Mutation of the Conserved Domains of Two Inositol Polyphosphate 5-Phosphatases†

Anne Bennett Jefferson‡ and Philip W. Majerus*

Division of Hematology−Oncology, Washington University School of Medicine, 660 South Euclid, Box 8125, St. Louis, Missouri 63110

Received February 2, 1996; Revised Manuscript Received April 3, 1996⊗

ABSTRACT: Two short amino acid motifs, WXGDXNXR and PXWCDRXL, define a large family of inositol polyphosphate 5-phosphatases. We tested the importance of seven of these conserved amino acids to substrate binding and catalysis by mutating each to alanine in the platelet 75 kDa inositol polyphosphate 5-phosphatase II (5-phosphatase II). Native and mutant forms of 5-phosphatase II were expressed in baculovirus-infected Sf9 cells, and the recombinant proteins were purified by Mono Q chromatography and studied for enzyme activity. Mutants D476A, N478A, D553A, and R554A had no detectable activity using all four known substrates for this enzyme. Mutants R480A, W551A, and I555A showed greatly reduced hydrolysis of Ins(1,4,5)P_3 when compared to native enzyme [K_m = 75 μM, V_m = 8300 nmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} (mg of protein)$^{-1}$]. Mutants W551A and I555A had a K_m for Ins(1,4,5)P_3 hydrolysis similar to that of the native enzyme (35 μM and 81 μM, respectively), suggesting that these amino acids do not play a role in binding substrate. By contrast, mutant R480A had both increased K_m (634 μM) and decreased V_m [855 nmol of Ins(1,4,5)P_3 hydrolyzed min^{-1} (mg of protein)$^{-1}$]. As judged by measurement of K_m, mutant R480A retained normal binding of Ins(1,3,4,5)P_4, suggesting that the arginine in motif 2 has a greater role in Ins(1,4,5)P_3 binding than in Ins(1,3,4,5)P_4 binding. Mutant I555A bound Ins(1,3,4,5)P_4 with 8-fold reduced affinity. These mutations markedly reduced 5-phosphatase II hydrolysis of the three other substrates, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. We also tested a mutation comparable to D553A, D460A, in the 110 kDa form of the signaling inositol polyphosphate 5-phosphatase (5SIP110). 5SIP110 D460A had no detectable enzyme activity but retained the ability to bind GRB2. These results are consistent with a role for these conserved amino acids in substrate binding and catalysis.

The inositol polyphosphate 5-phosphatase (5-phosphatase)[1] family of enzymes is defined by two conserved motifs, WXGDXNXR and PXWCDRXL (recently two yeast genes were found with I in the last position instead of L) (Laxminarayan et al., 1994; Jefferson & Majerus, 1995). cDNA clones for nine family members have been identified, five of which have been characterized with regard to substrate specificity. There are four substrates that are hydrolyzed by these enzymes including Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. The five characterized enzymes include 5-phosphatase I that was originally identified in human platelets amd was subsequently cloned from several tissue sources (Laxminarayan et al., 1994; Connolly et al., 1985, 1987; DeSmedt et al., 1994; Verjans et al., 1994). This enzyme hydrolyzes only Ins(1,4,5)P_3 and Ins(1,3,4,5)-P_4. 5-Phosphatase II was also first identified in platelets and hydrolyzes all four known 5-phosphatase substrates (Jefferson & Majerus, 1995; Matzaris et al., 1994; Jackson et al., 1995; Mitchell et al., 1989; Ross et al., 1991). OCRL, the 5-phosphatase mutated in Lowe syndrome, also hydrolyzes all four substrates although the lipid substrates are much preferred (Attree et al., 1992; Zhang et al., 1995; A. B. Jefferson, unpublished data). A novel 5-phosphatase which contains an SH2 domain and binds GRB2 (SHIP or 5SIP145) and a possible alternatively spliced version which lacks the SH2 domain (5SIP110) have recently been cloned (Kavanaugh et al., 1996; Damen et al., 1996). These 5-phosphatases hydrolyze only Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 (Kavanaugh et al., 1996; Damen et al., 1996). Synaptojanin is a 5-phosphatase that contains a region homologous to Sac 1, a yeast protein implicated in regulation of phospholipid metabolism and actin cytoskeleton. Synaptojanin hydrolyzes three of the four substrates [PtdIns(3,4,5)P_3 has not yet been tested with this enzyme] (McPherson et al., 1996).

The large number of enzymes with differing substrate preferences suggests that 5-phosphatases serve a variety of functions in intracellular signaling. The best known function is as a signal terminating enzyme in calcium mobilization mediated by Ins(1,4,5)P_3 (Majerus, 1992; Berridge, 1993). Other less well-defined functions include regulating Ras pathway activation via an Ins(1,3,4,5)P_4-binding GTPase activating protein 1 (GAP1) (Cullen et al., 1995) and hydrolysis of the 5-phosphate containing inositol lipids. PtdIns(4,5)P_2 serves as a pool for hydrolysis by phospholipase C, and both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 have also been implicated as signaling molecules (Lee & Rhee, 1995; Divecha & Irvine, 1995). Both may regulate cellular

† This research was supported by Grant HL14147 (Specialized Center for Research in Thrombosis) and Grant HL16634 from the National Institutes of Health.

* To whom correspondence should be addressed. Telephone: 314-362-8801. Fax: 314-362-8826. E-mail: phil@wums.wustl.edu.

‡ Current address: Chiron Corp., 4560 Horton St., Emeryville, CA 94608.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: 5-phosphatase, inositol polyphosphate 5-phosphatase; PTB, protein tyrosine binding; SH2, src-homology 2; β-ME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; OCRL, oculocerebrorenal lowe; 55IP110, signaling inositol polyphosphate 5-phosphatase; GST, glutathione *S*-transferase; HA, hemagglutinin; GRB2, growth factor receptor binding protein 2.

S0006-2960(96)00262-0 CCC: $12.00

secretion (Eberhard et al., 1990; Hay et al., 1995) and actin assembly (Jamney & Stossel, 1989; Jamney et al., 1992; Hartwig et al., 1995) and bind to proteins containing PTB, SH2, and pleckstrin homology domains (Harlan et al., 1994; Zhou et al., 1995; Rameh et al., 1995). PtdIns(3,4,5)P_3 may also regulate several enzymes including c-Akt, which is stimulated following activation of phosphatidylinositol 3-kinase, and members of the protein kinase C enzyme family (Franke et al., 1995; Burgering & Cotter, 1995; Nakanishi et al., 1993; Toker et al., 1994, 1995).

All of the 5-phosphatases contain two conserved motifs that may be involved in substrate binding and catalysis (Laxminarayan et al., 1994; Jefferson & Majerus, 1995). Because of the likely importance of members of this enzyme family in numerous cell signaling pathways, we undertook a mutational analysis of conserved amino acids in two 5-phosphatase enzymes.

EXPERIMENTAL PROCEDURES

Materials. ^{3}H-Ins(1,3,4,5)P_4 and ^{3}H-PtdIns(4,5)P_2 were purchased from Dupont/NEN. PtdIns(4,5)P_2 and anti-HA antibody 12CA5 were purchased from Boehringer Mannheim. Horseradish peroxidase-linked anti-mouse IgG was purchased from BioRad. Horseradish peroxidase-linked anti-rabbit IgG and ECL Western blotting detection reagents were purchased from Amersham Life Sciences, and the 9E10 anti-myc antibody was from Oncogene Science. Silica Gel 60 TLC plates (20 × 20 cm, 0.2 mm) were from Merck. The Mono Q HR 5/5 FPLC column was from Pharmacia.

Expression of Recombinant Proteins. Human 5-phosphatase II used in these studies was 5PtaseS consisting of amino acids 250−942 of the predicted amino acid sequence (Jefferson & Majerus, 1995). Site-directed mutagenesis of 5-phosphatase II was performed using synthetic oligonucleotides and the Transformer Mutagenesis Kit from Clontech. The 5-phosphatase cDNA in pQE-32 bacterial expression vector (Qiagen) was annealed with an oligonucleotide primer containing a mutation of a single amino acid to alanine and with a selection oligonucleotide primer eliminating an *Afl*II restriction site in the 3-prime untranslated sequence of 5-phosphatase. All mutants were verified by restriction enzyme digestion and DNA sequencing. 5-Phosphatase II cDNA mutants were subcloned into the pVL1393 baculoviral expression vector (PharMingen) and expressed in Sf9 cells using the BaculoGold transfection kit from PharMingen. The complete coding sequences of human 5SIP110 (Kavanaugh et al., 1996) and human GRB2 were expressed in Sf9 insect cells as GST fusion proteins with an intermediate hemagglutinin (HA) tag using the baculovirus expression vector pVIKS as described (Kavanaugh & Williams, 1994). 5SIP110 D460A was constructed according to the method of Higuchi (1990).

Baculovirus Expression, Purification, and Enzyme Activity. Sf9 insect cells were grown in TNM-FH medium with 10% heat-inactivated fetal calf serum and 100 μg of gentamicin/mL (Summers & Smith, 1987). Approximately 2 × 10^7 insect cells were infected with baculovirus encoding either native or mutant 5-phosphatase. After 3 days, cells were harvested from T175 flasks and sonicated 3 × 10 s at 100 W using a Biosonik II sonicator in 500 μL of 20 mM Tris, pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$, 2 mM EGTA, 10 mM β-mercaptoethanol (β-ME), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg of benzamidine/mL, 1 μM pepstatin A, and 10 μg of leupeptin/mL. Sonicates were centrifuged at 16000*g* for 10 min, and supernatants were filtered through a 0.45 μm spin filter (Millipore). Filtered supernatants were diluted to 4 mL in 20 mM Tris, pH 7.5, 3 mM $MgCl_2$, and 10 mM β-ME and loaded on a Mono Q HR 5/5 FPLC column. Protein was eluted with a 33 mL linear gradient of 0−0.5 M NaCl in the same buffer. Fractions containing native or mutant protein were detected by Western blotting with affinity-purified antibody to 5-phosphatase II (2 ng/mL).

Assay of 5-phosphatase activity using ^{32}P-Ins(1,4,5)P_3, ^{3}H-Ins(1,3,4,5)P_4, and ^{3}H-PtdIns(4,5)P_2 was as described (Connolly et al., 1985; Mitchell et al., 1989; Attree et al., 1992). PtdIns([3-^{32}P]-4,5)P_3 was prepared as described (Norris & Majerus, 1994) using PtdIns(4,5)P_2 and recombinant PtdIns 3-kinase (Hu et al., 1995). Hydrolysis was determined as described (Kavanaugh et al., 1996; Damen et al., 1996).

Western Blotting. Immunoblotting of native and mutant 5-phosphatase II was done with affinity-purified antibody to 5-phosphatase II raised against a recombinant antigen containing amino acids 233−428 (Ross et al., 1991). Secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG from Amersham (1:5000 dilution). Immunoblotting of 5SIP110 was done with anti-HA antibody used at 120 ng/mL. Secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG from BioRad (1:5000 dilution). All blots were developed using ECL (Amersham).

GRB2 Association. For experiments determining the association of 5SIP110 with GRB2, Sf9 cells expressing native or D460A mutant 5SIP110 (HA-tagged) or GRB2 (HA- and myc-tagged) were lysed in 20 mM Tris, pH 8, 140 mM NaCl, 10% glycerol, 1% Triton X-100, 3 mM $MgCl_2$, 1 mM PMSF, 10 μg of benzamidine/mL, 1 μM pepstatin A, and 10 μg of leupeptin/mL. Lysates were centrifuged at 16000*g* for 10 min, and the relative amount of recombinant protein in each lysate was determined by Western blotting with anti-HA antibody. Equimolar amounts of native or D460A mutant 5SIP110 and GRB2 were mixed with anti-myc antibody in a 1:50 dilution and incubated at 4 °C overnight. Protein A−Sepharose (20 μL 50%) was added for 3 h, and the protein A−Sepharose pellet was washed 3 times in the lysis buffer described above and 3 times in 50 mM Tris, pH 7.5, and 3 mM $MgCl_2$. Equal portions of each pellet were assayed for 5SIP110 by Western blotting with anti-HA antibody.

RESULTS AND DISCUSSION

Comparison of the amino acid sequence of the platelet inositol polyphosphate 5-phosphatases I and II shows only two regions of homology (Laxminarayan et al., 1994; Jefferson & Majerus, 1995). These two motifs are conserved in a number of other identified or putative inositol polyphosphate 5-phosphatases and allowed us to identify consensus sequences that may define regions that participate in substrate binding and/or catalysis (Figure 1). Totally conserved residues are noted although there are many other amino acids that are partially conserved. At the time these experiments were designed we thought that I555 was conserved, but we later found examples where V is in this position. We sought to confirm that amino acids conserved among the 5-phosphatases are critical for enzyme actvity by mutating a number of them to alanine in 5-phosphatase II

MOTIF 1															
5PTASE II	(472)	L	W	L	G	D	L	N	Y	R	I	E	E		
5PTASE I	(228)	F	V	F	G	D	F	N	F	R	L	D	S		
GA5PTASE	(370)	F	W	F	G	D	L	N	Y	R	V	D	L		
SYNAPTOJANIN	(726)	F	W	C	G	D	F	N	Y	R	I	D	L		
OCRL	(495)	I	W	L	G	D	L	N	Y	R	L	C	M		
YEAST-1	(708)	I	W	M	G	D	L	N	Y	R	I	T	L		
YEAST-2	(767)	F	W	L	G	D	L	N	Y	R	I	T	L		
YEAST-3	(742)	F	W	L	G	D	M	N	Y	R	I	N	L		
CONSENSUS					G	D		N	Y	R					
						↑		↑		↑					
MOTIF 2															
5PTASE II	(545)	K	C	R	A	P	A	W	C	D	R	I	L	W	K	G
5PTASE I	(341)	N	T	R	C	P	A	W	C	D	R	I	L	M	S	P
GA5PTASE	(452)	K	Y	N	L	P	S	W	C	D	R	V	L	W	K	S
SYNAPTOJANIN	(498)	K	C	R	T	P	A	W	T	D	R	V	L	W	R	R
OCRL	(568)	K	C	R	V	P	A	W	C	D	R	I	L	W	R	G
YEAST-1	(780)	K	M	R	I	P	A	W	T	D	R	I	L	S	R	G
YEAST-2	(842)	K	A	R	T	P	S	W	T	D	R	I	I	Y	K	G
YEAST-3	(818)	K	E	R	T	P	S	W	T	D	R	I	I	Y	K	G
CONSENSUS						P		W		D	R	(i/v)				
								↑		↑	↑	↑				

FIGURE 1: Two regions of amino acid similarity between human 5-phosphatase II, human 5-phosphatase I, 5SIP110 (designated as GA5PTASE), and other phosphatases. Amino acid sequence number is indicated in parentheses. Alignment was performed using the program ALIGN (Dayhoff et al., 1983). Consensus amino acids are based on the predicted sequence of all putative or demonstrated 5-phosphatases. Amino acids in 5-phosphatase II that were mutated to alanine are indicated by an arrow.

and characterizing the effect on enzyme function. In motif 1, we chose D476, N478, and R480 as targets because of their conservation among all of these proteins. Likewise in motif 2, we mutated W551, D553, R554, and I555. The cysteine at 5-phosphatase position 552 was not mutated because a yeast homolog with demonstrated 5-phosphatase activity (J. D. York, personal communication) contains a threonine at that position, and because 5-phosphatase II retains activity in *N*-ethylmaleimide which alkylates cysteine (data not shown).

We expressed each of these seven mutated 5-phosphatases using a baculoviral expression system in Sf9 cells, partially purified them and the native enzyme by elution from a Mono Q FPLC column, and identified fractions containing recombinant enzyme by immunoblotting with antibody to 5-phosphatase II. Figure 2A shows immunoblots of the elution of native and three representative mutant 5-phosphatases. These mutant enzymes elute at the same salt concentration as the native enzyme, as did the additional 5-phosphatase mutants not shown. This indicates that there are no gross differences in enzyme surface charge or protein folding. Figure 2B shows the Coomassie-stained peak Mono Q fraction of each native and mutant 5-phosphatase. The 5-phosphatase band is clearly visible as the major protein in each fraction. The amount of 5-phosphatase protein in each fraction was estimated by scanning densitomitry of the Coomassie-stained gel and comparison to phosphorylase *b* in molecular weight standards run on this gel and on another gel run in parallel.

We next assayed the native and mutant 5-phosphatases for their ability to hydrolyze ^{32}P-Ins(1,4,5)P_3 (Figure 3A). Only mutants R480A and I555A showed detectable, concentration-dependent hydrolysis of Ins(1,4,5)P_3, but the activity of these mutants was reduced 56- and 27-fold, respectively, compared to native enzyme. When larger amounts of enzyme were assayed, there was also detectable activity in the mutant W551A. Mutants D476A, N478A, and R554A had no detectable activity above a buffer blank, suggesting that mutation of these amino acids decreases activity by more than 100-fold in comparison to native enzyme. We next asked whether these mutations also had a similar effect on the hydrolysis of three other soluble and

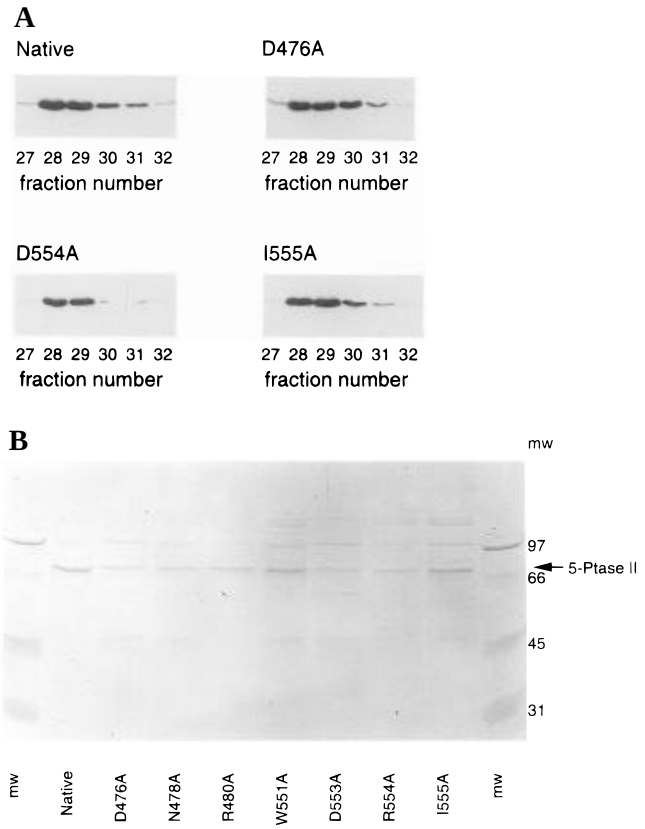

FIGURE 2: Partial purification of native and mutant 5-phosphatases. (A) Native and mutant 5-phosphatases were partially purified by Mono Q FPLC, and peak fractions were identified by immunoblotting with antibody to 5-phosphatase II. Shown are immunoblots of peak fractions of representative mutant and native 5-phosphataes. (B) Coomassie stain of peak Mono Q FPLC fractions of each native and mutant 5-phosphatase II.

lipid substrates. Hydrolysis of ^{3}H-Ins(1,3,4,5)P_4, ^{3}H-PtdIns-(4,5)P_2, and ^{32}P-PtdIns(3,4,5)P_3 was reduced by all mutations, and only R480A and I555A had any concentration-dependent hydrolysis of substrate at the enzyme amounts assayed (Figure 3B,C,D). Mutation of any of these amino acids in the conserved 5-phosphatase motifs impairs 5-phosphatase activity toward all four enzyme substrates. Interestingly, however, it appeared that mutant R480A had more activity than mutant I555A when used to hydrolyze Ins(1,3,4,5)P_4 and the lipid substrates.

We also did substrate concentration curves using Ins-(1,4,5)P_3 and Ins(1,3,4,5)P_4 using each partially active 5-phosphatase II mutant to determine whether K_m or V_{max} was affected by the mutation. Native recombinant enzyme hydrolysis of Ins(1,4,5)P_3 had a K_m of 75 μM and a V_m of 8300 nmol min^{-1} (mg of protein)$^{-1}$ as shown in Table 1. These values are similar to those of 24 μM and 25 000 nmol min^{-1} mg^{-1} for enzyme purified from platelets (Mitchell et al., 1989). Mutants W551A and I555A had a similar K_m for Ins(1,4,5)P_3 as the native enzyme but showed reductions of 488-fold and 42-fold, respectively, in V_m. By contrast, R480A had an approximately 8-fold increased K_m and a 10-fold decreased V_m when compared to native enzyme. Recombinant native 5-phosphatase also hydrolyzed Ins-(1,3,4,5)P_4 with K_m (4 μM) and V_m (1125 nmol min^{-1} mg^{-1}) similar to values reported for purified enzyme (7.5 μM and 1300 nmol min^{-1} mg^{-1}). Mutant R480A bound Ins(1,3,4,5)-P_4 with similar apparent affinity to native enzyme, but had even greater reductions in V_m for this substrate than for Ins-(1,4,5)P_3. This suggests the arginine in motif 1 has a more

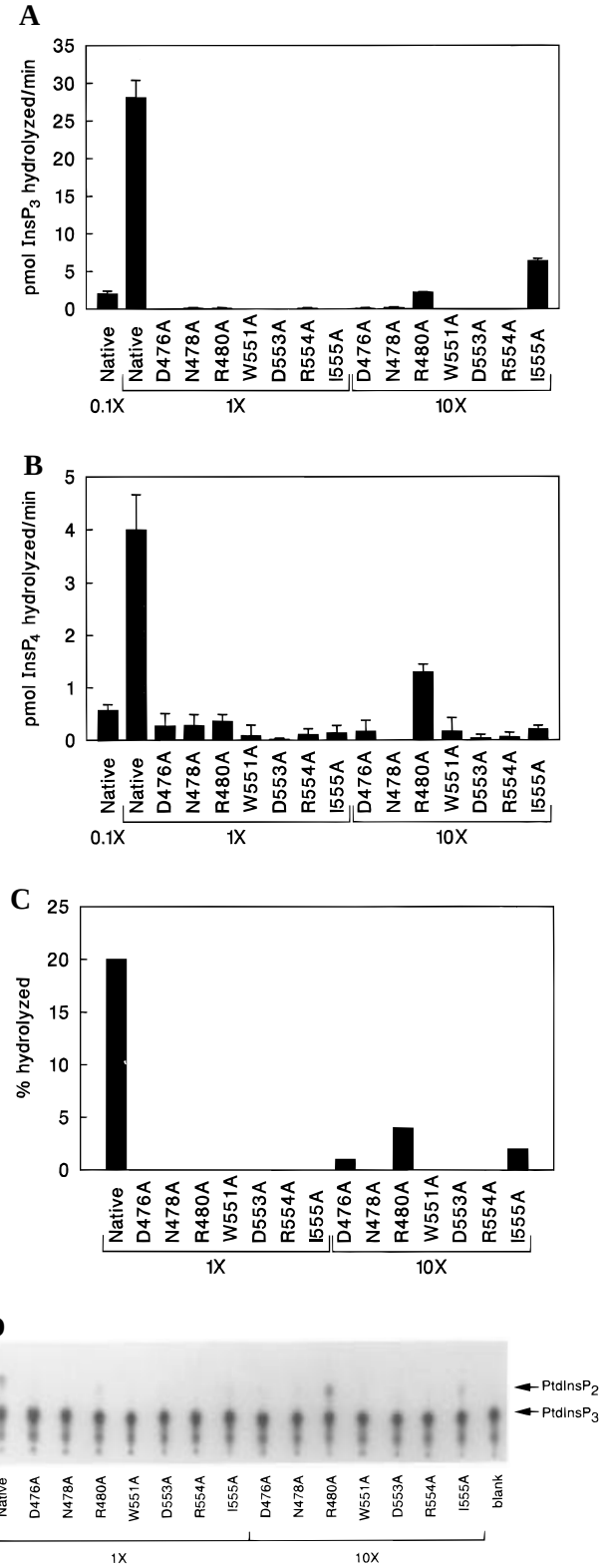

FIGURE 3: Hydrolysis of 5-phosphatase substrates by native and mutant 5-phosphatases. (A) Hydrolysis of ^{32}P-Ins(1,4,5)P_3 with 60 μM InsP_3 for 15 min at 37 °C. (B) Hydrolysis of ^{3}H-Ins(1,3,4,5)P_4 with 16 μM InsP_4 for 15 min at 37 °C. (C) Hydrolysis of ^{3}H-PtdIns-(4,5)P_2 with 45 μM PtdInsP_2 for 1 min at room temperature. (D) Hydrolysis of ^{32}P-PtdIns(3,4,5)P_3 with trace amounts of substrate for 30 min at 37 °C. Shown are the results of triplicate determinations for soluble substrates, single determinations for lipid substrates. Actual 5-phosphatase 1X protein amounts are: native, 32 ng; D476A, 16 ng; N478A, 15 ng; R480A, 14 ng; W551A, 24 ng; D553A, 7 ng; R554A, 4 ng; I555A, 20 ng.

active role in binding Ins(1,4,5)P_3 than Ins(1,3,4,5)P_4. Mutant I555A had a 9-fold reduction in K_m using Ins-(1,3,4,5)P_4 and over 1000-fold reduction in V_m. These results are consistent with the hypothesis that amino acids in motifs 1 and 2 are involved in 5-phosphatase substrate binding and hydrolysis.

Table 1: K_m and V_m for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 Hydrolysis by Native and Mutant 5-Phosphatases[a]

	Ins(1,4,5)P_3 hydrolysis		Ins(1,3,4,5)P_4 hydrolysis	
	K_m (μM)	V_m (nmol min^{-1} mg^{-1})	K_m (μM)	V_m (nmol min^{-1} mg^{-1})
native	75	8300	4	1125
R480A	634	855	11	13
W551A	35	17	ND[b]	ND
I555A	81	200	35	7

[a] Assays were performed in triplicate for 15 min at 37 °C. Results shown are representative of multiple determinations. For Ins(1,4,5)P_3 hydrolysis: native, five; R480A, five; W551A, two; I555A, five. For Ins(1,3,4,5)P_4 hydrolysis: two determinations for each native, R480A, and I555A. [b] ND, not determined.

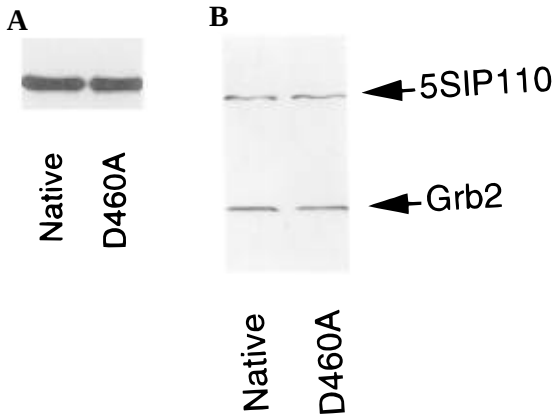

FIGURE 4: Expression and activity of native and D460A mutant 5SIP110. (A) Expression of native or D460A mutant 5SIP110. HA-tagged protein was expressed in Sf9 cells with a baculoviral expression system and was visualized in cell supernatants by immunoblotting with anti-HA antisera. (B) Association of native or D460A 5SIP110 with GRB2. HA-tagged native or mutant 5SIP110 was incubated with myc- and HA-tagged GRB2 and immunoprecipitated with anti-myc antiserum. The protein A−Sepharose pellet was subjected to SDS−PAGE, and associated 5SIP110s were visualized by immunoblotting with anti-HA antiserum.

We also tested whether a mutation in these conserved amino acids had a similar effect on 5-phosphatase activity in another 5-phosphatase family member. 5SIP110 binds GRB2 through association of proline-rich regions in its C-terminal portion and hydrolyzes Ins(1,3,4,5)P_4 and PtdIns-(3,4,5)P_3 but not Ins(1,4,5)P_3 or PtdIns(4,5)P_2 (Kavanaugh et al., 1996). Mutation of D460 to alanine, which corresponds to 5-phosphatase II mutant D553A, results in a protein that is expressed in Sf9 cells equally as well as the native 5SIP110 (Figure 4A). However there was no detectable hydrolysis of ^{3}H-Ins(1,3,4,5)P_4 using 5SIP110 D460A (i.e., <1% of native pSIP110). We next tested whether this mutation affected the ability to bind GRB2. We mixed native or D46OA 5SIP110 with GRB2, immunoprecipitated GRB2 with an antibody to its myc tag, and assayed for association of native or D460A 5SIP110 by Western blotting the protein A−Sepharose pellet with an antibody to an HA tag that is contained on each protein. As shown in Figure 4B, D460A 5SIP110 binds to GRB2 equally as well as native 5SIP110. Thus, mutation of this conserved amino acid also eliminates 5-phosphatase enzyme activity in a second 5-phosphatase without affecting its GRB2-binding ability.

The 5-phosphatase enzyme family now consists of at least nine distinct members based on the predicted amino acid sequence from cDNA clones and on the enzyme activity of partially purified proteins. In this study, we undertook a mutational analysis of amino acids in conserved domains of two 5-phosphatases. Our results are consistent with a role for these amino acids in substrate binding and catalysis. Identification of structural motifs responsible for differences in substrate specificity in the 5-phosphatases must await more detailed mutational or crystallographic analysis of these enzymes.

ACKNOWLEDGMENT

We thank David A. Pot and Lewis T. Williams of Chiron Corp. for baculovirus encoding GRB2, 5SIP110, and 5SIP110 D460A and Anke Klippel (Chiron Corp.) for recombinant PI3-kinase. We also thank F. Anderson Norris for helpful discussions on this work, and John D. York for sharing his unpublished data on yeast 5-phosphatases.

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BI9602627