Site-directed Mutagenesis of Diphosphoinositol Polyphosphate Phosphohydrolase, a Dual Specificity NUDT Enzyme That Attacks Diadenosine Polyphosphates and Diphosphoinositol Polyphosphates

(Received for publication, July 21, 1999, and in revised form, September 27, 1999)

Xiaonian Yang‡, Stephen T. Safrany, and Stephen B. Shears

From the Inositide Signaling Group, Laboratory of Signal Transduction, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Diphosphoinositol polyphosphate phosphohydrolase (DIPP) hydrolyzes diadenosine 5′,5″-P1,P6-hexaphosphate (Ap6A), a Nudix (nucleoside diphosphate attached-moiety) substrate, and two non-Nudix compounds: diphosphoinositol pentakisphosphate (PP-InsP5) and bis-diphosphoinositol tetrakisphosphate (PP2-InsP4). Guided by multiple sequence alignments, we used site-directed mutagenesis to obtain new information concerning catalytically essential amino acid residues in DIPP. Mutagenesis of either of two conserved glutamate residues (Glu66 and Glu69) within the Nudt (Nudix-type) catalytic motif impaired hydrolysis of Ap6A, PP-InsP5, and (PP)2-InsP4 >95%; thus, all three substrates are hydrolyzed at the same active site. Two Gly-rich domains (glycine-rich regions 1 and 2 (GR1 and GR2)) flank the Nudt motif with potential sites for catalysis and coordination and substrate binding. GR1 comprises a GGG tripeptide, while GR2 is identified as a new functional motif (GXGXG) that is conserved in yeast homologues of DIPP. Mutagenesis of any of these Gly residues in GR1 and GR2 reduced catalytic activity toward all three substrates by up to 95%. More distal to the Nudt motif, H91L and F84Y mutations substantially decreased the rate of Ap6A and (PP)2-InsP4 metabolism (by 71 and 96%), yet PP-InsP5 hydrolysis was only mildly reduced (by 30%); these results indicate substrate-specific roles for His91 and Phe84. This new information helps define DIPP’s structural, functional, and evolutionary relationships to Nudix hydrolases.

The mammalian diphosphoinositol polyphosphate phosphohydrolase (DIPP) was originally characterized (1) as being responsible for the dephosphorylation of PP-InsP5 and (PP)2-InsP4 (Fig. 1), which are the most highly phosphorylated members of the inositide signaling family of molecules (2). PP-InsP5 and (PP)2-InsP4 are widely distributed across the phylogenetic spectrum; they have been identified in mammals (3, 4), yeasts (5), slime molds (6–8), plants (9), and free living amoebae (10, 11). The dephosphorylation of PP-InsP5 and (PP)2-InsP4 results in a considerable free energy change, in no small part due to the relief of the severe electrostatic and steric constraints imposed by the high density of phosphate groups that are clustered around the inositol ring (7). Nevertheless, in vivo there is rapid turnover of both PP-InsP5 and (PP)2-InsP4, so the levels of these compounds must be continually replenished at a robust rate. This ATP-consuming process therefore places a substantial burden upon the cell’s energy reserves (12). These observations have led to the suggestion that PP-InsP5 and (PP)2-InsP4 turnover might act as a molecular switch for a very dynamic cellular process, for which vesicle trafficking has emerged as a candidate (13, 14). Support for the idea that PP-InsP5 in particular might regulate the movement of vesicles through the cell comes from demonstrations that some of the proteins that regulate this process bind PP-InsP5 very tightly; examples include coatomer (5, 15), AP-2 (see Ref. 3), and AP-3 (16). Another energy-consuming activity that may be nominated for being regulated by diphosphoinositol polyphosphates is mRNA export from the nucleus. Recent studies indicate that efficient mRNA export in yeasts requires the synthesis of inositol hexakisphosphate (17). However, rather than inositol hexakisphosphate participating in this process directly, as was originally proposed (17), the significance of inositol hexakisphosphate may actually be as the precursor of PP-InsP5 and (PP)2-InsP4 (12), the turnover of which can more readily account for some of the energy-dependent aspects of mRNA export.

The contention that it is the actual turnover of diphosphoinositol polyphosphates that is functionally significant is further supported by their cellular levels being regulated by specific cell-signaling events. These include the mobilization of certain categories of cellular Ca2+ stores, which inhibits the synthesis of PP-InsP5 (18). In addition, the metabolism of diphosphoinositol polyphosphates is regulated by receptor-mediated changes in levels of cAMP and cGMP (19). Since DIPP is the enzyme with primary responsibility for controlling the dephosphorylation of PP-InsP5 and (PP)2-InsP4 (1), this phosphohydrolase is a prime candidate for being regulated by these intracellular signaling processes.

Recently, the prominence of DIPP2 was further enhanced by...
Two complementary mutagenic primers, each containing one base mismatch (underlined in the codon for Gly99, Gly103, Gly159, Gly160, Gly161, Gly164, Phe166, or His175) were synthesized with a Beckman Oligo 10000M DNA Synthesizer. Only the sequences of the forward primers are shown. The E70Q mutant was prepared as previously described (1).

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**Experimental Procedures**

**Materials**—PP-[^3H]InsP₅ was obtained from NEN Life Science Products; (PP)₂[^3H]InsP₄ was prepared as described previously (20). Ap₆A was purchased from Sigma. The Partisphere 5-μm SAX column was purchased from Krackler Scientific (Durham, NC). NuPAGE gels were obtained from Novex (San Diego, CA).

**Site-directed Mutagenesis of Recombinant DIPP**—Wild-type, recombinant human DIPP was prepared as described previously (1). The recombinant DIPP mutants were prepared by using the Quick-Change™ site-directed mutagenesis kit from Stratagene, according to the manufacturer's instructions. Two complementary mutagenic primers, each containing one base mismatch, were synthesized with a Beckman Oligo 10000M DNA synthesizer (Table I); the preparation of the E70Q mutant was described previously (1). The cDNA construct for recombinant DIPP was used as template for PCR using the mutagenic primers. The wild-type strand in the resulting PCR product was removed by digestion with 10 units of DpnI at 37 °C for 1 h before being used to transform *Escherichia coli* XL1-Blue supercompetent cells. The colonies from the transformation plates were screened by colony PCR with two primers flanking the DIPP gene, and plasmids were prepared from the colonies that generated the expected PCR product. The presence of the correct mutation in all of the constructs was verified by DNA sequencing using a PCR-based dRhodamine fluorescent dye method (Perkin-Elmer).

**Expression and Purification of Recombinant DIPP and Its Mutants**—The cDNAs for DIPP and each of the mutants were transformed into M15 competent cells, which were incubated overnight at 37 °C in 10 ml of LB broth containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. The overnight culture was inoculated into 100 ml of the same medium, and the incubation was continued until A₆oo reached 0.9–1.0, whereupon 1.5 ml isopropyl-β-thiogalactopyranoside was added to initiate the expression. After an additional 5 h, the cells were harvested by
Experimental Procedures. Approximately 0.7 mg of purified recombinant DIPP and the mutants described in this study were expressed and purified with the nickel-nitrilotriacetic acid column as described under "Experimental Procedures." Approximately 0.7 μg of each purified protein was analyzed by SDS-polyacrylamide gel electrophoresis as described below. The gel was stained with Coomassie Blue dye. Molecular weight standards (low range) were from Bio-Rad. The purity of the E70Q mutant is described elsewhere (1).

The dialyzed protein was then filtered (0.45 μm) and dialyzed against 4 liters of 10 mM phosphate buffer (pH 7.5). The dialyzed protein was then filtered (0.45 μm), and its protein concentration was determined according to Beer's law. The extinction coefficient at 280 nm was calculated (27) to be 27,870 M⁻¹ cm⁻¹ for recombinant DIPP and its mutants (except F84Y, which was 28,950 M⁻¹ cm⁻¹). The final concentration of the dialyzed mutants was between 9.1 and 32.2 mM. The purity of each protein preparation was determined on 4–12% gradient NuPAGE gels (Fig. 2) with MES SDS running buffer.

Enzyme Assays—First-order rate constants for the dephosphorylation of PP-[3H]InsP₅ and (PP)₂-[3H]InsP₄ were determined as described previously (1). The dephosphorylation of 100 μM Ap₆A was measured in 100-μl assays (20) that were quenched with 50 μl of ice-cold 2 M K₂CO₃, 5 mM EDTA. Reaction products were resolved by high pressure liquid chromatography using a 4.6 × 125-mm Partisphere 5-μm SAX column, eluted at 1 ml/min by the following gradient generated by mixing buffer A (1 mM Na₂EDTA) and buffer B (buffer A plus 1.3 mM (NH₄)₂HPO₄, pH 3.85, with H₃PO₄): 0–1 min, 0% B; 1–31 min, 0–25% B; 31–51 min, 25–55% B; 52–62 min, 0% B. Metabolites were identified as described previously (28).

RESULTS AND DISCUSSION

Identification of Candidate Functional Elements of DIPP by Multiple Sequence Alignments—Mammalian DIPP has dual specificity, in that it hydrolyzes diphosphoinositol polyphosphates (1) and diadenosine polyphosphates (20). Two yeast proteins (Aps1 and YOR163w; see Ref. 20 and Fig. 3) have been identified that also hydrolyze these two different groups of substrates. The amino acid sequences of these yeast proteins diverge considerably from that of DIPP, except for a limited degree of conservation within the central core that includes the Nudt catalytic motif and short flanking regions (20). We focused on this homologous internal region of DIPP, and aligned it with a large number of Nudix hydrolases, including 40 that were listed in a recent study (29). A small subgroup of these proteins were identified, in which there are varying degrees of conservation of several interesting features (Fig. 3): (i) highly conserved glutamate residues within the Nudt motif; (ii) Gly-rich regions flanking the Nudt motif; (iii) a conserved phenylalanine residue; and (iv) a patch of amino acids with strongly positive electrostatic potential, in which a His residue is the most strongly conserved.

The identification of these conserved elements forms the basis for the mutagenic strategy that we have used in this study.

Glutamate Residues within the Nudt Motif Are Essential for the Hydrolysis of Diphosphoinositol Polyphosphates and Diadenosine Polyphosphates—In our sequence alignments (Fig. 3), five of the seven Glu residues in the Nudt region of DIPP are highly conserved; two of these aligned Glu residues have previously been shown to be catalytically essential in other Nudt contexts (Glu⁶⁵ in human MutT homologue type 1 (30) and Glu⁶⁷ in MutT (31)). We therefore targeted the equivalent Glu residues in DIPP (Glu⁶⁶ and Glu⁷⁰; see Fig. 3).

The E70Q mutant of recombinant DIPP had a greatly reduced rate of hydrolysis of PP-InsP₅ and (PP)₂-InsP₄ (Fig. 4 and Ref. 1). We have extended this observation by demonstrating that this same E70Q mutation also greatly impaired (by 98%) the ability of recombinant DIPP to hydrolyze Ap₆A (Fig. 4). The E66Q recombinant DIPP mutant also had no significant activity toward either PP-[³H]InsP₅ or (PP)₂-[³H]InsP₄, and hydrolysis activity against Ap₆A was found to be only 2% as active as the wild-type enzyme (Fig. 4). The catalytic paradigm for the Nudt motif envisages these two Glu residues participating in both binding of the Mg²⁺-substrate complex and in general base catalysis (31, 32). Our experiments are consistent with this catalytic process in DIPP being utilized for the hydrolysis of both diphosphoinositol polyphosphates and diadenosine polyphosphates.

We also used CD spectroscopy to examine whether these two mutations had any effect on the secondary structure of recombinant DIPP. The CD spectra of the E66Q and E70Q mutants were not significantly different from that of wild-type recombinant DIPP (data not shown). The CD spectrum for the wild-type enzyme (see below), when analyzed using the Selcon computer algorithm, indicated that the content of α-helix and β-pleated sheet content was obtained using the Selcon fitting program.
E70Q, F84Y, and H91L mutants of recombinant DIPP. Wild-type PP-InsP5 were assayed for phosphohydrolase activities against recombinant DIPP and E66Q, E70Q, F84Y, and H91L mutants of recombinant DIPP. The Nudt signature (26), in human DIPP (GenBank™ accession number AF062529), plus amino- and carboxyl-flanking sequences, were aligned with five orders. The Identification and Potential Significance of Conserved Glycine-rich Domains Flanking the Nudt Motif of DIPP—DIPP contains two short Gly-enriched regions (GR1 and GR2; see Fig. 3) that extend out from each end of the Nudt motif in DIPP. The hydrolysis of Ap6A was more substantially impaired in the H91L mutant (26% of the activity of the wild type enzyme; Fig. 4). However, the most profound effect of this mutation was observed with (PP)2-InsP4 as substrate; the H91L mutant expressed only 4% of the activity of the wild-type enzyme (Fig. 4). The hydrolysis of Ap6A was substantially impaired (by 63 and 75%, respectively; Fig. 4). In contrast, the rate of hydrolysis of PP-InsP5 was only slightly reduced in the F84Y recombinant DIPP mutant (by only 30%; Fig. 4), suggesting only a relatively minor role for Phe84 in the dephosphorylation of that particular substrate.

A recurring characteristic to emerge from our multiple sequence alignment (Fig. 3) is the presence of six of these eight proteins, of a patch of between two and five residues with strong positive electrostatic potential, beginning 16–23 residues downstream of the carboxyl terminus of the Nudt motif. This kernel of positive charge is a good candidate for having electrostatic interactions with the negative charges carried by the phosphate groups on DIPP’s substrates. The most conserved of this group of residues (His91) was chosen as a target for further study of this particular issue. An H91L mutant of recombinant DIPP was constructed; CD spectra indicated that this amino acid substitution had no significant effect on the overall secondary structure (data not shown). However, the rates of hydrolysis of (PP)2-InsP4 and Ap6A were substantially impaired (by 63 and 75%, respectively; Fig. 4). In contrast, the rate of hydrolysis of PP-InsP5 was only slightly reduced in the F84Y recombinant DIPP mutant (by only 30%; Fig. 4), suggesting only a relatively minor role for Phe84 in the dephosphorylation of that particular substrate.

The catalytic properties of wild-type (W.T.) and E66Q, E70Q, F84Y, and H91L mutants of recombinant DIPP. Wild-type recombinant DIPP and E66Q, E70Q, F84Y, and H91L mutants of recombinant DIPP were assayed for phosphohydrolase activities against PP-InsP5, gray bars; n = 3–5 for mutants, n = 14 for wild type) or (PP)2-InsP4, (black bars; n = 3–6 for mutants, n = 12 for wild type) or for hydrolysis activity toward Ap6A, (striped bars; n = 3–7 for mutants, n = 15 for wild type). S.E. values are also indicated. k1 is the first-order rate constant in the following rate equation: [S] = [S]eq – Δ[S]. The units for k1 are min⁻¹ µg⁻¹. Data for PP-InsP5 and (PP)2-InsP4 metabolism by the E70Q mutant are similar to those published previously (1).
lytic activity was severely compromised (Ala). This mutant of recombinant DIPP had the same CD spec-

above) was pursued by conservatively mutating this residue to Gly for Gly51 in DIPP (see above), then we can rationalize the effect of the G51A mutation; the steric bulk of the Ala side chain may prevent the cation from approaching the backbone amides, to which the ligand is gripped by hydrogen bonds (38, 39).

The rates of hydrolysis of diphospho-

in other Nudix hydrolases and corresponds to Gly51 in the GR1 region of DIPP (Fig. 3).

Second, since Ap6A is a nucleotide, it is significant that Gly-rich sequences are also a recurring feature of nucleotide-binding loops (36, 37). The absence of a side chain in the Gly residue permits a phosphate group to approach the backbone amides, to which this structural role may be to con-

GR1: Mutagenesis of Gly50, Gly51, and Gly52—Our proposi-
tion that Gly52 may be functionally significant in GR1 (see above) was pursued by conservatively mutating this residue to Ala. This mutant of recombinant DIPP had the same CD spectrum as the wild-type enzyme (data not shown), but its catalytic activity was severely compromised (>97% less than that of the wild-type enzyme; see Fig. 5). Assuming we are correct with our suggestion that the cation-binding function for the carbonyl carbon of Gly38 in the E. coli MutT protein is conserved (31, 32), this residue is conserved in other Nudix hydrolases and corresponds to Gly51 in the GR1 region of DIPP (Fig. 3). Since Ap6A is a nucleotide, it is significant that Gly-rich sequences are also a recurring feature of nucleotide-binding loops (36, 37). The absence of a side chain in the Gly residue permits a phosphate group to approach the backbone amides, to which this structural role may be to con-

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GR2: Mutagenesis of Gly72, Gly75, Gly78, and Gly82—Four separate Gly-to-Ala mutants of recombinant DIPP were constructed in the GR2 region: G72A, G75A, G78A, and G82A. The catalytic activity of the G78A mutant (Fig. 6) and its CD spectrum (data not shown) were not significantly different from that of the wild-type enzyme. This is an interesting observation, because Gly78 is the only one of these four Gly residues that is not conserved in Apa1 and YOR163w. Note, however, that the additional steric bulk introduced in a G78V mutant was not tolerated, and there was a substantial decrease in catalytic activity toward all three substrates (Fig. 6).

The CD spectrum obtained from the G82A mutant showed a substantial change from that of the wild-type enzyme (Fig. 7). The nadir in the spectrum at 203 nm broadened considerably and increased in value from −9000 to −3000 degrees cm^-2 dmol^-1 (Fig. 7). The Selcon computer algorithm estimated that, compared with the wild-type recombinant DIPP, the content of α-helix in the G82A mutant was 36% lower, while the β-sheet content was 30% higher. Considering the very conservative nature of the Gly-to-Ala substitution, the consequences for the overall secondary structure of the protein are very dramatic. Further structural analysis will be required in order to account for this effect. However, in view of the fact that Gly plays an important role in the architecture of certain polypeptide loops (36), such as those that generally flank the Nudt motif (31, 32, 34, 35), it is notable that there are examples of proteins where no other amino acid can replace Gly at particularly tight turns in a loop (40); this may be why the introduction of an G82A mutation promotes some unraveling of the normal secondary structure. Presumably as a consequence, the catalytic activity of this mutant recombinant DIPP is sub-
stantially impaired (Fig. 6).

The CD spectra of the remaining two mutants, G72A and G75A, were both very similar to that of the wild-type enzyme (data not shown), indicating that the mutations had no significance upon the secondary structure. However, the catalytic activities of the G72A and G75A mutants toward PP-InsP_5, (PP)_2-InsP_4, and Ap_5A were impaired by >95% (Fig. 6). Our mutagenesis of the GR2 region of DIPP has therefore identified a novel Gly-rich consensus, GXXGXG, that is essential for this enzyme’s dual specificity.

**General Conclusions**—The importance of this study primarily lies in its providing new information relevant to our understanding of the structural and functional relationship that DIPP has with Nudix hydrolases. This knowledge is particularly significant, because the metabolism of diphosphoinositol polyphosphates is an unorthodox activity for a protein that has a Nudt catalytic motif. Our study also breaks new ground by identifying some catalytically important residues that lie outside the Nudt consensus. It is these extramural residues that are believed to impart substrate specificity upon each of the individual members of this hydrolase family (26). We have found several residues in this category that make important contributions to the unusual dual catalytic specificity of DIPP.

A particularly striking illustration of these developments is our identification of a new functionally and structurally important array of Gly residues (the “GR2” motif, GXGXG) that projects out from the carboxyl terminus of the Nudt domain. The GR2 motif is conserved in Aps1 and YOR163w (Fig. 3), which are two yeast homologues of DIPP that also express dual specificity toward diadenosine polyphosphates and diphosphoinositol polyphosphates (20). Moreover, this GR2 motif is absent from two Nudix hydrolases that actively degrade diadenosine polyphosphates but do not attack diphosphoinositol polyphosphates, namely human APAH1 (20, 35) and the IaIAA invasion protein of *Bartonella bacilliformis* (20, 41, 42). These comparisons suggest that the emergence of the GR2 motif has been an essential factor for the evolution of dual specificity toward diadenosine polyphosphates and diphosphoinositol phosphates. In this case, the GR2 motif may be a signature sequence that helps predict which other proteins with a Nudt motif may have a similar catalytic activity. We have already identified one candidate: the putative slt1058 protein from *Cyanobacterium synechocystis* (Fig. 3).

Among other catalytically essential features of DIPP that we identified in this study, His^64^ was found to be of special significance; the mutation of this residue to Leu inhibited (PP)_2-InsP_4 dephosphorylation by 96%, yet PP-InsP_5 metabolism was only reduced by 30% (Fig. 4). One other residue, Phe^84^, was also found to contribute more to the hydrolysis of (PP)_2-InsP_4 compared with PP-InsP_5 (Fig. 4). These findings provide the first evidence for there being a substantial difference in active-site residues that participate in the hydrolysis of these two substrates. As such, this is an important step forward toward the goal of understanding how DIPP has the flexibility to express differential positional specificity toward these two inositol-based substrates (see Fig. 1).

**Acknowledgments**—We are indebted to Devon Allen for expert assistance with the CD measurements. We also thank Drs. Richard McKay and Kiyoshi Itagaki for helpful comments during the writing of this manuscript.

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Site-directed Mutagenesis of DIPP