The protein phosphatase encoded by coliphage lambda (PPA) was found to be the equivalent of the minimal catalytic core of serine/threonine protein phosphatases (PP) by biochemical and mutational criteria. Bacterially expressed truncated versions of PPA and PPA phosphatases, representing the catalytic cores homologous to PPA, exhibited potent phosphatase activity. Unlike full-length PPA, but like PPA, the recombinant cores could use casein, p-nitrophenyl phosphate, and a wide variety of peptides as substrates and were resistant to okadaic acid, microcystin-LR, and trypsin. Mutations of His173, Asp208, or Arg221 had little effect on the activity of the PPA1 core protein, indicating its closer identity with PPA than with full-length PPA. Terminal deletions of a few amino acids of the cores destroyed their activity, supporting their minimal nature. Analysis of PPA mutants suggested an influence of the substrate on metal ion binding. The minimal length of a phosphopeptide substrate of PPA appeared to be a phosphorylated serine/threonine flanked by 1 or 2 amino acid residues on either side, the N-terminal ones being more effective.

Protein phosphatases play essential roles in the reversible phosphorylation and dephosphorylation of proteins and have been implicated in an enormous variety of cellular events (reviewed in Refs. 1–4). However, studies of structure-function relationships in protein phosphatases have been initiated only recently. Multiple alignment of the primary sequences of Ser/Thr phosphatases [PP] including the bacteriophage phosphatase PPA (5–7) has revealed a highly conserved central region of about 250 residues that contained three invariant stretches of amino acids, viz. GDHXG, GDVXDRG, and RGNHE, separated by 25–30 residues (X representing any amino acid). Portions of the conserved region also exhibited significant similarity with various phosphatases that had no known protein phosphatase activity (5, 8).

Crystallographic analysis of eukaryotic Ser/Thr phosphatases PP1 and calcineurin have revealed the structure of the catalytic site and indicated possible roles for a number of invariant residues in catalysis and/or metal ion binding (9, 10). In both enzymes, the active site has been proposed to contain two metal ions that used the following amino acid ligands (PP1 numbers followed by corresponding numbers for calcineurin in parentheses), Asp64(69)*, His66(92)*, and Asp92(118)* for one metal ion and Asp92(118)*, Asn124(150)*, His173(199)*, and His248(281)* for the other metal ion. In addition, both metal ions also coordinate to water molecules and to the phosphate group of the substrate. Amino acid residues that played a direct role in phosphate binding are Arg96(122)*, Asn124(150)*, His173(199)*, Arg211(254)*, and Tyr272(311)*. In calcineurin, the residue equivalent to PP1-His125 (His151) has been proposed to coordinate to a third water molecule (10, 11). Asp96(122)* and Asp208(234)* were not directly involved in either metal or substrate binding but were likely to be hydrogen-bonded to His125(151)* and Arg211(254)*, respectively. It remains intriguing, therefore, that some of these residues are conspicuously absent in PPA; these include Arg221(254), His248(281), and Tyr272(311). In the absence of a crystal structure of PPA, it is not known whether unique PPA residues may serve the function of these residues.

Together, these studies have also spawned the notion that the central conserved region may constitute the primary catalytic domain of all phosphatases, whereas the exterior residues may be involved in secondary activities, such as interaction with activators and inhibitors (9, 11). Mutational evidence to support these analyses, however, remains fragmentary, primarily due to the difficulties in obtaining the catalytic subunits in large quantities and in soluble form. Recent cloning and expression of the bacteriophage lambda (λ) phosphatase (PPA) have helped to expedite biochemical and mutational analysis of Ser/Thr phosphatases (12, 13). Recombinant PPA, expressed in Escherichia coli, was shown to possess a phosphatase activity that was active against Ser(P), His(P), and Tyr(P) residues in a number of proteins, although dephosphorylation of Tyr(P) residues was pronounced slower than that of the other two (12, 13). The unique biochemical properties of PPA included resistance to okadaic acid and heat-stable inhibitors 1 and 2, sensitivity to orthovanadate, an absolute requirement of Mn2+, and Ni2+ that could not be substituted by Cu2+ or Mg2+, and an apparent absence of a regulatory subunit. Thus, despite the sequence homology with eukaryotic phosphatases, a biochemical classification of PPA has remained elusive (12). With regard to mutagenesis, deletion of one of the invariant peptides, viz. RGNHE, destroyed the protein phosphatase activity of recombinant PPA (12). Subsequently, detailed mutational analysis of PPA (8) suggested specific roles of the following invariant residues (the corresponding PPA numbers are shown in

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1 The abbreviations used are: PPA, protein Ser/Thr phosphatase; CNBr, cyanogen bromide; CK2, casein kinase 2 (II); CC, minimal catalytic core; Phos α, phosphorylase a; pNPP, p-nitrophenyl phosphate; PCR, polymerase chain reaction; RSV, respiratory syncytial virus; PPA, protein phosphatase lambda; PAGE, polyacrylamide gel electrophoresis.
parentheses, although mutagenesis of the PP1 residues have not been reported: Asp206, His209, Asp212, Arg215, His321, and Glu322 are involved in Mn$^{2+}$ binding, whereas Arg52, Arg55, and Arg73,122 are important in substrate binding. These studies were, however, carried out using the small molecule substrate, pNPP; the behavior of the mutants against phosphoprotein substrates remained unexplored.

Although the minimal substrate motifs of many protein kinases have been elucidated with a high degree of certainty, those of Ser/Thr phosphatases are essentially unknown (14). This is due to two major reasons: an apparent promiscuity of a given phosphatase against a variety of substrates, and the difficulty of chemically synthesizing phosphopeptides containing Ser(P). We have recently found that the P protein (phosphoprotein) of human respiratory syncytial virus (RSV) expressed by recombinant means is phosphorylated at a single serine (Ser$^{237}$) using cell extracts and that the resultant phosphoprotein is an excellent substrate for PPA in vitro.

Based on these findings, we have explored the availability of recombinant PPA and the recombinant P protein substrate to investigate the following fundamental aspects of Ser/Thr phosphatase function: (a) the boundaries of a minimal phosphatase; (b) biochemical properties and substrate specificities of the catalytic cores of eukaryotic phosphatases; (c) relationship of eukaryotic phosphatases to PPA; (d) mutational analysis of the catalytic core using defined phosphoprotein substrates; and (e) minimal length of a peptide substrate of PPA.

**EXPERIMENTAL PROCEDURES**

**Expression and Mutagenesis of Recombinant Proteins—**All phosphatases and RSV P protein were expressed in *E. coli* in soluble form using the pET-3a expression system as described (12, 15). Internal deletions and site-directed mutagenesis of the PPA gene were carried out in vitro using modified megaprimer methods based on PCR, as described (16). An initiator methionine was added to the N-terminal deletions. Bacterially expressed mutants of PPA were purified as described earlier, except that an additional phenyl-Sepharose chromatography was performed (13). The postulated cores of the catalytic subunits of PP1 (Lys$^{159}$-Ser-Arg-Glu-Ile-Ph-e-Leu . . . Val-Thr-Leu-Ph-e-Ser-Ala$^{241}$, 241 residues) (17) and PP5 (Val$^{51}$-Leu-Ser-Lys-Leu . . . Val-Thr-Ile-Ph-e-Leu$^{181}$, 228 residues) (18), homologous with PP2A, were amplified by reverse transcription-PCR using appropriate oligonucleotide primers such that the proteins had an initiator Met residue. The products were cloned into the Ndel and BamHI sites of pET-3a. *E. coli* BL21(DE3) cells containing pET-3a-PP5-CC or pET-3a-PP1-CC were grown in LB containing 4 μM MnCl$_2$ (19) at room temperature (25–28°C) to an $A_{600}$ of about 0.4. β-Isopropyl-thio-galacto- side was added to a final concentration of 0.2 mM, and growth was continued for another 1 h at room temperature. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris-Cl, pH 8.3, 50 mM NaCl, 2 mM EDTA, 2 mg of lysozyme per ml, 10% glycerol, and 2 mM dithiothreitol), and lysed by sonication in ice. MnCl$_2$ was added to the lysate to a final concentration of 10 mM, and the broken cells and debris were removed by centrifugation at 13,000 × g for 10 min. The supernatants containing soluble and active phosphatases were assayed using pNPP or phosphoproteins as substrate. Recombinant RSV P protein was purified as described (15).

**Generation of Truncated RSV Phosphoproteins—**Through site-directed mutagenesis, mutants of RSV P protein were made in which Ser$^{237}$ was first mutated to Ala (15), and then various amino acid residues at different distances upstream and downstream of the phosphorylatable Ser$^{237}$ residue (20) were mutated to Met using PCR. Expression and purification of recombinant mutant P proteins using the pET-3a expression system of E. coli were performed as described (15). 2–4 μg of purified protein was phosphorylated for 1 h using total HEp-2 cell extract as the source of CK2 and $[^{32}P]ATP$ as phosphate donor under standard kinase conditions as described earlier (15). Unincorporated ATP was then removed by inclusion in a gel filtration column. The excluded fraction was heated at 60°C for 10 min to inactivate the CK2. Wherever needed, P protein was concentrated in a Centricon 10 concentrator (Amicon).

To generate phosphopeptide fragments of the P protein, the phosphorylated P protein was cleaved at specific Met residues as follows.

The 32P-labeled P protein was incubated with CNBr (66 μg/ml) in 70% formic acid for 16 h at room temperature in the dark. The reaction was evaporated to dryness and contained essentially pure peptides. Peptidemdescribed in Table III were purchased from Research Genetics (Huntsville, AL). The majority of the peptides was further purified by reversed-phase HPLC and ion-exchange chromatography as described. The purity of the peptides was ascertained by a combination of these methods, by amino acid analysis, and by SDS-PAGE as follows. Peptide$\ldots$ were electrophoresed in discontinuous Laemmli gels (40% acrylamide; 12% bisacrylamide) (21) overnight at a constant 70 V until the dye front just ran out of the gel, followed by direct autoradiography and densitometric scanning of the autoradiograph. These gels resolved peptides as short as trimers and separated two peptides differing by a single amino residue.

**Phosphatase Reactions—**RSV-P and phosphorylase b proteins were phosphorylated by HEp-2 extract and the catalytic subunit of PKA, respectively, as described (20). The exact stoichiometry of phosphorylation in each substrate preparation was calculated from the known molecular weights of the substrates and the specific activity of ATP used. In different preparations, the stoichiometry ranged between 0.2 and 0.8, generating about 1000–4000 cpm per pmol substrate. For none of these substrates did the presence of excess non-phosphorylated substrate have any effect on phosphatase activity, confirming an earlier observation (22).

A standard phosphatase reaction (12) contained 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM diithiothreitol, 20 mM MnCl$_2$, 2–2.0 mg of enzyme (depending on activity), and pNPP (20–30 μM) or phosphorylated proteins (0.5–100 μM) as substrates. The pNPP reactions were carried out in 0.5 ml, terminated by the addition of 0.5 ml of 1 N NaOH, and quantitated by measuring absorbance at 420 nm. For 32P-labeled substrates, reaction volumes were 20 μl, and at various time points, 5-μl portions were taken out for analysis. The liberated 32P was quantitated with the phosphomolybdate assay using carrier orthophosphate, essentially as described (22). In addition, portions of the same phosphatase reactions were sometimes analyzed by SDS-PAGE as described above. For wild type enzymes and for mutants having near wild-type activity, reactions were carried out for a few minutes only, whereas reactions using the highly defective PP mutants were typically continued for a few hours and/or overnight. The $K_{m}$ values of substrates and Mn$^{2+}$ were determined by Edie-Hofstee (23, 24) plot using initial rate constants, when no more than a fifth of the substrate had been hydrolyzed. In the pNPPase assay, 1 unit of phosphatase was defined as the activity hydrolyzing 1 μmol of pNPP per min at 37°C (13).

**RESULTS AND DISCUSSION**

**PPA Represents a Minimal Catalytic Core—**As alluded to earlier, all PPs contain a region of about 250 amino acids long, which is homologous to PPA, and which we will refer to as the minimal catalytic core. The relative location of this core in the different phosphatases varies greatly. Thus, PPZ1 has about 400 residues upstream (N-terminal) and 50 residues downstream (C-terminal) of its core (25), whereas in PP2B, there are only about 70 residues upstream of the core but about 200 residues downstream (26). Because of this large variation in the total length of the catalytic subunit of phosphatases, it was important to first determine the limits of the minimal functional core of these enzymes. Thus, we carried out systematic deletions at either terminus of PPA and determined their effects on enzyme activity. While deletion of up to 2 residues (Arg-Tyr) from the N terminus had no effect on activity, deletion of 3 residues (Arg-Tyr-Tyr) reduced the enzyme activity by about 80%, and deletion of 4 residues (Arg-Tyr-Tyr-Glu) abolished activity completely. The C terminus was even more sensitive to deletions. A single residue (Ala) deletion did not have any appreciable effect on activity, whereas deletion of 2 residues (Gly-Ala) reduced enzyme activity by about 98%. Further deletions from either end, as expected, produced inactive mutants. Essentially similar results were obtained using RSV phosphoprotein as substrate (Fig. 1, lanes 1–9), demonstrating the authenticity of the pNPPase assays.

Once the minimal functional length of PPA was known, we were in a position to extend the analysis to larger, eukaryotic...
phosphatases. Specifically, we wanted to test whether the PPα-related core regions of the eukaryotic phosphatases, when expressed by recombinant means, retain catalytic activity. We chose two phosphatases, viz. PP1 and PP5, for this purpose, since they are phylogenetically distant, differ in length, and possess variable numbers of amino acids upstream and downstream of the postulated core region (17, 18). We opted to keep a few extra amino acids on either side of the core, since (a) the homology of eukaryotic phosphatases with PPα falls off toward the terminus of the core, which makes the borders of the core region somewhat imprecise, and (b) the extreme intolerance of PPα to terminal deletions (Fig. 1) made it likely that the same might hold true for the core regions. Thus, we cloned and expressed the region Lys41-Ser-Arg-Glu-Ile-Phe-Leu..... Val-Thr-Val-Phe-Ser-Ala441 of PP5 with an initiator Met in front of the reading frame. The recombinant core polypeptides (Fig. 2) were tested for phosphatase activity in vitro using pNPP as a substrate. Based on the quantitation of the expressed proteins in stained SDS-polyacrylamide gels, we estimated specific activities of about 3200 and 4100 units per mg of PP1 and PP5 core region proteins, respectively, that are comparable (albeit somewhat lower than) with that of PPα (~6,000 units/mg). As shown later, both cores also possessed substantial casein phosphatase activity (Table III). Deletion of 10 amino acid residues from either the N or the C terminus of the PP1 core polypeptide destroyed the pNPPase activity (data not shown), strengthening the essential and minimal nature of this region in the catalytic function of PP1. Thus, residues outside the core region, even if they may be conserved between eukaryotic phosphatases (e.g. His248, Ser-Ala-Pro-Asn-Tyr272 etc.), may not be absolutely essential in catalysis. In what follows, the equivalence of the catalytic cores of different phosphatases is further strengthened by biochemical studies.

Resistance of the Catalytic Cores to Trypsin and Inhibitors— Crystallographic studies have indicated that PP1 possesses a condensed central scaffold of alternating α and β regions held together by two metal ions. Digestion of PP1 catalytic subunit with trypsin was previously shown to produce a ~33-kDa fragment that appeared to retain most of the phosphatase activity (27, 28). Although the sites of cleavage were not mapped, it is tempting to speculate that the trypsin-resistant fragment included the catalytic core region of PP1 that we have cloned and expressed here. In order to test this and also to establish a convenient structural criterion for the core polypeptide, we tested the effect of trypsin on PPα and the core PP1 and PP5 recombinant proteins. No detectable change in the mobility of PPα in SDS-PAGE or its phosphatase activity was observed even after 30 min of pretreatment with stoichiometric amount of trypsin (data not shown). Essentially similar resistance to trypsin was observed using the cores of PP1 and PP5 (data not shown), demonstrating a highly compact nature of the catalytic region of the phosphatases.

To determine whether the similarity among the core polypeptides also extends to their accessory properties, we tested the sensitivity of the enzymes to phosphatase inhibitors. Both PP1 and PP5 are known to be sensitive to nanomolar concentrations of okadaic acid and microcystin-LR (18, 29), whereas PPα is completely resistant to micromolar concentrations of either inhibitor (12, 13). When the PP1 and PP5 core enzymes were tested at a high dilution against [32P]casein as substrate, both enzymes were also found to be resistant to okadaic acid and microcystin-LR at concentrations as high as 1.2 μM (data not shown).

Mutational Analysis of the Catalytic Core—To ascertain whether the invariant amino acid residues within the minimal catalytic region play essential roles in the phosphoprotein phosphatase activity, we have carried out deletion and site-directed mutagenesis analysis of the core proteins. The Kcat and Km values of two phosphoprotein substrates, viz. phospho-rylase α and RSV P protein, were determined for each recombinant mutant enzyme as described under “Experimental Procedures.” The relative activities of the various mutants closely paralleled those obtained with pNPP as substrate, although in general, the defects appeared to be more severe when phosphoprotein substrates were used. These results are presented in Table I, and a few selected mutants are also represented in Fig. 1 (lanes 10–13). We mutated two additional residues, viz. Asn75, which belongs to the invariant stretch RGNHE (Asn244 in PP1), and His140, which is not invariant, but its closest equivalent in PP1 (His173) has been postulated to be involved in metal binding in PP1 (9). Mutation of Asn75 to Asp reduced the pNPPase activity by 85% (data not shown) and the protein phosphatase activities by more than 99%. However, there was little effect on the Km for Mn2+ measured with either kind of substrate (Table II), suggesting that this residue is not involved in binding the metal ion and may be more important in catalysis and/or substrate binding. Mutation of His140 to Gln had a moderate effect on catalytic activity (Table I); there was

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* T. Ansai, L. C. Dupuy, and S. Barik, unpublished results.
no effect on $K_m$ of Mn$^{2+}$ using pNPP as substrate; however, using phosphoprotein substrates, a 4–6-fold increase in $K_m$ was observed (Table II). Likewise, mutation of the corresponding His$^{177}$ in the PP1 core polypeptide also had very little effect on either the catalytic parameters (Table I) or the metal ion $K_m$ (Table II).

The metal $K_m$ values of Asp$^{75}$ and His$^{140}$ mutants of core PP1 presented above (Table II) are in apparent conflict with structural predictions for full-length PP1, whereby the equivalent residues Asn$^{124}$ and His$^{177}$ have been shown to coordinate the second of the two metal ions that were fitted into the crystal structure (M2 in Fig. 2 of Ref. 9). Asn$^{124}$ was additionally involved in bonding to one of the oxygens of the phosphate group. Thus, we entertain the possibility that M2 may be involved in bonding to one of the oxygens of the phosphate group. However, as shown in Table I, PP1 core mutants D208N and R221A both showed essentially wild type activity. Since the C terminus of PP1 is dissimilar with PP1, there are no obvious analogs of these residues in PP1. Our ability to produce active PP1 and PP5 cores devoid of Tyr$^{272}$ also suggests a lack of its importance in basic catalysis, whereas in full-length PP1, this residue is postulated to be involved in coordinating a water molecule and the phosphate group (9). Based on these results and the overwhelming biochemical similarity between PP1 and the catalytic cores of PP1 and PP5 presented earlier, we suggest that a similar three-dimensional structure may be operative for the active site of all these core polypeptides, which is somewhat different from that of the full-length proteins. In other words, removal of the extraneous residues may alter the structure of the active site of the eukaryotic phosphatases.

The mutations described above generally had a more severe effect on the phosphoprotein phosphatase activity than on pNPPase activity. For example, the $K_{cat}/K_m$ values for D52N and E77Q mutants were about 8 and 2% of wild type activity, respectively, using pNPP as substrate (8), but had negligible activity against the phosphoproteins (Table I). Interestingly, the $K_m$ values for Mn$^{2+}$ were always significantly higher when the phosphoprotein was used as substrate than when pNPP was the substrate. Table II shows the mutants with the most significant substrate-dependent differences of metal ion $K_m$. The difference is particularly dramatic for the D52N mutant, which had a near normal Mn$^{2+}$ $K_m$ using pNPP, but about 17-fold higher Mn$^{2+}$ $K_m$ using the phosphoprotein. Taken together, these results lead to the obvious conclusion that the nature of the substrate may influence the interaction of the metal ion with the enzyme. Although we do not know the exact reason for this phenomenon, it is consistent with the demonstration that the metal ion, the phosphatase group of the substrate, and the enzyme form a tripartite complex in the three-dimensional structure of PP1, and thus, interaction between any two components of the complex can have an effect on interaction with the third component. It is also possible that the metal ion directly affects the structure of the phosphoprotein substrate. At the same time, this raises caution in interpreting the role of a specific residue of an enzyme in metal binding using only one kind of substrate.

| Mutant | $K_m$ (Mn$^{2+}$ (μM)) | $K_m$ (pNPP) | $K_m$ (RSV-P) | $K_m$ (Phos a) | $K_m$ (pNPP) | $K_m$ (RSV-P) | $K_m$ (Phos a) |
|--------|------------------------|--------------|--------------|---------------|--------------|--------------|---------------|
| PPA    |                        |              |              |               |              |              |               |
| Wild type | 0.22 ± 0.04             | 240 ± 4     | 1090 ± 15   | 0.11 ± 0.03   | 180 ± 4      | 1636 ± 24    |               |
| D20N   | 0.41 ± 0.06             | <0.004      | <0.008      | 0.25 ± 0.05   | <0.003       | <0.006       |               |
| H22Q   | 0.34 ± 0.05             | <0.008      | <0.02       | 0.14 ± 0.04   | <0.005       | <0.018       |               |
| D49N   | 0.52 ± 0.05             | <0.08       | 0.15 ± 0.04 | 0.61 ± 0.05   | <0.095       | 0.13 ± 0.02  |               |
| D52N   | 2.5 ± 0.2               | <0.006      | <0.005      | 1.8 ± 0.2     | <0.004       | <0.002       |               |
| R53K   | 5.0 ± 0.5               | <0.003      | <0.005      | 3.8 ± 0.3     | <0.004       | <0.0005      |               |
| R73K   | 4.0 ± 0.3               | 1.2 ± 0.2   | 0.3 ± 0.04  | 5.2 ± 0.4     | 1.4 ± 0.04   | 0.27 ± 0.04  |               |
| N75D   | 5.5 ± 1.0               | <0.005      | <0.005      | 4.1 ± 0.2     | <0.005       | <0.0008      |               |
| H76Q   | 0.2 ± 0.03              | <0.004      | <0.018     | 0.3 ± 0.04   | <0.003       | <0.006       |               |
| E77Q   | 4.6 ± 0.2               | <0.003      | <0.0005    | 4.8 ± 0.2     | <0.003       | <0.0005      |               |
| H140Q  | 0.5 ± 0.1               | 224 ± 5     | 448 ± 12    | 0.15 ± 0.03   | 143 ± 5       | 953 ± 14     |               |
| PP1 CC |                        |              |              |               |              |              |               |
| Wild type | 0.35 ± 0.03             | 156 ± 5     | 445 ± 10    | 0.22 ± 0.03   | 115 ± 4       | 523 ± 12     |               |
| D208N  | 0.45 ± 0.04             | 124 ± 6     | 276 ± 8     | 0.25 ± 0.03   |               |               |               |
| R221A  | 0.30 ± 0.03             | 118 ± 3     | 393 ± 12    | 0.24 ± 0.04   |               |               |               |
| H173Q  | 0.32 ± 0.04             | 143 ± 5     | 447 ± 14    | 0.28 ± 0.05   |               |               |               |
Synthetic peptides were phosphorylated in the presence of [γ-32P]ATP by the catalytic subunit of protein kinase A, except for RREEETEEEEAA, which was phosphorylated by purified CK2. Partially dephosphorylated α-casein was phosphorylated by HEP-2 cell extract. Phosphatase reactions were carried out as described under “Experimental Procedures” except that the 32P-labeled peptide concentration was 2 μM (calculated from specific radioactivity), and assays were carried out for 10 min at 30 °C in the presence of 20 mM Mg2+ and at an enzyme concentration of 30 μM (15 ng of enzyme in a 20-μl reaction). PP2A results were taken from Ref. 22; therefore, reaction conditions and enzyme concentrations were also adopted from that paper. The reaction rate remained unchanged over a substrate concentration range of 2–200 μM. The labeled [32P]phosphate was quantitated using the phosphomolybdate assay as described under “Experimental Procedures.” Activities are expressed in pmol phosphate liberated per min per ml (22). The (?) mark indicates that the substrate was slightly different from the one in Ref. 22, where the CK2 peptide had an extra Arg residue at the N terminus, and the mixed casein was phosphorylated by CK2.

| Peptide    | PP2A | PP1 | PP1-CC | PP5-CC | PP6 |
|------------|------|-----|--------|--------|-----|
| RRASVA     | 12   | 0   | 50     | 46     | 72  |
| RRRAASVA   | 375  | 11  | 634    | 347    | 400 |
| RRATVA     | 320  | 0   | 144    | 223    | 322 |
| RRPTVA     | 316  | 0   | 165    | 310    | 256 |
| RPRTVA     | 0    | 0   | 0      | 0      | 0   |
| RREEETEEEEA| 200  | (?) | 123    | 117    | 168 |
| (2/3)α-casein| 22  | (?) | 144    | 116    | 160 |

studies by Pinna and co-workers (22) have recently established a collection of synthetic peptides that served to distinguish between PP1, PP2A, and PP2C. PP1 was shown to be ineffective against all of these peptides, whereas PP2A was more active than PP2C against all peptides tested. Interestingly, however, the core PP1 enzyme was found to be highly active against these peptides and also against phosphorylated casein (Table III). Moreover, it is clear that both the PP1 and PP5 core enzymes had very similar overall substrate profile that is comparable with that of PP6 (Table III) which once again supports the biochemical equivalence of these enzymes. While it has not been reported whether PP5 displays any casein phosphatase activity, its activity against phosphorylase a has been shown to be 100-1000-fold lower than that of PP1 or PP2A (18). If this is also true for casein, then the increased activity of the core PP5 enzyme toward casein must be a result of truncation of the enzyme.

A comparison of the substrates confirms earlier results and extends them to the core enzymes including PP6. Comparison between RRASVA and RRATVA, for example, suggests that phosphothreonine is a much better substrate than phosphoserine (22). A proline on the C-terminal side of the phosphothreonine completely abolished substrate activity by all phosphatases tested so far, inclusive of PP6 (Table III). Within a given class, bigger peptides were generally better than the smaller ones (compare RRRAASVA to RRASVA). As shown below, this is also in agreement with the lower limits of a functional substrate.

The Minimum Length of a Phosphatase Substrate—Since phosphopeptides containing phosphoserine residues are difficult to synthesize by chemical means, we took advantage of the fact that recombinant P protein of RSV was phosphorylated in vitro at the single Ser residue 232 by the casein kinase II (CK2) activity in HEP-2 cell extracts, is a potent substrate for PP6. The minimal recognition motif of CK2 has been found to consist of a Ser (or Thr) residue at site n (to be phosphorylated) followed by an acidic residue (Asp or Glu) at n + 3 on the C-terminal side, SXXXD/E. Since Ser232 can also act as a phosphoacceptor site under certain conditions (15), we mutated this site to Ala to limit phosphorylation exclusively at Ser232 and then used this mutant as the starting material to construct all other mutants described here. Thus, the relevant region, close to the C terminus of the 241-residue long RSV P protein, has the sequence Gly-Asn-Asp-Asp232Asp-Asn-Asp-Leu-Ala-Leu-Glu-Ala-Phe, the underlined Ser being the phosphorylation site. To generate shorter phosphopeptides, at first C-terminal deletion mutants of P protein were made by simply bringing the termination codon UGA closer to Ser232 by PCR mutagenesis. The resultant shorter proteins were expressed in E. coli, purified, phosphorylated by cell extract, and used as substrate in phosphatase studies. As shown in Table IV, PP6 could efficiently dephosphorylate a P protein in which the phosphoserine has only 3 amino acid residues on the C-terminal side (peptide number 4). Mutants with deletions closer to Ser232 could not be phosphorylated by the cell extracts. Thus, to get closer to Ser232 from the C-terminal end, and to produce deletions on the N-terminal end, we devised a different strategy. By site-directed mutagenesis, desired residues on either side of Ser232 were first mutated to Met. The recombinants were purified, phosphorylated by cell extract, and then cleaved at Met residues using CNBr. The resultant phosphopeptides were purified and then used as substrate for PP6 as described under “Experimental Procedures.” Results clearly show (Table IV) that there is a gradual loss of substrate function as the deletions approach closer to the phosphoserine from either the N- or the C-terminal end and that a phosphopeptide of the minimal sequence XS(P)X is a reasonably efficient substrate. Further deletion to produce dipeptides S(P)X or XS(P) (where S(P) represents phosphoserine, and X represents any residue) results in sharp drop of substrate activity, although XS(P) is a somewhat better substrate than S(P)X. Just serine phosphatase, by itself, was not a substrate (12, 13). Thus, one amino acid on either side of phosphoserine appears to be necessary and sufficient for phosphatase action, although 1 or 2 extra residues considerably improves the efficiency.

We would like to note that during the above studies we unraveled some of the sequence specificities of CK2, based on the efficiency with which various mutant recombinant RSV P protein acted as substrates for CK2. The relevant sequence regions of the mutants and their relative substrate activities, measured in a standard kinase reaction as under “Experimental Procedures,” were as follows (the phosphorylation site is underlined; mutations are in bold): the wild type P protein (GNDSDNDLNALEDPF), and two mutants with substitutions of hydrophobic residues at n + 2 position (GNDSDLNLAEFD, and GNDSDLNLALAEFD) were excellent substrates; a deletion mutant with no amino acids beyond n + 3 (GNDSDND), a mutant with a Met replacing the n + 1 acidic residue Asp (GNDSDLNLALAEFD), and a mutant with a basic residue (Lys) at n + 4 (GNDSDLNLDLAEFD) were all moderately good substrates; finally, mutants with a Lys or Leu at n + 1 (GNDSDLNLALAEFD, GNDSDLNLALAEFD), or a Lys at n + 2 (GNDSDLNLALAEFD), or mutants with one or no residue following Ser (GNDSDND, GNDSDND) could not be phosphorylated by CK2 at all. Detailed studies of Kd and Vmax of these substrates should shed light on the contribution of the specific residues in substrate activity. The inability to phosphorylate the last group of deletion proteins further underscored the need for our CNBr cleavage strategy in generating phosphopeptides for phosphatase reactions.

The principal conclusions of this communication are as follows. (i) PP6 homology regions of eukaryotic phosphatases are necessary and sufficient for phosphatase activity and, therefore, represent the minimal catalytic domain or the catalytic core of Ser/Thr phosphatases. (ii) Divalent cation cofactors and substrates most likely bind to the catalytic core of the phos-
Phosphorylation of bacterially made RSV-P protein or its site-directed Met mutants and CNBr cleavage of the $^{32}$P-labeled protein were carried out as described under “Experimental Procedures.” Phosphatase reactions were performed essentially as in Table III. The values were expressed relative to that using full-length RSV-P protein, taken at 100 (about 580 pmol of $^{32}$P liberated/ml/min). The phosphorylated Ser residues are underlined. The various phosphopeptides were produced by CNBr cleavage at the flanking methionine residues, either present in wild type P protein (Met$^{266}$) or introduced through mutagenesis. The only exception was peptide 8, whose C-terminus is the result of a termination codon. All peptides were used at a concentration of 100 μM, the stoichiometry of phosphorylation ranging between 0.5 and 0.8 (mol/mol).

| Peptide no. | RSV-P fragment | Length (in number of amino acids) | Relative activity of PP1 |
|------------|----------------|----------------------------------|-------------------------|
| 1.         | Full length P  | 241                              | 58                      |
| 2.         | $\ldots \ldots \ GNDSDND$ | 235                              | 75                      |
| 3.         | Ala$^{207} \ldots GNDSDNDLALDF$ | 35                               | 100                     |
| 4.         | Ala$^{207} \ldots GNDSDND$ | 29                               | 83                      |
| 5.         | Ala$^{207} \ldots GNDSD$  | 27                               | 64                      |
| 6.         | Ala$^{207} \ldots GND$  | 26                               | 54                      |
| 7.         | DSDNDLALDF       | 11                               | 62                      |
| 8.         | DSDNDLA          | 7                                | 28                      |
| 9.         | DS             | 3                                | 18                      |
| 10.        | DS$^*$          | 2                                | 8                       |
| 11.        | SDNDLALDF       | 10                               | 29                      |
| 12.        | SD             | 2                                | 2                       |
| 13.        | S$^*$           | 1                                | 0                       |

**Table IV: Minimum substrate length for PP1**

Phosphatase in an interactive manner. (iii) The biochemical properties of the catalytic cores of different phosphatases are highly similar, despite the differences in the full-length enzymes. (iv) The structure of the catalytic site in the core polypeptide may be different from that of the full-length phosphatases, perhaps involving only one metal ion instead of two. (vi) The minimal substrates for the core phosphatase, in order of efficiency, appears to be X(S)P(X > X)S(P) > S/PX. Random mutagenesis of amino acid residues neighboring the phosphoserine in the RSV P protein should produce a more detailed picture of the substrate sequence specificity of PP1. This is currently under way.

A variety of earlier observations are either explained by or consistent with our results. Deletion of 33 amino acid residues from the C terminus of recombinant PP1 had no effect on enzyme activity or on the binding of okadaic acid or inhibitor-2 (30, 31). In the three-dimensional structure of the full-length PP1, residues C-terminal to 272 are progressively further away from the catalytic pocket and were considered to be unlikely to participate in catalysis (9). The resistance of the catalytic core polypeptide to phosphatase inhibitors is consistent with recent studies attempting to map the inhibitor-binding domains. Mutational and structural analyses of PP1, for example, have suggested an essential role of Gly$^{274}$-Glu-Phe-Asp in binding microcystin and okadaic acid (32) and of Cys$^{273}$ in covalent linkage to microcystin (9). In PP2B (calcineurin A), the binding site for calcineurin B was shown to be at the end of a C-terminal groove, immediately following the catalytic domain (11).

It should be noted that despite the close similarity among the core enzymes, they differ in specific activity against a given substrate and thus, specific unique residues within the core domain must also contribute to optimal phosphatase activity. For example, although mutation of Cys$^{138}$ to Phe reduces PPA activity (12), the role of this Cys residue must be unique in PPA, since it is absent in all other PPases. Recent mutagenesis studies of recombinant PP1 in fact demonstrated that no Cys residue of PP1 is important for enzymatic activity (34). Clearly, analysis of co-crystals of the full-length and core phosphatases with their phosphopeptide substrates would be needed to determine the exact structure of the catalytic site and the role of various residues in catalysis. It will be also interesting to decipher the effect of the PPA mutations on its phosphohistidine-phosphatase activity reported earlier (13).

An increasing number of reports have demonstrated the regulation of eukaryotic phosphatases by direct phosphorylation; although the sites of phosphorylation generally remained unknown, in a recent study, cdc2-mediated inhibition of PP1α has been shown to result from phosphorylation at the Thr$^{220}$ residue of PP1α (33), which is outside the catalytic core region. In an attempt to answer whether such phosphorylations could occur within PP1 or PP1-CC, we have used a variety of potential sources of kinase and reaction conditions, followed by analysis of the reactions in SDS-PAGE and autoradiography. These studies have only produced negative results (data not shown). In brief, attempts to autophosphorylate PP1 or PP1-CC, or to phosphorylate them with bacterial or HEp-2 cell extracts, in the presence or absence of various divalent cations (Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$) and/or cAMP, using either $[\gamma^{32}\text{P}]\text{ATP}$ or $[\gamma^{32}\text{P}]\text{GTP}$ as phosphate donor, failed to demonstrate any phosphorylation. Phosphorylation could not be achieved even in the presence of orthovanadate or okadaic acid, or by using phosphatase-defective PPA mutants. To investigate phosphorylation in vitro, we subjected E. coli cells expressing either wild type or phosphatase-defective recombinant PP1 to metabolic labeling with $[\text{32P}]\text{orthophosphate}$, followed by immunoprecipitation with anti-PP1 antibody. Again, no $^{32}$P labeling was detected in the PP1 protein band. Using $[\gamma^{32}\text{S}]\text{GSH}-\text{ATP}$ as the (thio)phosphate donor, a stoichiometric thiophosphorylation of PP1 could be demonstrated in vitro; however, this was judged to be nonspecific and nonenzymatic on the following grounds. (a) Thiophosphorylation occurred within a few seconds at 0 °C. (b) Essentially all proteins could be thionophosphorylated under these conditions, including all the protein bands in a total E. coli extract. Thus, we conclude that regulatory phosphorylation, like other regulatory features of phosphatases, may map outside of the catalytic core.

Phosphate transfer reactions catalyzed by a variety of phosphatases proceed through a covalent phosphoryl-enzyme intermediate. Examples of such enzymes and the corresponding residues include protein tyrosine phosphatase (Cys) (35), alkaline phosphatase (Ser) (36), acid phosphatase (His) (37, 38), and low molecular weight phosphatases (Cys/His) (37, 38). For the Ser/Thr phosphatases, however, a phosphoenzyme intermediate has yet to be documented. Our attempts to detect such an intermediate using PPA and various $^{32}$P-labeled substrates under different conditions of trapping have been unsuccessful, suggesting that a covalent intermediate, even if it exists, must be extremely unstable. Biochemical and kinetic studies of PP2B (calcineurin) have in fact suggested a direct hydrolysis of the phosphoester bond without the generation of a phosphoryl-enzyme intermediate (40, 41).

Lastly, the true physiological substrate(s) of most phosphatases remains elusive. PPA mutants defective in the transfer of phosphates but not in the binding of phosphopeptide substrates, e.g. D20N, H22Q, and D49N, may be useful tools in identifying its physiological substrate(s) by affinity chromatography.

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