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FOR THE RECORD

An unexpected structural relationship between integral membrane phosphatases and soluble haloperoxidases

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Abstract: The mechanism of a membrane-bound enzyme important in phospholipid signaling, type 2 phosphatidic acid phosphatase, is suggested by sequence motifs shared with a soluble vanadium-dependent chloroperoxidase of known structure. These regions are also conserved in other soluble globular and membrane-associated proteins, including bacterial acid phosphatases, mammalian glucose-6-phosphatases, and the *Drosophila* developmental protein Wunen. This implies that a similar arrangement of catalytic residues specifies the active site within both soluble and membrane spanning domains.

Keywords: Aur1p; bromoperoxidase; Dri42; PAP2; PgpB; vanadate

Phosphatidic acid phosphatase (PAP) plays a crucial role in signal transduction (Exton, 1994; Brindley & Waggoner, 1996; Kocsis & Weselake, 1996). PAP converts phosphatidic acid (PA) into diacylglycerol, a lipid second messenger that activates protein kinase C (Nishizuka, 1984). Moreover, PAP isolated from rat liver plasma membranes was recently found to be a lipid phosphomonoesterase that hydrolyzes (in addition to PA) three other phospholipids: ceramide 1-phosphate, lysophosphatidate, and sphingosine 1-phosphate (Waggoner et al., 1996). PA and these phospholipids are also thought to be involved in signal transduction and are bioactive when added externally to cells (Brindley & Waggoner, 1996; English et al., 1996; Flores et al., 1996; Moolenaar, 1995a, 1995b; Moolenaar et al., 1992), and two products of these reactions, ceramide and sphingosine, are also potent signaling molecules (Brindley & Waggoner, 1996). Hence, PAP performs an important function in signal transduction by regulating levels of these phospholipids and their dephosphorylated derivatives.

Plasma membrane bound (type 2) phosphatidic acid phosphatase (PAP2) is the isoform of PAP thought to be involved in cellular signal transduction. The mouse PAP2, which was recently sequenced (Kai et al., 1996), is closely related to two other membrane proteins: the *Drosophila* protein Wunen, which is responsible for guiding germ cell migration from the gut into the gonads during embryonic development (Zhang et al., 1997), and the rat Dri42 protein, whose expression is increased during epithelial development and differentiation (Barila et al., 1996). Thus, these PAP2 homologs are also likely to function as phosphatases in phospholipid metabolism and signaling.

Recently, Stukey and Carman (1997) described shared signature motifs in a super-family of proteins that includes PAP2 and vanadium-dependent chloroperoxidase (CPO), whose structure is known (Messerschmidt & Wever, 1996). Here, their findings are corroborated using a statistically based search and multiple sequence alignment program Probe (Neuwald et al., 1997) and extended through further analysis. Surprisingly, this analysis suggests that membrane-associated PAP2-like proteins share conserved structural elements and a similar catalytic mechanism with related soluble globular enzymes.

Starting with the mouse PAP2 sequence, Probe returned an alignment of three conserved regions (Fig. 1) corresponding to structural elements and catalytic residues near the active site of CPO (Fig. 2a). These regions were previously found to be conserved between CPO and bromoperoxidase (Messerschmidt & Wever, 1996), and contain the signatures reported by Stukey and Carman. Also, the second of these regions coincides with a conserved pattern (GSYPSGHT) found in bacterial acid phosphatases (Thaller et al., 1994), which belong to this super-family. Many highly conserved positions in the alignment correspond to residues directly interacting with vanadate at the active site of CPO (see below).

In addition to PAP2, its close homologs, and bacterial phosphatidylglycerophosphatase, other putative integral membrane proteins in the superfamily (whose functions are unknown) may also be involved in phospholipid metabolism. For example, two closely related bacterial proteins from *Rhodococcus erythropolis* (g1666180 and g1666188) contain a second domain with highly significant similarity ($p < 10^{-10}$) to sequence motifs (not shown) conserved in diacylglycerol kinases, which catalyze the reverse reaction from PAP (Kanoh et al., 1996). Furthermore, the presence of PAP2-like motifs in the yeast putative transmembrane protein Aur1p suggests a link between phospholipid metabolism and the organization and stabilization of microtubules (inactivation of the *aur1* gene results in the disappearance of microtubules) (Hashida-Okado et al., 1996). However, another PAP2-related protein, glucose-6-phosphatase, appears to be membrane-associated only to ensure tight coupling of its phosphatase activity to glucose transport (Lei et al., 1996).

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protein	start	Motif A		Motif B		Motif C	end		description
1VNC (pdb)	338	VDVACTDAGIFSWKEKWEF-EFWRP	(37)	AYPSGHATFGGAVFQMVRRY	(63)	LMFENAISRIFLGVHWRFDAAAARDILIP	510	(99)	chloroperoxidase (Curvularia inaequalis)
BRPO	9	LIGAAELAQRSSWYQNWQVHRFARP	(62)	SYPSGHATQNGAFATVLKAL	(38)	LAVNVAFGRQMLGIHYRFDGIQGLLLGET	182	(56)	bromoperoxidase (Ascophyllum nodosum)
P26976	108	KNLLTMGGYYATASAKKYY-MRTRP	(21)	SYPSGHTAYGTLLALVLSEA	(10)	RGWEFGQSRVICGAHWQSDVDAGRYVGAV	211	(39)	acid phosphatase (Salmonella typhimurium)
g532976	109	TQVLQDSHDYAVRNAKEYY-KRVRP	(21)	SYPSGHASFGWAVALILAEI	(10)	RGYEFGESRVICGAHWQSDVEAGRLMGAS	212	(34)	ATP diphosphohydrolase (Shigella flexneri)
g1487873	105	FLFGVSASQSLTDIAKYTI-GSLRP	(37)	SFYSGHSSFSMYCMLFVALY	(23)	FSIYVGLSRVSDYKHHWSDVTVGLIQGAA	237	(46)	PAP2 (mouse)
g1769525	117	YAFGAVLSQLTTDIAKYSI-GRLRP	(46)	SFPSGHSSFTFFAMVYLALY	(23)	VAWYTALSRVSDYKHHWSDVLAGSLIGSI	258	(42)	Wunen (fly)
g1684745	134	FLFGCAISQSFTDIAKVSI-GRLRP	(37)	SFFSGHASFSMFTMLYLVLY	(23)	MAFYTGLSRVSDYKHHPSDVLAGFAQGAL	266	(46)	Dri42 (rat)
Q10022	129	SQIGFVMNIALNIVTKHVV-GRLRP	(39)	SFYSGHSAVSLYCATWSALY	(24)	IGLGISFSRITDNKHHWSDVLVGIFIGIF	264	(77)	Ysx3 (worm)
g1072342	146	YLLMYAACTFAMEFLKCYV-GRLRP	(42)	SFPSGHTAAAFHVFLFVYIY	(26)	WTVFCAVTRVTDNWHFPTDVLGGVILAVV	286	(32)	F13E6.5 (worm)
g790400	150	YLAGLLIVQIFVDTIKLMT-GYQRP	(42)	TFPSLHAVVSSYAACFASLY	(23)	LCIVDSFSRINGYKNHWRDIWVAWVIGIF	287	(109)	T06D8.3 (worm)
g927773	121	LMLIISINAALTGALKLII-GNLRP	(39)	STPSGHSSFIVSTMGFTYLW	(17)	LALVVMVSRVIDHRHHWYDVVSGAVLAFL	249	(25)	D9719.9 (yeast)
g1332640	103	LSLAWFSTSFFTNFIKNWI-GRLRP	(37)	TTPSGHSSESFAGLGYLYFW	(25)	GAALIALSRTQDYRHHFVDVILGSMLGYI	237	(52)	ORF (yeast)
P18201	82	LAAAILVGQGVKSWIKDKV-QEPRP	(52)	AFPSGHTMFAASWALLAVGL	(15)	WATGVMGSRLLLGMHWPRDLVVATLISWA	221	(33)	PgpB (E. coli)
g1666188	66	MLALAGSSALANGILKPLF-PRRRP	(18)	SFPSGHSSSAAAFTAAVAME	(10)	LAAAVAYSRVHNGVHWPSDVFAGLAVGGA	166	(321)	ORF (Rhodococcus erythropolis)
P35575	61	LLWVAVIGDWLNLVFKWIL-FGQRP	(29)	GSPSGHAMGTAGVYYVMVTS	(28)	VQLNVCLSRIYLAAHFPHQVVAGVLSGIA	190	(167)	G6Pase (human)
S01073	63	TVLATTASGALNLALKHLF-NTPRP	(10)	GFPSGHAQVSASFWSILTLL	(12)	MVTGVSLSRLYLRAHYPIDVAGGVVIGLT	157	(68)	ORF (Desulfurococcus mobilis)
g409159	68	LAYAAAFTSGLNGGIKRIL-KIPRP	(15)	STPSGHSSISAFIYPAVLFY	(33)	LPLLVGFSRVYLGVHYPTDVLLGWGLGAF	188	(154)	phosphatase (Treponema denticola)
g1001247	119	VFATLGILLVIDQVLKPFF-NRTRP	(10)	SFPSGHAAGGVVFYFYLAFL	(18)	WVGFIGIASMYCRVHWATDILAGYGVGFI	219	(21)	ORF (Synechocystis sp.)
S32217	92	FTAVMVGSPLLNLMVKLFF-QRARP	(11)	SFPSGHAMNAFSLYGILTFL	(20)	MILSIGISRIYLGVHYPSDIIAGYLAGGC	195	(21)	ORF (Bacillus megaterium)
g1049369	68	LNLGLYFDLILIAIIKFYF-HRERP	(15)	SFPSGHSSRAAMLLVMAYNA	(10)	FPLVVGLSRVALGRHYITDVLAGIFIGYL	165	(180)	T13C5.6 (worm)
P53223	63	VAFGQLMNEIFNNVIKNII-KQPRP	(16)	GMPSAHSQFMGFCFTYNSLK	(22)	LSFCVCFSRVYLHYHNLDQVIVGFSVGAL	173	(66)	Yg1p (yeast)
P47013	113	MVYILGYSIYLSGFFKDYW-CLPRP	(18)	GAPSSHTANATGVSLLFLYN	(21)	YYMTLVFGRIYCGMHGILDLVSGGLIGIV	224	(185)	Yjn4p (yeast)
g1788500	79	CSLLAMGMLMLSFWLKENA-PGRRR	(54)	SFPGDHGMMLLIFSAFMWRY	(11)	IFVVFAFPRVMIGAHWFTDIIVGSMTVIL	216	(33)	ORF (E. coli)
g1515352	250			AFPSLHSGCATMEALFFCYC	(10)	YVCWLWWSTMYLTHHYFVDLMAGSVLSYV	308	(93)	Aur1p (yeast)
P38954	293			AVPSLHSAIAFQCFLFLVSR	(132)	YIILQWWATMYLDHHYRFDLFVGVLYAMT	473	(54)	Yd05p (yeast)
g1787679	129			QSPSLHIILCWLLWRHFRQH	(11)	WFLLIAISTLTTWQHHFIDVITGLAVGML	188	(242)	ORF (E. coli)
g747659	70			TGFSGHSALSAAFWPIFLWL	(19)	LAAVVGYSRLVIHAHSFSEVIAGLLLGAA	137	(72)	ORF (Klebsiella pneumoniae)
g1591375	142			EIPSLHTAYSFLLALHFKDE	(9)	LAILIPISTLIMGMHWIVDVITGVLYGYI	199	(40)	ORF (Methanococcus jannaschii)
P42334	102			SFPSDHTILFFSIGFLIFLF	(10)	LAFAVGISRIWSGVHYPLDVAAGALLGVL	160	(43)	BcrB (Bacillus licheniformis)

Fig. 1. Alignment of representative sequences in the PAP2 super-family. In all about 50 proteins were detected in the February 10, 1997, release of the NCBI non-redundant database (those sequences not shown in the figure, which are mostly hypothetical proteins, are closely related to at least one of the sequences shown). Conserved residues are highlighted (residues with catalytic side chains in CPO are highlighted in red). Known non-integral membrane proteins are boxed. Numbers in parentheses are gap lengths. Sequence similarities are significant at the $p \leq 0.01$ level; p-values are based either on pairwise similarity (Karlin & Altschul, 1993) or on similarity to an alignment model (Neuwald et al., 1995) that lacks the sequence in question and pairwise similar sequences. The partial sequence for *Ascophyllum nodosum* bromoperoxidase (BRPO) (Vilter, 1995) lacks a statistically significant match to motif A; the region shown (which matches motif A with a p-value of 0.5) was manually aligned by Messerschmidt and Wever (1996) based on structural considerations. This region appears to be obscured by an insertion and by a replacement of the functional lysine residue with an asparagine, which can also hydrogen bond to vanadate. Nevertheless, BRPO has clearly significant matches to motifs B and C ($p \approx 10^{-9}$ based on a single sequence comparison to a Probe alignment model constructed without the haloperoxidases). Several other proteins also lack significant matches to motif A, possibly due to similar structural rearrangements.

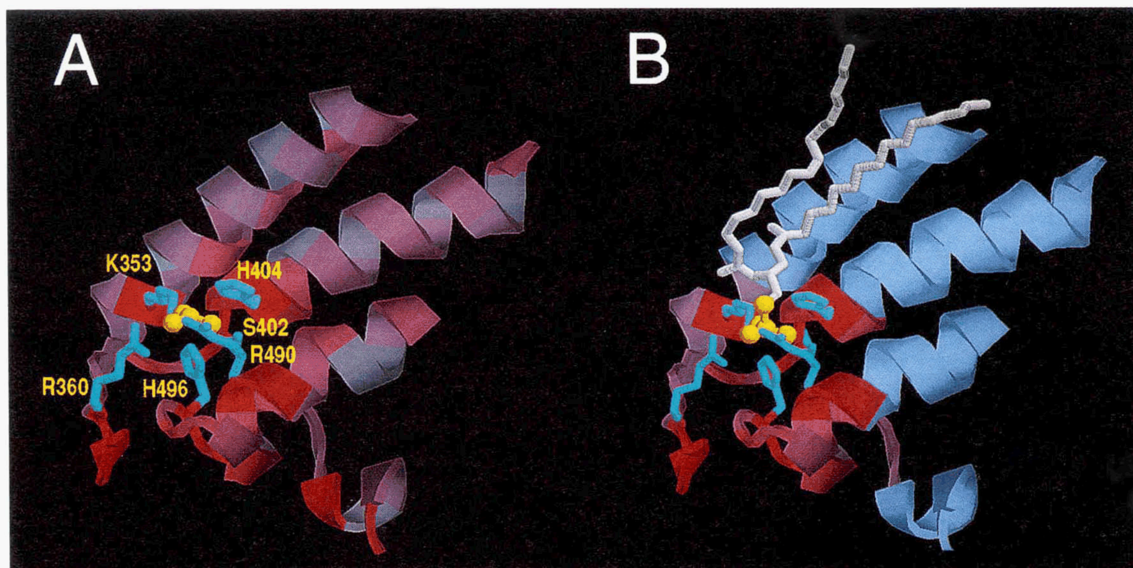

Fig. 2. **A:** Structure of the vanadium binding domain of chloroperoxidase (pdb: 1VNC). The vanadate ion (orange) is bound from below by His-496 and from above by azide (not shown). The backbone ribbon is shaded proportional to the degree of conservation at those positions in the alignment (Fig. 1) with red being most highly conserved. **B:** Hypothetical model of the active site of mouse PAP2 based on the CPO structure. Coloring scheme: vanadate (in **A**) and transition state phosphate (in **B**), orange (the central atoms correspond to vanadium and phosphorus, respectively); residue sidechains interacting with vanadate or phosphate, cyan; PAP2 regions (in **B**) predicted by the PHD program (Rost et al., 1995) to span the membrane, blue; diacylglycerol moeity of phosphatidic acid, light gray.

The conserved active site regions of CPO occur near the ends of three α-helices that are part of a four-helix bundle (Fig. 2a), the second of two four-helix bundles that make up the main structural components of CPO (Messerschmidt & Wever, 1996). Thus, these conserved structural elements constitute a subdomain of CPO, rather than a distinct domain. The residues in CPO that directly interact with vanadate or the peroxide substrate (Messerschmidt & Wever, 1996) are: a histidine (His-496) that directly binds to the vanadium atom; several residues hydrogen bonded to vanadate oxygens (Lys-353, Arg-360, Ser-402, the backbone nitrogen of Gly-403, and Arg-495); and the catalytic histidine (His-404), which is the only invariant residue. These residues are highly conserved in the PAP2 alignment (Fig. 1) and are completely conserved in PAP2, Wunen, Dri42, and the *Escherichia coli* PgpB protein, another lipid phosphomonoesterase with phosphotidylglycerophosphate, PA, lyso-PA, and diacylglycerol pyrophosphate phosphatase activities (Dillon et al., 1996).

As previously suggested (Stukey & Carman, 1997), the fact that residues critical for the function of CPO are conserved implies that these other proteins have similar structures and catalytic mechanisms. Such similarity between vanadium-binding peroxidases and phosphatases is not unexpected inasmuch as vanadate readily adopts a pentacoordinate structure that resembles the transition state structure of phosphate (Lindquist et al., 1973; Denu et al., 1996). In fact, vanadate is known to be an inhibitor of phosphate metabolizing enzymes including glucose-6-phosphatase and acid phosphatases (Stankiewicz et al., 1995).

A feasible catalytic mechanism for phosphomonoester hydrolysis can be devised based on the CPO structure. In this mechanism, three of the phosphate oxygens hydrogen bond to residues corresponding to the CPO vanadate oxygen binding sites (Fig. 2b); this is important for transition state stabilization during the reaction. Hydrolysis proceeds through two steps (Fig. 3). In the first step, a charge-relay system—which involves residues corresponding to Asp-500 and His-496 of CPO—establishes a histidine to phosphate N-P bond, while the catalytic histidine (His-404 in CPO) acts as an acid to facilitate phosphoester bond cleavage and release of the dephosphorylated substrate. In the second step, the catalytic histidine residue (now acting as a base) facilitates nucleophilic attack upon phosphorus by a water oxygen atom and cleavage of the intermediate N–P bond. This releases orthophosphate from the enzyme and frees up the active site for another round of catalysis.

Considerable portions of the conserved regions within PAP2 (Kai et al., 1996), Wunen (Zhang et al., 1997), and Dri42 (Barila et al., 1996) are predicted to span the membrane (Fig. 2b). This is surprising, inasmuch as the same arrangement of catalytic residues specifies the active site within soluble globular domains (e.g., the haloperoxidases, the bacterial acid phosphatases, and ATP diphos-

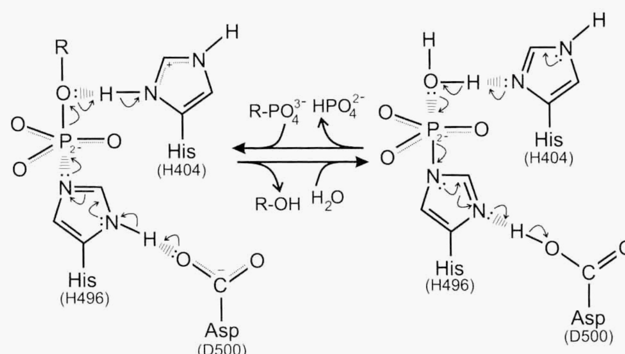

Fig. 3. Proposed catalytic mechanism for PAP2-related phosphatases. Residue positions in parentheses indicate corresponding active site residues within CPO.

phohydrolase). Assuming divergent evolution, this implies that catalytic activity was somehow retained during relocation of an ancestral enzyme from the membrane to a water-soluble environment, or vice versa. Indeed, the presence of collinear conserved motifs makes this more likely than convergent evolution, which often leads to distinct structural folds having similar active site configurations. This is seen, for instance, among structurally diverse hydrolases sharing similar serine protease-like catalytic triads (Ollis et al., 1992; Tong et al., 1996).

Because there appear to be no other reported examples of this kind, it is important to determine whether the six predicted membrane spanning regions (H1–H6) in these PAP2-like proteins are real. Fortunately, the Dri42 membrane spanning regions have been confirmed by in vitro co-translational insertion of Dri42–CAT fusion proteins into microsomal membranes (Barila et al., 1996). Based on the patterns of CAT and Dri42 postranslational glycosylation (which normally occurs within the lumen of the endoplasmic reticulum), the six predicted membrane spanning regions were indeed found to function either as signal/anchor (H1, H3, H5) or halt transfer signals (H2, H4, H6).

Thus, a model for PAP2, and related integral membrane proteins can be constructed by threading their sequences through the conserved structural elements of CPO (Fig. 2b). This suggests how hydrolysis of PA (or other phospholipids) could occur: by lateral diffusion through the membrane PA comes into contact with PAP2 such that the glycerolphosphate group (which is near the membrane surface) docks at the active site. The fatty acid tails of PA would then be positioned in the membrane near the PAP2 membrane spanning helices. After phosphate hydrolysis (by the mechanism described above) diacylglycerol would diffuse away—freeing the PAP2 active site for further catalysis.

Those regions that are not conserved throughout the PAP2 superfamily may be involved in regulation or substrate specificity. For example, two residues between motifs A and B in CPO (Trp-350 and Phe-397) provide a hydrophobic environment presumably to stabilize chloride binding (Messerschmidt & Wever, 1996). Similarly, several regions within PAP2 that are unconserved in the super-family as a whole contain patterns that are conserved in Wunen, Dri42, and Ysx3 (a closely related hypothetical roundworm protein). The most notable of these is the pattern RG[IF][FY]C[ND]D[ED]S[IL][KR] which, based on the predictions of Zhang et al. (1997) and Barila et al. (1996), is located between the first two transmembrane domains. This is on the same side of the membrane as the proposed active site, which corresponds to regions between the third and fourth and between the fifth and sixth transmembrane domains. This pattern is likely to specify a distinct function in these proteins, which may be linked to the developmental roles of the Wunen and Dri42 proteins.

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