Structures of P-type Transporting ATPases and Chromosomal Locations of Their Genes

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ABSTRACT. P-type ATPases (E₁E₂-ATPases) are primary active transporters which form phospho-intermediates during their catalytic cycle. They are classified into P1 to P4 based on the primary structure and potential transmembrane segments. Although the classic P-type ATPases are cation transporters, two new members have recently been found; one is a flippase catalyzing the flip-flop movement of aminophospholipids, but the substrate and function of the other one remain unknown. It would be interesting to determine whether the cations and aminophospholipids are transported by similar or different mechanisms. P-type ATPases are believed to have been derived from a common ancestor, and their genes are found to be distributed in various chromosomal loci. However, gene duplication events can be traced from the tandem arrangement of genes and their linkage map. Na⁺/K⁺- and H⁺/K⁺-ATPases have not only closely related α subunits but also similar β subunits. Renal Na⁺/K⁺-ATPase has an additional subunit γ . Similar small polypeptides (phospholemman, Mat-8 and CHIF), which induce Cl⁻ and K⁺ currents, have been found. The idea of their functional and structural coupling with P-type ATPases, especially with H⁺/K⁺-ATPase, is intriguing. Each P-type ATPase must have specific domains or sequences for its intracellular trafficking (sorting, retention and recycling). Identification of such regions and studies on the molecules playing role in their recognition may facilitate the unveiling of various cellular processes regulated by P-type ATPases.

Prologue

It is now widely accepted that the hydrolysis of ATP is coupled to the transport of varieties of inorganic and organic ions across the various membranes surrounding cell and cytoplasmic organelle compartments. The transporting ATPases (or pump ATPases) play essential roles in this process. These ATPases are classified into four groups: P-type, F-type and V-type ATPases (1), and ABC-transporters (2). Information on the primary structures of these ATPases and the chromosomal locations of their genes is accumulating with the progress of the genome projects and the identification of the genes responsible for various human genetic

disorders (3). In this review, we focus on the P-type ATPases, which are believed to be derived from a common ancestral gene (4). Well-known P-type ATPase members such as Na⁺/K⁺-ATPase and Ca²⁺-ATPase play critical roles in regulating the intracellular ionic balance and driving physiological or biological processes (1).

P-type ATPases have the unique property of the formation of a high-energy phosphate-bond with the carboxyl moiety of a conserved aspartic acid residue. The term of "P-type" is derived from this phospho-intermediate, whose bond energy is utilized to drive cation movement through the membrane domain of the ATPase (1). P-type ATPase is a primary active transporter previously called E₁E₂-ATPase, since two conformational states (E₁ and E₂) were indicated by kinetic studies (4). However, novel members whose substrates are aminophospholipids instead of cations have been found (5, 6). We will discuss the structural features of P-type ATPases, which are classified into three (7) or four (5) groups, the chromosomal locations of

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Abbreviations used: FISH, fluorescence *in situ* hybridization; PCR, polymerase chain reaction; PMCA, plasma membrane Ca²⁺-ATPase; RACE, rapid amplification of cDNA ends; SCAMP, secretory carrier membrane protein; SERCA, sarco/endoplasmic reticulum Ca²⁺-ATPase; SNAP25, synaptosome-associated protein; V-AMP, vesicle-associated membrane protein.

their genes, and then small single transmembrane polypeptides.

Structural features of P-type ATPases

Each member of the P-type ATPase family transports specific cations coupled with ATP hydrolysis and the formation of a high-energy phospho-intermediate through the DKTG sequence. Although the molecular weights of P-type ATPases are diverse (70–200 k), they have conserved motifs, such as [TGE(S/A)] in the conformationally flexible loop, [DKTG(S/T)] in the phosphorylation domain, (TGDN) in the ATP-binding domain, and (MXGDGXNDXP) in the junction between the ATP-binding domain and the transmembrane segment (7). Recently, P-type ATPases were classified into P1 to P3 (Fig. 1) based on the type of transported cations and the putative transmembrane domains (7).

The P1-ATPases involved in the transport of heavy metals (Cd^{2+} , Cu^{2+} and Hg^{2+}) have only two transmembrane segments following the ATP-binding region, whereas P2-ATPases have six (M5–M10). P2-ATPases have four transmembrane segments (M1–M4) preceding the ATP-binding region. However, two hydrophobic regions, which are potentially transmembrane, are further inserted prior to the TGE(S/A) sequence in P1-ATPases. P1-ATPases are also unique in that they have metal binding motifs (one to six of GMXCXXC or MXXMDHSXM sequences) in their amino-terminal regions, and have a transmembrane CP(C/H) sequence and a HP(L/V) sequence, each of which precedes and follows a phosphorylation site (5, 8–12). It would be interesting to know how these motifs and sequences participate in metal transport. Although P1-ATPases generally transport heavy metals from the cytoplasm to the outside, there is a bacterial enzyme which catalyses the uptake of Cu^{2+} from outside (9). P1-ATPases are also named CP $\times$ -type ATPases (10).

P2-ATPases (non-heavy-metal-transporting P-type ATPases) have four additional transmembrane segments (M7–M10) compared with P1-ATPases. P2-ATPases are classified into two subgroups. The Na^+/K^+ - and H^+/K^+ -ATPase subgroups have a catalytic α subunit and a glycosylated β subunit. They exchange intracellular Na^+ or H^+ with extracellular K^+ . Four and three distinct Na^+/K^+ - and H^+/K^+ -ATPase α subunits, respectively, have been reported in mammals (Table I and the following). From recent studies, it was established that H^+/K^+ -ATPase α isoforms are expressed tissue-specifically in the stomach ($\alpha 1$) (13), colon and kidney ($\alpha 2$) (14, 15), and skin, kidney and brain, but not in the colon ($\text{ATP1A1}=\alpha 4$) (15, 16). The mRNAs for two splicing isoforms of H^+/K^+ -ATPase $\alpha 2$ increase coordinately in the kidney in response to chronic K^+ deprivation (14, 15). $\alpha 2$ is assem-

P1-ATPase

P2-ATPase

P3-ATPase

Fig. 1. Model of the transmembrane orientation of P-type ATPases. The transmembrane orientation of P1, P2 and P3-ATPases is shown (modified from ref 7). M₁–M₁₀ indicate transmembrane segments. Unique transmembrane segments for each group are indicated by closed boxes. However, the authors of Ref. 10 reported that the 1st and 2nd transmembrane segments of P1-ATPase are unique. Thus, the roles of these unique segments should be determined carefully in future studies. The positions of conserved amino acid sequences among P-type ATPases are schematically shown by small closed boxes with lower case letters [a, TGE(S/A); b, DKTG(S/T); c, TGDN; d, MXGDGXNDXP]. Characteristic sequences of P1-ATPase are shown by open boxes [I, CP(C/H); II, HP(L/V)]. The ovals are metal binding motifs located in the amino-terminal region of P1-ATPase. P4-ATPase has four or six transmembrane segments downstream of the phosphorylation site (small closed box "b"), and the extracellular segment between M1 and M2 is extremely long (100–200 amino acid residues) (5).

Table I. CHROMOSOMAL LOCATIONS OF P-TYPE ATPASE GENES.

ATPase	Subunit	Gene	Human	Mouse	Comments	Ref.
Na ⁺ /K ⁺	$\alpha 1$ (αA)	ATP1A1	1p11–13	3	ubiquitous	(26)–(36)
	$\alpha 2$ (αB)	ATP1A2	1cen–q32	1	neural, muscle	
	$\alpha 3$	ATP1A3	19q13.2	7	neural	
	αD	ATP1AL1	13q12–14		brain, kidney, skin H ⁺ /K ⁺ -ATPase ($\alpha 4$)	
	αC	ATP1AL2	1q _(next to $\alpha 2$)		testis	
	$\beta 1$	ATP1B1	1q	1		(27), (29),
	$\beta 2$	ATP1B2		11		(30), (36)
	$\beta 3$	ATP1B3		7		
	$\beta 1^*$	ATP1BL1	4		pseudogene	
H ⁺ /K ⁺	α ($\alpha 1$)	ATP4A	19q13.11	7		(34), (37)
	β	ATP4B	13q34	8		
Cu ²⁺		ATP7A	Xq13.3	X	Menkes disease	(3), (37)–(40)
		ATP7B	13q14.3	8	Wilson's disease	
Ca ²⁺ (sarco/endo-plasmic reticulum)		ATP2A1 (SERCA1)	16p12.1–12.2	7	Brody disease fast-twitch [adult (1a), neonatal (1b)]	(41)–(44)
		ATP2A2 (SERCA2)	12q23–24.1		cardiac/slow-twitch (2a), smooth/non-muscle (2b)	
		ATP2A3 (SERCA3)	17p13.3		non-muscle cells	
Ca ²⁺ (plasma membrane)		ATP2B1 (PMCA1)	12q21–23		ubiquitous	(45)–(47)
		ATP2B2 (PMCA2)	3p25–26		neuronal tissues	
		ATP2B3 (PMCA3)	Xq28		neuronal tissues	
		ATP2B4 (PMCA4)	1q25–32		ubiquitous	

bled with Na⁺/K⁺-ATPase $\beta 1$ in the renal medulla and distal colon (17). A structurally different H⁺/K⁺-ATPase α has been reported in the toad bladder (18). Members of the other P2-ATPase subgroup transport divalent (Ca²⁺ or Mg²⁺) or monovalent (Na⁺ or H⁺) cations from the cytoplasm to the extracellular compartment. The plasma membrane (calmodulin-activated PMCA1, 2, 3 and 4) and sarco-endoplasmic reticulum (thapsigargin-sensitive SERCA1, 2 and 3) Ca²⁺-ATPases have not only isoforms but also splicing variants derived from a single gene (19, 20). The residues important for cation binding and transport by SERCA1 have been carefully studied and identified to be Glu³⁰⁹ in M4, Glu⁷⁷¹ in M5, Asn⁷⁹⁶, Thr⁷⁹⁹ and Asp⁸⁰⁰ in M6, and Glu⁹⁰⁸ in M8 (20).

KdpB-ATPase, which is a part of a complex involved in K⁺ uptake by *Escherichia coli* (21), is the only member of the P3-ATPase family. The carboxy-terminal region of KdpB is short, as indicated by the reduced number of transmembrane segments compared

with P2-ATPases. The amino-terminal hydrophilic region of P2-ATPases is suggested to be important for intramolecular interactions which could be essential for the conformational transition associated with cation efflux (7). However, this region of KdpB is also truncated.

Expansion of P2-type Ca²⁺-ATPases

Phylogenetic analysis suggests that there are two further groups of Ca²⁺-ATPases in addition to PMCA and SERCA (22). A third group [yeast Golgi PMR1 (plasma membrane H⁺-ATPase related) (5) and rat SPCA (secretory pathway Ca²⁺-ATPase) (23)] comprises the secretory pathway Ca²⁺-ATPases, which may represent the most ancient and widespread class of Ca²⁺-ATPases despite its being the least recognized. Golgi calcium, which seems to be important in the exocytic or secretory pathway, is stored by this Ca²⁺-ATPase. A fourth group found only in lower eukaryotes and chloroplasts exhibits significant similarities to PMCA, al-

though it lacks a calmodulin-binding regulatory domain (22). The members are all located in intracellular acidic compartments such as vacuoles, suggesting their roles in the detoxification of high concentrations of Ca^{2+} .

Finding of novel P-type ATPases

The above P-ATPases are all cation transporters. However, flippase (aminophospholipid translocase) found in bovine chromaffin granules (ATPase II) surprisingly belongs to the P2-ATPase family (6). Flippase catalyses the flip-flop movement of aminophospholipids such as phosphatidylserine and phosphatidylethanolamine between the inner and outer leaflets of a phospholipid bilayer coupled with ATP hydrolysis (24, 25). Similar ATPases are Drs2 (*yeast*), pfATPase 2 (*Plasmodium falciparum*), and the *Caenorhabditis elegans* ATPase on the CELT24H7.5 locus. The hydrophilic residues, important for cation binding and transport determined for SERCA1, are difficult to assign in the transmembrane regions (M4 and M6) of flippase (5, 6), suggesting that the transport mechanisms for cations and phospholipids could be different. Thus, it would be of interest to examine the mechanism underlying phospholipid flip-flop by P2-ATPases.

A total of 16 open reading frames encoding P-type ATPases have been identified in the yeast genome (5). Two members whose substrates are unknown are classified as P4-ATPases, and are homologous to ATPase I from *P. falciparum* and the *C. elegans* ATPase on the CEWO8D2 (5). P4-ATPases have an unusually long extra-membrane segment of 100–200 residues between transmembrane segments M1 and M2. The rest of the ATPases are P1-ATPases (2 members) and P2-ATPases (12 members). P2-ATPases could be transporters for either H^+ (2 members), Ca^{2+} (2 members), Na^+ (3 members), or possibly aminophospholipids (5 members including Drs2). Why the yeast has so many flippases and how these proteins participate in physiological processes such as vesicular sorting and membrane rearrangement are unknown.

Chromosomal location of P-type ATPase genes

It is interesting to consider how divergent P-type ATPase genes are dispersed from the view-point of chromosomal evolution and whether their evolution can be traced by comparing their locations on chromosomes. We compiled the gene mapping data for human and mouse P-type ATPases in Table 1 (3, 26–47). As for human Na^+/K^+ -ATPase, four genes (αA , αB , αC and αD) were identified in an early study (26). However, it is now believed that αD (=ATP1AL1) encodes H^+/K^+ -ATPase (16). Two Na^+/K^+ -ATPase genes [αB (= $\alpha 2$, ATP1A2) and αC (=ATP1AL2)] are located in tandem, suggesting that a gene duplication event could

have occurred. Furthermore, closely related linkage groups which carry genes for the immunoglobulin superfamily members (BCM1, CD2 and LFA3) and ATPases (ATP1A2 and ATP1A1) were demonstrated by genetic linkage studies (31, 48); one linkage group possesses the BCM1 and ATP1A2 genes, which are conserved in mouse chromosome 1 and human chromosome 1q. Another group carries the genes for CD2 and LFA3, and the ATP1A1 gene on chromosomes 3 (mouse) and 1p (human). These findings suggest that a chromosome region including the precursors of the genes for the immunoglobulin superfamily members and ATPase duplicated and gave rise to the linkage groups now observed. The duplicated regions may have stayed together on chromosome 1 in man (with the insertion of a centromere), while they seem to have dispersed with the formation of chromosomes 1 and 3 in the mouse. Similarly, the rat ATP1A1 and ATP1A2 genes are located on chromosomes 2 and 13, respectively (49, 50).

A member of the H^+/K^+ - and Na^+/K^+ -ATPase subfamily has a small β subunit in addition to the catalytic α subunit. The gene organization of the corresponding subunits of both ATPases is highly conserved (51, 52), suggesting that the α and β subunit genes, respectively, evolved from the same ancestral gene. Actually, both genes encoding the gastric H^+/K^+ -ATPase α and Na^+/K^+ -ATPase $\alpha 3$ subunits (ATP4A and ATP1A3, respectively) are located on the human chromosome 19 and mouse chromosome 7 (34). In this study, we precisely mapped the human gastric H^+/K^+ -ATPase α subunit gene to chromosome 19q13.11 (Fig. 2). The genes for the Na^+/K^+ -ATPase $\beta 1$ and H^+/K^+ -ATPase β subunits (ATP1B1 and ATP4B, respectively) are associated with the α subunit genes (ATP1A2 and ATP1AL2, and ATP1AL1, respectively). Other P-type ATPase genes (for catalytic subunits) are also often distributed on the same chromosomes: gene pairs ATP1AL1 and ATP7B, ATP1A2 and ATP2B4, ATP2A2 and ATP2B1, and ATP7A and ATP2B3 are located on chromosomes 13q, 1q, 12q, and Xq, respectively.

Small single transmembrane polypeptides and P-type ATPases

The presence of a γ subunit in addition to α and β subunits has long been discussed for Na^+/K^+ -ATPase (53). Recently, a polypeptide consisting of 58 amino acids was reported to be a potential γ subunit for various vertebrate Na^+/K^+ -ATPases (54–56). This polypeptide having a single transmembrane domain is colocalized with the α subunit in the basolateral membrane of renal epithelial cells (54), and is copurified with the α and β subunits, and forms the cardiac glycoside binding site together with the α subunit (54, 55). The

Fig. 2. Localization of the human gastric H^+/K^+ -ATPase α subunit gene on R-banded metaphase chromosomes determined by FISH. From a phage clone (51), we prepared a DNA probe for mapping, which is based on FISH combined with replicated prometaphase R-bands (74, 75). To suppress repetitive sequences contained in the cosmid clones, we used 2-fold excess human Cot-1 DNA (76). Microphotography (Filter combination, Nikon B-2A) was performed with Provia 100 film (Fuji, ISO 100). We observed 100 typical R-banded chromosomes. The signals of the clone were localized to the p13.11 band of chromosome 19. No signal was detected on other chromosomes. Thus, the human H^+/K^+ -ATPase α subunit gene was assigned to chromosome 19q13.11.

peptide stabilizes the E_1 conformation of Na^+/K^+ -ATPase (57). Cardiac sarcoplasmic reticulum Ca^{2+} -ATPase is inhibited by a small membrane protein composed of 52 amino acids, phospholamban (58). The

phosphorylation of phospholamban by cAMP-dependent protein kinase and Ca^{2+} /calmodulin-dependent protein kinase reverses the inhibition. However, there is no sequence similarity between the Na^+/K^+ -ATPase γ subunit and phospholamban (54). Furthermore, the membrane topologies of these proteins are different: the carboxy- and amino-terminal regions of the γ subunit and phospholamban, respectively, are postulated to be in the cytoplasm (58, 59).

Three polypeptides closely related to the γ subunit are now known. The major sarcolemmal substrate for cAMP-dependent protein kinase and protein kinase C in the myocardium is phospholemman, a small transmembrane protein (59). A mature form (72 amino acid residues) is formed through the cleavage of 20 amino terminal residues. Mat-8 (mammary tumor, 8 kDa) is a marker of a cell type preferentially transformed by *Neu* or *Ras* oncoproteins (60, 61). CHIF (channel-inducing factor) is induced in the colon by corticosteroids (62). The extracellular and transmembrane domains of phospholemman, Mat-8 and CHIF are homologous to those of the γ subunit of Na^+/K^+ -ATPase (Fig. 3). Interestingly, phospholemman (63) and Mat-8 (61) induce a Cl^- current while CHIF (62) induces a K^+ current, when expressed in *Xenopus* oocytes. Since it seems unlikely that they form membrane pores by themselves, they may function as modulators capable of activating endogenous oocyte channels. However, the roles of these small polypeptides *in vivo* remain unknown.

Although it has not been determined whether or not the γ subunit of Na^+/K^+ -ATPase exhibits similar activity, it is tempting to postulate that these small single transmembrane polypeptides are associated with cation

Fig. 3. Alignment of the amino acid sequences of Na^+/K^+ -ATPase γ subunits and related small proteins. The amino acid sequences of the Na^+/K^+ -ATPase γ subunits (NK- γ) [sheep, mouse, rat, bovine (54), and human (55)], phospholemman (PLM) [rat and human (77), and canine (59)], CHIF (62), and Mat-8 [mouse (60), human (61), and pig (this study)] are aligned [b, bovine; c, canine; h, human; m, mouse; p, pig; r, rat; s, sheep]. The potential transmembrane domain was thick-underlined. Conserved residues are indicated by asterisks. The arrowhead indicates the cleavage site of the signal sequence of canine phospholemman. The amino acid sequence of pig Mat-8 was deduced in this study from cDNA, which was prepared from total RNA of pig gastric mucosa (78). The oligonucleotide mixture [3'-gg(gatc)aa(ag)at(ag)at(ag)ct-5'] corresponding to the conserved PFYYDYE sequence was synthesized and used as the PCR-primer for 5'-RACE. The full-length cDNA was further amplified by PCR using the 5'-side primer (designed from the amplified sequence) and an oligo-dT₁₄ primer. The nucleotide sequence has been submitted to the GenBank Data Bank with accession number AB015759.

transport by P-type ATPases and modulate channel(s). In gastric parietal cells, the coupling of Cl^- and K^+ movements through channels with the antiport of H^+ and K^+ by H^+/K^+ -ATPase is essential for acid (HCl) secretion (64, 65). Mouse Mat-8 is transcribed at high levels in the uterus, stomach and colon, while phospholemman is transcribed in the heart, skeletal muscle and liver (63). Here we have cloned a cDNA for Mat-8 from pig gastric mucosa by means of a homology cloning method with degenerate primers designed for conserved regions of the γ subunit, phospholemman and Mat-8 (Fig. 3). Thus it would be interesting to determine whether or not such small single transmembrane polypeptides, especially Mat-8, function with the gastric H^+/K^+ -ATPase to secrete gastric acid from parietal cells.

Epilogue

The aspect that should be pointed out lastly is the mechanisms which govern the distribution of various P-type ATPases in specific organelles and membrane domains. Such localization is clearly important for regulation of the transport functions of the ATPases (66). P-type ATPase genes have not only been distributed to different chromosomal loci but also potentially evolved by capturing the DNA sequences, which encode specific domains or amino acid sequences for sorting ATPases to different cellular locations. Na^+/K^+ -ATPase seems to interact with the intracellular sorting machinery and to be targeted to the basolateral surface, where the cytoskeleton can directly participate in retention of the Na^+/K^+ -ATPase (66). Residues near the carboxy-terminal of the Na^+/K^+ -ATPase α -subunit are necessary for the formation of the α - β complex (67). In the Na^+/K^+ - and gastric H^+/K^+ -ATPases, the amino terminal regions of the α subunit are rich in lysine residues (68). The functional significance of phosphorylation or dephosphorylation of Ser and Tyr residues in this region of H^+/K^+ -ATPase for catalysis and/or sorting is not known (69), although removal of the Lys-rich sequence of Na^+/K^+ -ATPase affected the conformational equilibrium which resulted in a change in the apparent affinity for K^+ (70).

Tubulovesicles enriched in gastric H^+/K^+ -ATPase fuse with the apical plasma membrane of parietal cells to form secretory canaliculi in response to secretagogues. This fusion event inserts the H^+/K^+ -ATPase into the apical surface to secrete HCl into the lumen of the gastric gland (71). A sorting signal has been delimited to a sequence of eight amino acids present in the fourth predicted transmembrane domain of the α subunit, and apical delivery of the subunit may be determined by the ability of this sequence to be partitioned into glycosphingolipid-rich membrane patches (66). The cessation of acid secretion is associated with the re-

trieval of the H^+/K^+ -ATPase from the cell surface and the regeneration of tubulovesicles that are competent for another round of fusion (71). The gastric H^+/K^+ -ATPase, VAMP-2, Rab11, Rab25 and SCAMPs, but not syntaxin 1A/1B or SNAP25, are present on the tubulovesicles, supporting the apical membrane recycling model of parietal cell secretion (72). The cytoplasmic tail of the H^+/K^+ -ATPase β subunit includes the sequence, FRQY, which is an internalization signal (YXRF or FRXY), and the ATPase no longer could return to the tubulovesicles when this signal was disrupted (73).

Each P-type ATPase member shows a high degree of structural similarity and commonly forms a phospho-intermediate. The interesting points are the finding of aminophospholipid flippases as well as members of the P-type ATPase family with unknown functions. It must be determined in the near future whether or not the recognition of substrates (phospholipids and cations) and their transport occur through essentially the similar processes or complete different mechanisms. Examination of the functional and/or structural coupling between small membrane proteins and P-type ATPases as well as of the mechanisms of their intracellular trafficking is also important for understanding cellular physiological and biological processes regulated by P-type ATPases.

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