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Protein pyrophosphorylation is by inositol pyrophosphates is a posttranslational event

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In a previous study, we showed that the inositol phosphophosphate diphosphoinositol pentakisphosphate (IP5) is one of the constitutively expressed kinases (IP6Ks) (5–7). IP6K and inositol polyphosphates participate in diverse physiologic activities including endocytosis (8, 9), apoptosis (10, 11), chemotaxis (12), and telomere elongation (13, 14). Recently, a novel inositol phosphophosphate, 4,6-PP-IP3, synthesized by the Vip1 protein in Saccharomyces cerevisiae, was identified by York and associates (15, 16) and shown to be involved in regulating cell growth, morphology, and phosphate homeostasis in yeast.

The pyrophosphate bond of IP7 has a calculated phosphorylation potential that equals or exceeds that of ATP, suggesting that it could serve a similar function (4, 17). We demonstrated that IP7 phosphorylates mammalian and yeast proteins. We now report that this phosphorylation potential reflects pyrophosphorylation. Thus, proteins must be prephosphorylated by ATP to prime them for IP7 phosphorylation. ATP phosphorylates synthetic phosphoproteins but not if their phosphates have been masked by methylation or phosphorylation.

Moreover, IP7 phosphorylated peptides are more acid-labile and more resistant to phosphatases than ATP phosphorylated peptides, indicating a different type of phosphate bond. Pyrophosphorylation may represent a novel mode of signaling to proteins.

Inositol phosphophates have diverse biologic roles with the best known, inositol-1,4,5-trisphosphate (IP3), releasing intracellular calcium (1). Inositol pyrophosphates (also designated inositol diphosphates), exemplified by diphosphoinositol pentakisphosphate (5-PP-I(1,2,3,4,6)P5, hereafter called IP5) and bis-diphosphoinositol tetrakisphosphate ([PP]2-IP4, IP8) (2–4) are formed by a group of three inositol hexakisphosphate (IP6) kinases (IP6Ks) (5–7). IP6K and inositol pyrophosphates participate in diverse physiologic activities including endocytosis (8, 9), apoptosis (10, 11), chemotaxis (12), and telomere elongation (13, 14). Recently, a novel inositol phosphophosphate, 4,6-PP-IP3, synthesized by the Vip1 protein in Saccharomyces cerevisiae, was identified by York and associates (15, 16) and shown to be involved in regulating cell growth, morphology, and phosphate homeostasis in yeast.

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Results and Discussion

In our earlier study, we showed that 5β[32P]IP7 phosphorylates a wide range of proteins in both the yeast, S. cerevisiae, and in mammalian tissues (18). We characterized in detail phosphorylation of several of these proteins, such as the yeast proteins NSR1 and SRP40. To obtain large amounts of these proteins, we expressed them in Escherichia coli (Fig. 1a). Whereas these proteins purified from yeast are robustly phosphorylated by 5β[32P]IP7, the corresponding proteins purified from bacteria display no phosphorylation. What property of yeast but not bacterial extracts conveys the capacity for IP7 phosphorylation? One distinction is that proteins in yeast may be prephosphorylated whereas eukaryotic proteins are unlikely to be phosphorylated in E. coli. We examined whether yeast extracts influence IP7 phosphorylation of proteins purified from E. coli (Fig. 1b). Although yeast extracts alone do not augment IP7 phosphorylation, yeast extracts plus ATP allow IP7 phosphorylation of proteins purified from bacteria comparable to phosphorylation of the same proteins purified from yeast. Boiled yeast extracts fail to prime proteins for IP7 phosphorylation, indicating that the critical factor may be a protein, possibly an enzyme (Fig. 1c).

Several lines of evidence indicate that prephosphorylation of proteins primes them for IP7 phosphorylation. ATP and GTP, which can donate phosphate groups, provide priming activity, whereas AMP-PNP, which is nonhydrolyzable, is inactive, as is UTP, a poor substrate for protein kinases (Fig. 1d). CK2 (formerly casein kinase-2) is one of the few protein kinases for which GTP donates phosphate to a comparable extent as ATP (19). Because GTP primes IP7 phosphorylation as well as ATP, we examined the priming activity of CK2 on the IP7 phosphorylation of NSR1 (1–50), a fragment of NSR1 which is an excellent substrate for IP7 phosphorylation (supporting information (SI) Fig. 5) and has a strong consensus motif for CK2 phosphorylation. CK2 robustly primes IP7 phosphorylation (Fig. 1e). Furthermore, the IP7 substrate NSR1 (27–50) (SI Fig. 5), can be directly phosphorylated by IP7 when purified from E. coli that coexpress the catalytic subunit of CK2 (Fig. 1f). If prephosphorylation is the critical priming event for IP7 phosphorylation, then dephosphorylation should prevent such priming. Dephosphorylation of NSR1 (51–166) or NSR1 (1–50) by X-phosphatase greatly reduces IP7 phosphorylation (Fig. 1g). Protein kinase-mediated priming for IP7 phosphorylation is observed for all IP7 substrates tested, including yeast SRP40, NSR1, YGR130c, and...
APL6 and mammalian Nopp140, AP3β3A, and TCOF1 (data not shown).

The requirement for priming by prephosphorylation suggests that either (i) ATP phosphorylation at certain sites allosterically facilitates IP7-mediated phosphorylation at other sites, or (ii) IP7 directly phosphorylates amino acids that have been previously ATP-phosphorylated and hence provides a pyrophosphorylation or diphosphorylation modification. Earlier, we noted that IP7 phosphorylation occurs primarily at stretches of serines flanked by acidic amino acids and concluded that phosphorylation occurs on the serine residues (18) (also see SI Fig. 6). In Nopp140, deletion of individual serine residues in such a stretch (amino acid 80–100) decreases IP7 phosphorylation, implying that IP7 can phosphorylate several of them (Fig. 2a). Substitution of the single threonine residue in this sequence has no effect on IP7 phosphorylation. Because prephosphorylation by CK2 leads to IP7-mediated phosphorylation of such sites, Nopp140 fragments containing phosphates at the CK2-preferred sites should be substrates for IP7 phosphorylation in the absence of any other priming. To identify the preferred sites for CK2 phosphorylation, we phosphorylated an E. coli-expressed Nopp140 fragment comprising amino acids 80–100 and identified the major phosphorylation sites by mass spectrometry as serines 88 and 91 (data not shown). We synthesized a peptide with both of these serines prephosphorylated (Fig. 2b). This phosphopeptide is robustly phosphorylated by 5[32P]IP7, whereas the corresponding unphosphorylated peptide is resistant to 5[32P]IP7 phosphorylation (Fig. 2c). Using two peptides with phosphates at serines 88 and 91, respectively, we observe substantially greater phosphorylation at position 91 than 88 (Fig. 2c). This differential influence on discrete sites supports the specificity of IP7-mediated phosphorylation. Both the unphosphorylated and phosphorylated peptides are phosphorylated by [γ-32P]ATP and CK2 (Fig. 2d) on one or more of the available serine residues (data not shown).
phosphorylation of the prephosphorylated but not the unphosphorylated Nopp140 peptide is consistent with pyrophosphorylation. This possibility is further supported by mass spectrometric analysis showing no phosphorylation by IP7 of the unphosphorylated Nopp140 peptide but addition of one or two phosphates to the prephosphorylated peptides (SI Table 1). The maximum number of phosphates accepted by a peptide from IP7 equals the number of preexisting phosphate groups on the peptide; single phosphoserine-containing peptides accept one phosphate from IP7, whereas the peptide containing two phosphoserine residues accepts one or two phosphates.

The phosphorylated Nopp140 peptide might facilitate IP7 phosphorylation of nearby serines or might be pyrophosphorylated. To distinguish those alternatives, we synthesized the same phosphopeptide in which the two phosphates have been methylated to prevent pyrophosphorylation (Fig. 2b). The methylated phosphopeptide is not phosphorylated by 5β[32P]IP7 (Fig. 2c and SI Table 1), although it can be phosphorylated by γ[32P]ATP and CK2 (Fig. 2d).

The failure of the methylated phosphopeptide to be IP7-phosphorylated strongly implies that IP7 provides pyrophosphorylation. To further test this possibility, we synthesized the same Nopp140 peptide in which both serines 88 and 91 are pyrophosphorylated. This pyrophosphorylated peptide resists IP7 phosphorylation, although it can be phosphorylated by CK2 (Fig. 2c and d and SI Table 1). This further supports the notion that IP7 pyrophosphorylates its targets.

If IP7 phosphorylates proteins on serines in the same fashion as ATP, then the properties of the phosphoserine bond should be the same with IP7 and ATP phosphorylation. By contrast, the chemical properties of a pyrophosphorylated serine should differ from those of a conventional phosphoserine. We examined the acid sensitivity of the phospho-Nopp140 peptide after phosphorylation by γ[32P]ATP and CK2 or 5β[32P]IP7 (Fig. 3a and b). Phosphorylation by 5β[32P]IP7 is more labile to treatment with 3 M HCl compared with phosphorylation by γ[32P]ATP and CK2. Furthermore, we subjected the CK2 and ATP or IP7-phosphorylated peptides to hydrolysis in the presence of 6 M HCl to release single amino acids. Hydrolysis liberates a prominent peak of [32P]phosphoseryl from the ATP-phosphorylated peptide but not from the IP7-phosphorylated peptide (Fig. 3c–e). Thus, the pyrophosphate modification on serine is more labile to acid than the conventional phosphoserine elicited by ATP-mediated phosphorylation.

In contrast to the greater lability to acid treatment, the IP7-phosphorylated peptide is more stable to enzymatic dephosphorylation (Fig. 4a). As observed earlier (Fig. 1g), λ-phosphatase treatment before adding IP7 prevents IP7 phosphorylation of NSR1 (1–50). After IP7 phosphorylation, however, the protein is completely resistant to λ-phosphatase. λ-Phosphatase greatly reduces phosphorylation of CK2 and ATP phosphorylated NSR1 (27–50) or Nopp140 peptide but fails to affect the IP7-phosphorylated peptides (Fig. 4b and c). The same protein fragments are resistant to λ-phosphatase when purified from yeast labeled with inorganic orthophosphate [32PO4] (Fig. 4d), implying that these proteins exist within yeast cells in a pyrophosphorylated form.

These experiments suggest that the pyrophosphoserine bond is resistant to λ-phosphatase. We directly tested this possibility using the Nopp140 peptide containing pyrophosphorylated serines at position 88 and 91 compared with the same peptide with phosphorylated serines at these positions. λ-Phosphatase abolishes phosphorylation of the phospho-Nopp140 peptide but does not affect the pyrophosphorylated Nopp140 peptide (Fig. 4e and SI Table 2).

We also examined the sensitivity of the IP7-phosphorylated NSR1 (27–50) fragment to other protein phosphatases. The following protein phosphatases are completely inactive: calcineurin, protein phosphatase-1, and alkaline phosphatase (SI Fig. 7a). In addition, the pyrophosphatase enzymes, thermolabile inorganic pyrophosphatase and tobacco acid pyrophosphatase, fail to dephosphorylate the IP7-phosphorylated peptide (SI Fig. 7a). Because these enzymes hydrolyze inorganic pyrophosphate (PPi), it is likely that they do not recognize pyrophosphates linked to a serine residue. We also studied DIPP (diphosphoinositol polyphosphate phosphohydrolase), an enzyme that Shears and associates (20, 21) have shown to physiologically dephosphorylate IP7. As expected, pretreatment of the reaction mix containing IP7 lowers phosphorylation, whereas treatment with DIPP after IP7 phosphorylation has no effect (SI Fig. 7b).

In summary, we report that IP7 pyrophosphorylates proteins. Evidence includes the requirement of prephosphorylation by ATP to prime proteins for IP7 phosphorylation and the selective phosphorylation by IP7 of synthetic phosphopeptides but not of...
such peptides that are “blocked” by methylation or are already pyrophosphorylated. Moreover, the properties of IP$_7$-phosphorylated peptides differ markedly from ATP-phosphorylated peptides in terms of acid lability, which is greater with IP$_7$-phosphorylated peptides, and sensitivity to phosphatases, which is much less with IP$_7$-phosphorylated peptides.

We wondered whether 4/6-PP-IP$_5$, the novel isomer of IP$_7$ identified by York and colleagues (15), and IP$_8$ are able to phosphorylate proteins in a manner similar to 5-PP-IP$_5$. Radiolabeled 4/6-PP-IP$_5$ and IP$_8$ phosphorylate proteins in extracts obtained from $S$. cerevisiae (SI Fig. 8), the pattern of phosphorylated proteins being identical to that obtained with the CK2 and ATP phosphorylated sample due to incomplete removal of 5P[IP$_7$].

Fig. 3. The properties of pyrophosphoserine differ from those of phosphoserine. (a and b) NOP-2[pS] peptide was phosphorylated by CK2 and y[32P]ATP (a) or by 5P[IP$_7$]IP$_7$ (b), then treated with HCl and subjected to NuPAGE; Coomassie G250 staining (Left) and autoradiography (Right). (c) NOP-2[pS] peptide was phosphorylated by either CK2 and y[32P]ATP or by 5P[IP$_7$]IP$_7$, as described in SI Materials and Methods. A fraction of the phosphorylated peptides was subjected to NuPAGE to ensure equivalent phosphorylation under both conditions; Coomassie G250 staining (Left) and autoradiography (Right). (d and e) The remaining CK2 phosphorylated peptide (d) and IP$_7$-phosphorylated peptide (e) were hydrolyzed with 6 M HCl, and the resulting amino acids were resolved by HPLC. Data collected during the first 40 min of the HPLC run are presented; elution profiles of the first 3 aa (Upper) and corresponding radioactivity in each 1-ml fraction (Lower). Note that high levels of $^{32}$P in the IP$_7$-phosphorylated sample compared with the CK2 and ATP phosphorylated sample are due to incomplete removal of 5P[IP$_7$].

Fig. 4. $\lambda$-phosphatase does not dephosphorylate IP$_7$-phosphorylated proteins. (a) GST-tagged NSR1 fragment (amino acids 1–50) purified from $S$. cerevisiae was incubated without or with $\lambda$-phosphatase and then phosphorylated by 5P[IP$_7$]IP$_7$ (lanes 1 and 2). Alternatively, the protein was first phosphorylated by 5P[IP$_7$]IP$_7$ and then incubated without or with $\lambda$-phosphatase; Coomassie G250 staining (Left) and autoradiography (Right). (b) GST-tagged NSR1 fragment (amino acids 27–50) purified from $S$. cerevisiae was phosphorylated either by CK2 and y[32P]ATP (Left) or by 5P[IP$_7$]IP$_7$ (Right) and then incubated with or without $\lambda$-phosphatase; Coomassie G250 staining (Left) and autoradiography (Right). (c) NOP-2[pS] peptide bound to streptavidin agarose beads was phosphorylated by either CK2 and y[32P]ATP (Left) or by 5P[IP$_7$]IP$_7$ (Right) and then incubated with or without $\lambda$-phosphatase as in b. (d) $S$. cerevisiae expressing GST-tagged Nopp140 fragment (amino acids 80–100) (Left) or NSR1 fragment (amino acids 27–50) (Right) were labeled with inorganic orthophosphate [32PO$_4$]. Purified radiolabeled proteins were incubated with or without $\lambda$-phosphatase; autoradiography (Upper) and immunoblotting (Lower). (e) NOP-2[pS] peptide (Left) or NOP-2[ppS] peptide (Right) were treated with or without $\lambda$-phosphatase, resolved by NuPAGE, and stained with either Coomassie G250 or Pro-Q Diamond phosphoprotein gel stain, as indicated.
Therefore, it is likely that the principal known inositol pyrophosphates are able to pyrophosphorylate proteins.

Nonenzymatic pyrophosphorylation is thermodynamically feasible. Semiempirical calculations show that, whereas the free enthalpy (ΔH) of reaction for transfer of the β-phosphate from IP₇ to a simple primary alcohol is pH- and counterion-dependent, ΔH values at pH 6.8 can reach ~38.3 kcal/mol (17). This strongly exothermic reaction equals or exceeds ΔH for phosphorylation by ATP. The high phosphorylation potential is attributable to the sterically and electronically crowded environment of the IP₇ pyrophosphate group. The phosphoserine to pyrophosphoserine conversion by IP₇ will require more advanced modeling, but two important clues emerge from the protein substrates for IP₇ that suggest how the 3D structures of the polypeptide may organize reagent and substrate to provide an entropic driving force to contribute to the overall free energy (∆G) for the reaction. First, the polypeptides must have Asp or Glu residues present for pyrophosphorylation to occur. Second, the pyrophosphorylation shows an absolute requirement for divalent cations, with Mg²⁺ preferred (18). Both of these features are reminiscent of the prenyl diphosphate synthases, which feature DDXXD motifs that coordinate Mg²⁺, which in turn activates the pyrophosphate as a leaving group (22).

For serine pyrophosphorylation, one can imagine IP₇ phosphates organized by H-bonds within a polySer tract, whereas the IP₇ pyrophosphate could be activated and targeted to a particular phosphoserine phosphate with the assistance of an Asp/Glu-chelated Mg²⁺.

What might be the physiologic role of pyrophosphorylation? Classic protein kinase-mediated phosphorylation modifies the conformation of proteins, enhancing or decreasing catalytic activity, determining protein localization, or altering protein–protein interactions. Presumably pyrophosphorylation also elicits such alterations in protein function. ATP-mediated phosphorylation is typically regulated by dephosphorylation, whereas pyrophosphorylated proteins are resistant to known protein phosphatases. This suggests that serine pyrophosphorylation may be a more stable modification within the cell, even though it is thermodynamically more unstable compared with phosphoryserine. Such a conclusion is consistent with the fact that some of its such alterations in protein function. ATP-mediated phosphorylation, protein interactions. Presumably pyrophosphorylation also elicits such alterations in protein function. ATP-mediated phosphorylation is typically regulated by dephosphorylation, whereas pyrophosphorylated proteins are resistant to known protein phosphatases. This suggests that serine pyrophosphorylation may be a more stable modification within the cell, even though it is thermodynamically more unstable compared with phosphoserine. Such a conclusion is consistent with the fact that some of its modifications of an earlier method (26). Full experimental details are provided in SI Materials and Methods.

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Materials and Methods

Synthesis of Inositol Pyrophosphate. The inositol pyrophosphate used throughout these studies was 5-PP-I(1,2,3,4,6)₄P₅ (referred to as IP₇). The synthesis and purification of radiolabeled 5β²[P]IP₇ was carried out as described earlier (18). By using these procedures, 5β²[P]IP₇ was obtained at a specific activity of 60 Ci/mmol (1 Ci = 37 GBq) and used at 1–2 μCi per reaction for protein phosphorylation. The synthesis of ²³⁷²[^]P-labeled 4/6-PP-IP₃ and IP₅ is described in SI Materials and Methods. Unlabeled 5-PP-I(1,2,3,4,6)₄P₅ was prepared by total synthesis by using SI Materials and Methods.

Preparation of Recombinant Proteins. The expression and purification of all recombinant proteins are described in SI Materials and Methods.

Protein and Peptide Phosphorylation Assays. Cell lysates were obtained by resuspending yeast cells in ice-cold lysis buffer [20 mM Hepes (pH 6.8)/5 mM DTT/1 mM EGTA/1 mM EDTA/0.1% CHAPS, protease inhibitor mixture, and 200 mg/liter phenylmthanesulfonyl fluoride] and vortexing the sample in the presence of glass beads. The homogenates were centrifuged for 20 min at 15,000 × g, and supernatants were used in the assay. In experiments where lysates were used directly for phosphorylation, protein extracts (10–20 μg) were incubated in IP₇ phosphorylation buffer [25 mM Hepes (pH 7.4)/50 mM NaCl/6 mM MgCl₂/1 mM DTT] and 1 μCi 5β²[P]IP₇ for 15 min at 37°C. The reactions were heated at 95°C for 5 min in sample buffer before separation by NuPAGE (Invitrogen, Carlsbad, CA). The gels were either stained and dried, or transferred to a PVDF membrane. Radiolabeled proteins were detected by autoradiography. Immunoblotting using anti-Xpress (Invitrogen) or anti-GST (Sigma, St. Louis, MO) antibodies, was performed according to standard procedures.

GST- or hexahistidine-tagged proteins purified from S. cerevisiae were used directly for phosphorylation by 5β²[P]IP₇. The protein (1–2 μg) was incubated in the presence of 5β²[P]IP₇, and the reaction was performed as described above. Where indicated, the purified proteins were preincubated with α-phosphatase (400 units; New England Biolabs, Beverly, MA) at 30°C for 1 h, washed twice with PBS, and then used in a 5β²[P]IP₇ phosphorylation assay. GST fusion proteins purified from E. coli, were coupled to glutathione beads, and either incubated directly with 5β²[P]IP₇, as above or were first preincubated with 12 μg of extract prepared from S. cerevisiae (strain DDY1810) at 30°C for 1 h in IP₇ phosphorylation buffer with or without 1 mM ATP or other nucleotides. Where indicated, the S. cerevisiae extract was incubated in boiling water for 5 min, cooled, and then used for the preincubation with purified protein. After incubation with yeast extract, the glutathione beads were washed twice with PBS and once with IP₇ phosphorylation buffer and used for phosphorylation by 5β²[P]IP₇ as described above. In some cases, proteins purified from E. coli were first preincubated with 250 units of CK2 (New England Biolabs) in the supplied CK2 reaction buffer, along with 1 mM ATP at 30°C for 1 h, and then washed and used for 5β²[P]IP₇ phosphorylation. NR1 amino acids 27–50 purified from E. coli cells coexpressing human CK2A1 was used directly in 5β²[P]IP₇ phosphorylation assays.

Peptides were synthesized as described in SI Materials and Methods. Synthetic peptides were used for both CK2 and ATP or IP₇-mediated phosphorylation assays. Peptide (3–6 μg) was incubated with 250 units of CK2 in CK2 phosphorylation buffer, 200 μM ATP, and 1 μCi ³²[^]P]ATP at 30°C for 15–30 min. For IP₇ phosphorylation, 3–6 μg of peptide was added to IP₇ phosphorylation buffer and 1–2 μCi of 5β²[P]IP₇ at 37°C for 15 min, followed by incubation at 95°C for 5 min. The peptides were mixed with sample buffer and resolved on a 12% gel by using the NuPAGE system. The gels were stained by using Safe Stain (Invitrogen), dried, and used for autoradiography. Although peptide phosphorylation by IP₇ was routinely performed at 95°C to maximize the extent of phosphorylation, a time-dependent increase in phosphorylation is observed at 37°C (SI Fig. 9).

Acid-Sensitivity Assay. To determine the sensitivity of CK2 and ATP or IP₇-phosphorylated NOP-2[pS] peptide to treatment with acid, peptides were phosphorylated as described above and then incubated with either 1 M HCl at 50°C, with 3 M HCl at 25°C, or without any acid at 25°C, for 1 h. After incubation, the acid was neutralized with an appropriate volume of 10 M NaOH, and the reaction was mixed with sample buffer and subjected to NuPAGE. Phosphorylation was detected as described above. Peptide hydrolysis in the presence of 6 M HCl, followed by separation of amino acids by HPLC, was performed as described in SI Materials and Methods.

α-Phosphatase-Sensitivity Assay. Purified GST fusion proteins or NOP-2[pS] peptide were phosphorylated by CK2 and γ³²[^]P]ATP or by 5β²[P]IP₇ as described above. After phosphorylation, the
biotinylated NOP-2[pS] peptide was bound to streptavidin agarose beads (Sigma) and washed with PBS. Protein or peptide was then incubated in the presence of λ-phosphatase (400 units; New England Biolabs) in supplied buffer according to the manufacturer’s instructions at 30°C for 1 h, mixed with sample buffer, resolved by NuPAGE, and subjected to autoradiography. Expression and purification of 32PO4-labeled GST fusion proteins and their treatment with phosphatase is described in SI Materials and Methods.

The Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) was used to determine relative levels of phosphate on the NOP-2[pS] or NOP-2[ppS] peptides before or after treatment with λ-phosphatase. NOP-2[pS] peptide (6 μg) or NOP-2[ppS] peptide (24 μg) were incubated with 400 units of λ-phosphatase for 1 h at 30°C and then resolved by NuPAGE and stained with Pro-Q Diamond stain according to the manufacturer’s instructions.

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