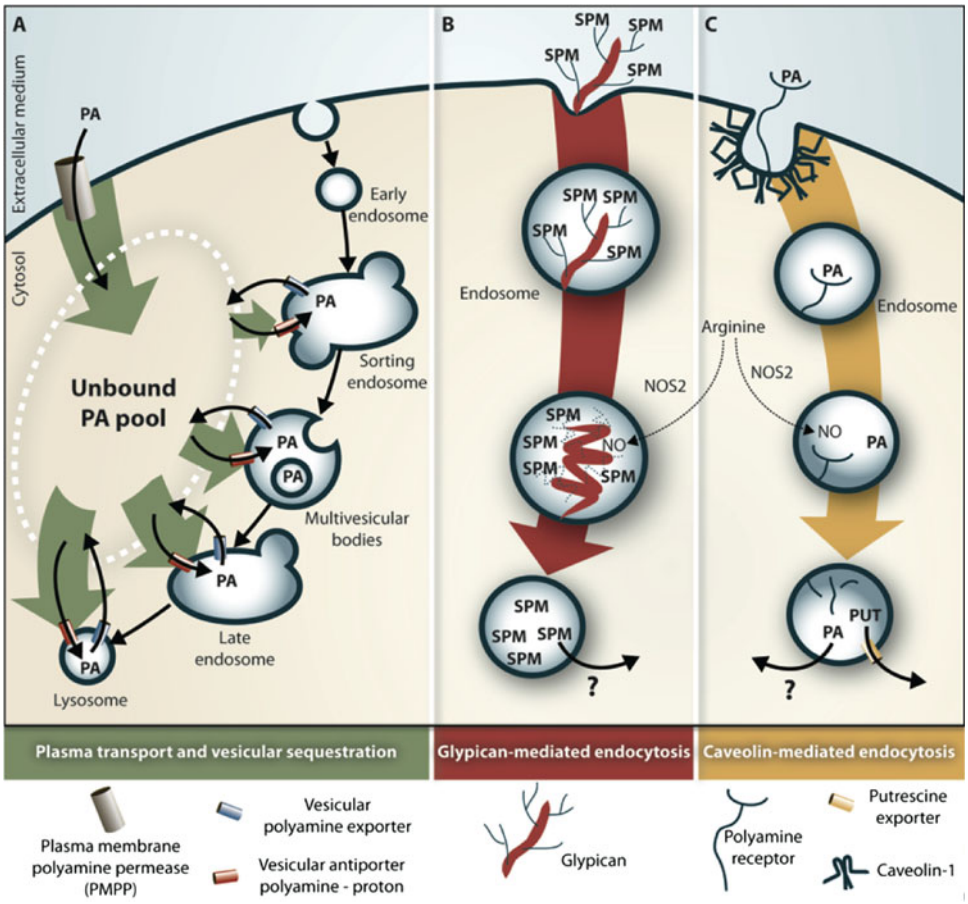

Fig. 1 Putative models for the mechanism of polyamine accumulation and sequestration into vesicles in mammalian cells. **a** Model adapted from (Soulet et al. 2004) illustrating a two-step mechanism for polyamine transport and vesicular sequestration. **b** Model adapted from (Belting et al. 2003) proposing that spermine first binds to heparan sulfate groups in glypican-1 and is subsequently internalized and freed from glypican via NO-mediated oxidation. *NOS2* nitric oxide synthase-2, *PA* polyamines, *PUT* putrescine, *SPM* spermine. **c** Model based on (Uemura et al. 2010) suggesting a caveolin-1-dependent internalization of polyamines bound to a putative "polyamine receptor". Putrescine (but not higher polyamines) might exit from the vesicles via the SLC3A2-bound diamine exporter

high affinity, which then triggers RME and internalizes spermine, thereby accounting for PSV formation (Fig. 1b); (Belting et al. 2003). Additional features to their model include nitrosylation of free glucosamine groups in glypican-1 via NO synthase 2 (NOS2) activity, followed by secondary release of NO within endosomes which, together with the action of Cu^{2+}, disrupts spermine binding to glypican-1 and catalyzes the degradation of endocytosed glypican-1. However, no candidate permease or mechanism was proposed for an exiting mechanism for vesicle-sequestered spermine. One serious disadvantage of that model is that it cannot thus far apply to either putrescine or spermidine. Therefore, although spermine-mediated endocytosis of glypican-1:spermine complexes might contribute a significant fraction of overall spermine accumulation in some cell types, it is difficult to invoke such a mechanism to account the activity of the general PTS.

To obviate the substrate limitations inherent to a heparan sulfate-based mechanism, Gerner and colleagues have attributed polyamine-induced RME to as yet unknown "polyamine receptors" located in caveolin-1-enriched regions of the plasma membrane (Uemura et al. 2010). Similarly to the latter glypican-based model, an NOS2 activity-dependent mechanism would somehow destabilize the putative spermine:receptor complexes. In this alternative model (Fig. 1c), caveolin-1 acts in fact as a regional tag for the receptors (and indirectly, for polyamine uptake) but, surprisingly enough, inhibits putrescine transport in a manner that is counteracted by its phosphorylation by K-Ras. Interestingly, one possible candidate permease gene, namely the putative diamine exporter that forms a heterodimer with SLC3A2 (Uemura et al. 2008), was proposed as allowing the exit of putrescine from caveolin-dependent endosomes by operating in the reverse direction (Uemura et al. 2010). Although the latter model provides advantages over the glypican-based mechanism by invoking a more general initial binding step, the problem of exporting true polyamines (i.e., spermidine and spermine) from the endosomes remains unsolved.

Taking baby steps: the riddle of the identity of polyamine permeases

As it must have seemed obvious since the beginning, we are still in the prelude of identifying the permeases

responsible for the general PTS activity. However, a few encouraging breakthroughs have been made that fare well for unmasking the actual culprits in the near future.

We will not review the advances made in yeast polyamine import, as a recent review already covers the topic and the permeases involved do not have close homologs in animal cells (Aouida et al. 2005; Igarashi and Kashiwagi 2010). Likewise, recent exciting progress made in the molecular identification of distinct diamine and polyamine permeases in trypanosomatids is unlikely to have much resonance for animal systems as the permeases identified in *Leishmania* spp. and *Trypanosoma* spp. (Hasne and Ullman 2011) are more closely related to transporter families found in vascular plants.

A few relatively recent reports have assigned polyamine transport activity to known permeases in human cells. First, kinetic evidence in favor of a common uptake of basic amino acid substrates of the y^+ system (now assigned to the CAT (SLC7) permease family) and polyamines was described (Sharpe and Seidel 2005). Indeed, the SLC7 permeases exhibit some properties consistent with the general PTS such as Na^+ independence and $\Delta\Psi_{pm}$ dependence, but the presence of a carboxyl group in classic substrates of SLC7 would preclude these transporters from catalyzing genuine PTS activity. Against a role of SLC7A1 as a polyamine permease, transfection of SLC7A1 or OAZ1 had no effect on polyamine and lysine transport activity, respectively (Sharpe and Seidel 2005).

A second putative permease unexpectedly exhibits spermidine and spermine and, to a lesser degree, putrescine transport activity, namely the *a* splice variant of human SLC12A8 (=CCC9), a member of the cation:Cl^- cotransporter family that is usually involved in inorganic ion transport (Daigle et al. 2009). The isoform has a reasonably wide distribution in human tissue and organ types, and elevated polyamine transport could be demonstrated in cells transfected with CCC9a. However, the CCC9a-mediated spermidine transport activity was stimulated by a number of amino acids (especially glutamate, aspartate, and lysine), unlike the endogenous PTS, and reciprocally CCC9a catalyzes amino acid transport in a polyamine-stimulated fashion. Furthermore, the canonical, high PTS activity found in ZR-75-1 human breast cancer cells was found to be completely insensitive to loop diuretics (bumetanide, furosemide) that strongly depress CCC9a activity, as with most members of the SLC12 family (Poulin et al. 1995b).

More recently, SLC22A16 (=OCT6 or CT2), a member of the large SLC22 family that regroups organic cation/anion/zwitterion transporters, has been characterized as a high-affinity polyamine and carnitine permease that also recognizes substrates having apparently little in common, such as bleomycin A_5 and doxorubicin (=adriamycin) (Aouida et al. 2010). SLC22A16 has a rather limited tissue distribution in human adults as it is exclusively found in Sertoli cells, principal cells of the epididymis, the endometrium (under progestin regulation), and cells of hematopoietic lineage, especially CD34(+) populations (Koepsell et al. 2007; Aouida et al. 2010). A particular feature of SLC22A16 is that it can accommodate other quaternary amines such as the carnitine precursor, betaine, as well as wide variations and additions of even bulky side groups to the structure of the carboxyl group-bearing end of the carnitine backbone (Aouida et al. 2010). The remarkable tolerance of the substrate recognition site is further illustrated by the fact that SLC22A16 imports bleomycin A_5 (but not other bleomycin isoforms) that bears a spermidinyl side chain, with relatively high affinity. Since bleomycin extracted from natural sources is a complex mixture of isoforms that include the A_5 as a minor species, cells expressing SLC22A16 are targets of the drug, whereas cells deficient in expression of the permease display marked resistance. The latter finding has considerable therapeutic significance. Bleomycin is a first-line chemotherapeutic drug for the treatment of testicular cancer, and the dependence of its pharmacological activity on SLC22A16 expression strongly suggests that the spermidinyl moiety of bleomycin-A_5 is crucial for its import into the testis. Likewise, the ability of SLC22A16 to import doxorubicin makes it a prognostic tool of choice for predicting the response to that drug in several leukemias (Aouida and Ramotar 2010). However, despite its high affinity for polyamines, the ability of SLC22A16 to accept quaternary amino acids and large amphipathic substrates such as doxorubicin, as well as its very restricted distribution, make it a typical zwitterion/cation permease that is unlikely to correspond to the general PTS. The testicular localization of SLC22A16 immediately raises the hypothesis that it might be a polyamine/carnitine permease under the specific regulation by OAZ3 and indirectly, AZIN2, which are both testis specific. That possibility is rather unlikely, however, as the OAZ3-AZIN2 tandem is strictly limited to germ cells, in which SLC22A16 is absent (Lopez-Contreras et al. 2008).

Nevertheless, the finding that a member of the OCT/OAT permease family has high-affinity spermidine transport activity has intriguing implications. Indeed, the physiological properties of SLC22 permeases make them ideal candidates based on the "identikit picture" of the mammalian PTS. Most of the organic cation transport members of the family are Na^+ independent but can function as antiporters, using a local H^+ countergradient that drives cationic substrate efflux at the expense of H^+ influx, as found in the human kidney. Moreover, several members exhibit a pH and $\Delta\Psi_{pm}$ dependence that is highly similar to that of the mammalian PTS (Busch et al. 1996;

Koepsell et al. 2007). In fact, the strong voltage response of SLC22A1 (=OCT1) enables the permease to behave as a channel able to permeate low but significant polyamine fluxes (Busch et al. 1996). Finally, evidence has implicated SLC22A2, in the bidirectional transport of both agmatine and/or putrescine, whereas SLC47A1 (=MATE1), a member of the multidrug and toxin extrusion (MATE) family of drug exporters, is specific for the influx/efflux of agmatine (Winter et al. 2011). Since there are a number of orphan SLC22 permeases, including putative organic cation transporters, the now recognized ability of at least three members of that family to catalyze diamine/polyamine import and/or export warrants a systematic assessment of the potential ability of the uncharacterized permeases of that group to drive polyamine import.

Finally, an interesting development in the molecular identification of polyamine permeases in lower eukaryotes may have a potential impact on the similar quest in mammalian cells. In *Caenorhabditis elegans*, a member of the P_{5B} type of P-ATPases, CATP-5, has been identified using a genetic screen in mutants deficient in the uptake of *sym*-norspermidine, which is not only toxic to the nematode, but also impairs RNA interference, a fortuitous finding that was key to the screening method used (Heinick et al. 2010). CATP-5 has been authenticated as a PMPP present in the apical membrane of intestinal cells and the excretory cell, two cell types where high polyamine influx would be expected. A potentially significant implication of that finding is that at least three relatively close CATP-5 homologs, namely ATP13A2, -3 and -4, are found in the human genome but have not yet been assigned a clear function. Based on work in yeast, P_{5B}-type ATPases have been assigned a subcellular (e.g., endoplasmic reticulum, Golgi), but the yeast P_5-type homolog, Spf1p, belongs to the quite distinct P_{5A} subtype. Therefore, the question as to the possible function of the mammalian P_{5B} homologs of CATP-5 in mammalian polyamine remains open and constitutes a most interesting potential avenue.

Conclusion

This review was meant to convey an optimistic note in the sometimes weary refrain that polyamine biologists often sing when addressing the topic of this minireview. Not only have specific polyamine permease genes been identified (albeit their role might be of only peripheral importance), but some of our recent work has begun to tie up several loose threads that connect polyamine transport to intracellular vesicle trafficking and transport across vesicular membranes (i.e., VPAs). We are indeed characterizing the role of novel as well as known components of the *e*ndosomal *s*orting *c*omplexes *r*equired for *t*ransport (ESCRT) system (Hurley and Hanson 2010) identified via two independent screening processes (unpublished results). The genes thus identified clearly play an essential role in the regulation of polyamine transport, likely via an interaction with VPAs that virtually disrupts the PTS system completely, resulting in a phenotype almost undistinguishable from that of the well-known CHO-MG cells. We believe that this work will provide an important framework to the identikit picture of the mammalian PTS, including the definitive molecular identification of the relevant "general" permeases of both the PMPP and VPA types.

Acknowledgments Part of the work mentioned in this review that was performed by the authors was made possible through various grants from the Natural Sciences and Engineering Research Council of Canada, the Canadian Institutes of Health Research, the Cancer Research Society-Quebec and the National Institutes of Health (USA).

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