Structural and Functional Characterization of a Phosphatase Domain within Yeast General Transcription Factor IIIC

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RNA polymerase III (Pol III)² is the most complex of the three eukaryotic RNA polymerases and is responsible for the synthesis of various small, non-coding RNAs including tRNAs (1). Pol III-dependent transcription of tRNA genes controlled by type 2 promoters requires the transcription factor IIIC (TFIIC) to bind to two gene-internal elements named the A-box and B-box (2), thereby inducing the recruitment of the TFIIIC to bind to two gene-internal elements named the (1). Pol III-dependent transcription of tRNA genes controlled synthesis of various small, non-coding RNAs including tRNAs three eukaryotic RNA polymerases and is responsible for the transcription of tRNA genes controlled synthesis of various small, non-coding RNAs including tRNAs. The TFIIIC subunit complex of 0.5 MDa consisting of the two subcomplexes A (harboring the 131, 95, and 55 subunits) and B (harboring the 138, 91, and 60 subunits) that bind to the A and B boxes, respectively (4). The S. cerevisiae 55 protein consists of an N-terminal domain predicted to belong to the histidine phosphatase family and a C-terminal domain necessary for its interaction with 95. The N-terminal histidine phosphatase domain of the 55 protein (55-HPD) is only found in hemiascomycetes, whereas corresponding subunits in other eukaryotes only contain the C-terminal moiety required for the interaction with 95 orthologues (5). Because of a gene duplication event, S. cerevisiae has also evolved a parologue of the 55-HPD as an independent protein, called Huf.

Both proteins, 55-HPD and Huf, belong to the first branch of the histidine phosphatase family that has been originally termed the phosphoglycerate mutase family, according to its founding member, the eponymous enzyme, which catalyzes the conversion of 2-phosphoglycerate to 3-phosphoglycerate (6, 7). The first branch of the histidine phosphatase family contains enzymes with a broad range of enzymatic activities, including the phosphoglycerate mutase and the fructose-2,6-bisphosphatase enzymes, as well as the protein phosphatases Sts-1 (suppressor of T-cell signaling-1) and Sts-2, which also represent the closest homologues of 55-HPD in higher eukaryotes (8). In the second branch, the enzymatic activities are less diverse, including mainly representative of the acid phosphatase and thymidate categories. The two families have been linked through their characteristic short RHG peptide motif at the N-terminal end of their amino acid sequences (9).

The presence of an enzyme fused to a transcriptional regulator subunit has been unexpected. Because no enzymatic activity had been detected for 55 and because 55-HPD is nonessential and not required for TFIIC activity, only an auxiliary structural role of 55-HPD in stabilizing TFIIC had been suggested (10). However, partial deletions of 55-HPD result in a reduced growth rate at 30 °C or in thermo-sensitive phenotypes in glycerol- or ethanol-containing media, suggesting a possible role for 55-HPD in directly linking Pol III transcription with metabolic pathways. In addition, S. cerevisiae 55 and 95 form a complex separate from TFIIC and not required for TFIIC activity that has been also suggested to exert additional metabolic functions (10). Finally, the functional role of the 55-HPD parologue Huf and possible redundancies with 55-HPD have conserved phosphosite targets in vivo.

55-HPD and its closely related paralogue Huf, belong to the first branch of the histidine phosphatase family that has been originally termed the phosphoglycerate mutase family, according to its founding member, the eponymous enzyme, which catalyzes the conversion of 2-phosphoglycerate to 3-phosphoglycerate (6, 7). The two families have been linked through their characteristic short RHG peptide motif at the N-terminal end of their amino acid sequences (9).

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also not been explored. There is increasing awareness about the cross-talk between the metabolic state of the cell and Pol I and Pol III transcription allowing cells to adapt to changing environmental conditions. We therefore set out to further explore the functional roles of 55-HPD and its parologue Huf by characterizing their potential phosphatase activities and substrate preferences in vitro and by identifying potential in vivo phospho-target sites by performing comparative phosphoproteomic analyses. In a first step, we solved the crystal structures of S. cerevisiae 55-HPD at 1.5 Å and Huf at 2.0 Å resolution, respectively. Both structures show a canonical HPD fold, harboring the conserved catalytic active site residues. Consistently, we could observe phosphatase activity for both of the purified enzymes, which could be abolished by mutating conserved active site residues. Based on the 55-HPD crystal structure, we subsequently performed in silico docking studies of phosphocontaining small molecules and phospho-di- and tripeptides to identify potential substrates. Several known transcription-related phosphopeptides were tested in in vitro dephosphorylation assays using purified 55-HPD and Huf proteins. In an independent unbiased approach, we also compared the phosphoproteomes of wild type and double knock-out (55-HPDΔhufΔ) yeast strains to identify phosphosites that are under the direct and indirect regulation of these two potentially redundant phosphatases in vivo.

**EXPERIMENTAL PROCEDURES**

**Yeast Strain Preparation**—SCCM3 (ade2–101, his3–Δ200, lys2–801, trp1–Δ63, ura3–52, tfc7Δ::HIS3 pHA-Tfc7 (cen; TRP1)) and SCCM8 (ade2–101, his3–Δ200, lys2–801, trp1–Δ63, ura3–52, hufΔ::KanMX4, tfc7Δ::HIS3 pHA-Tfc7ΔNN (cen; TRP1)) were the yeast strains analyzed for differences in their phosphoproteomes. To obtain SCCM3, the TFC7 plasmid in YM2 (ade2–101, his3–D200, lys2–801, trp1–D63, ura3–52, tfc7D::HIS3 pTFC7 (2μ; URA3)) was substituted for pHA-Tfc7 (TRP1) by plasmid shuffling on plates with 5-fluoro-orotic acid. For construction of SCCM8, a DNA fragment bearing the KanMX4 marker flanked by HUF upstream and downstream sequences was cut out from pYCG-YNL108c (EUROSCARF) and transformed into YNM2. KanMX4-positive cells were selected on plates with G418, and deletion of the HUF open reading frame was confirmed by colony PCR. Subsequently, the pNM2 plasmid was substituted for pHA-Tfc7ΔN (TRP1) by plasmid shuffling on plates with 5-fluoro-orotic acid. For construction of SCCM8, a DNA fragment bearing the KanMX4 marker flanked by HUF upstream and downstream sequences was cut out from pYCG-YNL108c (EUROSCARF) and transformed into YNM2. KanMX4-positive cells were selected on plates with G418, and deletion of the HUF open reading frame was confirmed by colony PCR. Subsequently, the pNM2 plasmid was substituted for pHA-Tfc7ΔN (TRP1) by plasmid shuffling on plates with 5-fluoro-orotic acid.

**Protein Preparation**—Full-length untagged 55 protein was expressed in High-Five insect cells using the baculovirus system (virus kindly provided by J. Acker) and purified by anion exchange chromatography (Q Sepharose FF 16/10 column; GE Healthcare) and gel filtration chromatography (HiLoad 26–600 Superdex 200; GE Healthcare) to homogeneity. 55 was treated overnight at 4 °C with trypsin, added in a mass-to-mass ratio of 1:1000. After the limited proteolysis step, the resulting proteolytic fragment was separated, purified, and concentrated to 18.5 g/liter. For biochemical assays, 55-HPD (residues 1–270) was cloned into the pETM10 vector, giving rise to a noncleavable, N-terminal His tag on the resulting protein. The protein was expressed in SoluBL21 Escherichia coli cells (co-transformed with a pRare plasmid), purified by nickel-nitrotriacetic acid affinity chromatography followed by anion exchange using a Q Sepharose HiTrap column and gel filtration on a HiLoad 26/600 Superdex 200 column, and concentrated. The gene encoding Huf was also cloned into the pETM10 vector. Expression was performed overnight at 18 °C in E. coli BL21 Star pRare cells. The protein was purified similarly to 55-HPD, but bypassing the anion exchange step. The active site mutants of 55-HPD and Huf were prepared using site-directed mutagenesis and purified as described for the wild type HPD proteins.

**Protein Crystallization**—Crystals of 55-HPD were obtained using the hanging drop vapor diffusion method at a protein concentration of 18.5 g/liter using a reservoir solution of 0.77 m NaH2PO4 and 1.23 m K2HPO4. Crystals were cryoprotected with 30% glycerol and flash frozen in liquid nitrogen. Crystals of Huf were grown at 4 °C by hanging drop vapor diffusion, at a protein concentration of 6.2 g/liter with a reservoir solution consisting of 0.2 m magnesium formate and 26% PEG 3350. Cryoprotection of the crystals was performed by a soak in mother liquor supplemented with 8% glycerol, after which they were flash frozen in liquid nitrogen.

**X-ray Structure Determination**—Data for native 55-HPD and mercury-derivative crystals (soaked in 1 mm ethyl-mercury chloride) were collected at ESRF Beamline BM14. The 55-HPD structure was solved by the single isomorphous replacement with anomalous scattering method using data from the mercury-soaked crystal and refined against the best native data set to 1.50 Å resolution. Data for the Huf crystals were collected at ESRF Beamline ID29, and the structure was solved by molecular replacement using the 55-HPD structure as a search model and refined to 2.05 Å resolution.

**Docking**—To investigate potential substrates in an unbiased fashion (i.e., without limiting the docked molecules to phosphorylated compounds), 15,402 molecules of the Kyoto Encyclopedia of Genes and Genomes database (11, 12) of natural compounds were used. For a more focused docking of possible peptide substrates, an in-house generated library of phosphorylated dipeptides and selected tripeptides was used that corresponded to the central portions of the peptides that were also tested in vitro. Docking calculations were performed with DOCK3.5 (13–16). During docking, every molecule was fitted onto predetermined anchor points in the binding site. Each molecule pose was minimized for 25 steps with the simplex method.

**Phosphatase Activity Assays**—Phosphatase activity assays were performed using 3-O-methyl-fluorescein (Sigma) as substrate. Kinetic parameters ($K_m$, $V_{max}$ and $k_{cat}$) were analyzed with Origin 7.0 using nonlinear regression analysis and Michaelis-Menten equation. For peptide phosphorylation assays, free phosphate levels were detected using PiColorLock Gold Assay kit (Innova Biosciences) according to the manufacturer’s instructions.

**Comparative Phosphoproteomic Analysis**—Strains were grown in YPDA medium until optical density of 1.0 at 30 °C. The cells were harvested and lysed in buffer A (50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM NaF, 0.5 mM orthovanadate, phosphatase (PhosSTOP), and protease inhibitors) using glass beads. Lysate was cleared by centrifugation.
The samples were either directly used for protein digestion or used for heparin affinity chromatography, where 100 mg of cleared lysate was bound to a HiTrap Heparin HP column (GE Healthcare) washed with 50 ml of buffer A and eluted using buffer B (buffer A containing 1200 mM NaCl). Eluate was collected and used for protein digestion. Proteins were reduced and carbamidomethylated with dithiothreitol and iodoacetamide, respectively. Subsequently, proteins were digested with lysyl endopeptidase (Wako) for 4 h at 37 °C followed by a trypptic (Promega) digestion overnight at 37 °C. Peptides were desalted and differentially dimethyl-labeled (light and intermediate) on Sep-Pak tC18 1-cc 100-mg cartridges (Waters) as described (17). Replicate samples were labeled with the inverse labels (label swap), meaning the WT was labeled light in one replicate and intermediate in the other replicate and vice versa for r55-HPDΔ hufA. The labeled WT sample was mixed with the corresponding labeled r55-HPDΔ hufA sample, and an aliquot was analyzed by LC-MS/MS. The rest of the mixture was concentrated and enriched for phosphopeptides. TiO2 phosphopeptide enrichment by Ti (IV) immobilized metal affinity chromatography was carried out as described (18). The eluate was acidified with 10% formic acid and was either directly analyzed or further concentrated and diluted with 1% formic acid prior to LC-MS/MS analysis. Peptides were analyzed on a nanoAcquity UPLC system (Waters) directly connected to an LTQ Orbitrap Velos Pro (Thermo Scientific) via a Proxeon nanospray source. The utilized columns were a nanoAcquity Symmetry C18, 5 μm, 180 μm × 20 mm (Waters) trapping column and a nanoAcquity BEH C18, 1.7 μm, 75 μm × 200 mm (Waters) analytical column. The mobile phases A and B consisted of 0.1% formic acid in water and acetonitrile, respectively. The applied three-step gradients varied in length between 120 and 360 min, ranging from 3 to 85% mobile phase B at a constant flow rate of 0.3 ml/min. The eluent was directly introduced into the mass spectrometer via a Pico-Tip Emitter 360-μm outer diameter × 20-μm inner diameter; 10-μm tip (New Objective) with 2.2 kV applied spray voltage and capillary temperature of 300 °C. MS analysis was performed in positive ion mode. Full scan MS spectra were recorded from 300 to 2000 m/z in profile mode in the Orbitrap. Resolution in MS mode was set to 30,000. Lock mass correction using a background ion at m/z 445.12003 was used for internal calibration. The 10 most intense parent ions were subjected to fragmentation by higher energy collisional dissociation with the normalized collision energy set to 40%. Charge state screening was enabled to prevent fragmentation of singly charged ions. The resolution for MS/MS was set to 7500.

Heparin-enriched samples were digested, dimethyl-labeled, mixed, and subjected to strong cation exchange chromatography. Strong cation exchange chromatography was performed on an UltiMate 3000 HPLC (Dionex) coupled to a CapLC 2487 UV detector (Waters) using a 50×1-mm polysulfoethyl A column with 3-μm particles, pore size of 300 Å, at a flow rate of 75 μl/min. Buffer A consisted of 1 mM KH$_2$PO$_4$, 0.05% formic acid, and 30% acetonitrile, buffer B of 350 mM KCl, 5 mM KH$_2$PO$_4$, 0.05% formic acid, and 30% acetonitrile. Only one early fraction enriched in neutral or acidic phosphopeptides as described in Ref. 19 was concentrated, desalted, and subjected to either direct LC-MS/MS analysis or following TiO2 enrichment.

Raw data were processed with Proteome Discoverer software version 1.3.0.339 (Thermo Scientific). The data were filtered with a Top N peaks filter set to 10 with a 100-Da mass window prior to database searching. The filtered data were searched against the UniProt yeast database (6878 sequences). Mascot version 2.2 (Matrix Science) was used as search algorithm with the following parameters: trypsin allowing two missed cleavages; precursor mass tolerance, 20 ppm, fragment mass tolerance, 0.02 Da; carbamidomethylation as static modification for cysteine; oxidation on methionine, phosphorylation on serine, threonine, and tyrosine, light and intermediate dimethylation on lysine and the N terminus as dynamic modifications. Algorithm phosphoRS was used to score phosphorylation sites of the phosphopeptides and quantification node implemented in Proteome Discoverer for quantification. Mass precision herein was set to 2 ppm, and retention time tolerance of isotope pattern multiplets was set to 0.5 min. The percolator implemented in Proteome Discoverer was used to calculate the false discovery rate of 1%. Only peptides with high confidence (q value of Percolator below or equal to 0.01) were taken for peptide and protein reporting. For protein grouping only peptide spectrum matches with high confidence and a ΔCn better than 0.15 where selected, and strict maximum parsimony principle was enabled. The proteins were only considered to be regulated when they had a score greater than or equal to 20, were identified with at least three unique peptides, and were at least 2-fold up- or down-regulated in both replicates. Phosphopeptides were considered to be regulated only when they were identified in both replicates with a q value below 0.01 and were at least 2-fold up- or down-regulated in both replicates.

**RESULTS**

**Structure Determination of r55-HPD and Its Paralogue Huf—**Full-length r55 protein was overexpressed in insect cells and could be purified to homogeneity. Limited proteolysis

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**TABLE 1**

Crystallographic statistics for r55-HPD

| Data collection | Native | r55-HPD |
|-----------------|--------|---------|
| Space group     | P2,2,2 | P2,2,2  |
| Cell dimensions |        |         |
| a, b, c (Å)     | 80.6, 86.7, 44.3 | 80.5, 86.6, 44.4 |
| Wavelength (Å)  | 1.00849 | 1.00514 |
| Resolution (Å)  | 50.0-1.50 (1.54-1.50) | 50.0-1.95 (2.00-1.95) |
| R$_{cryst}$ (%)  | 16.1 (98.7) | 17.4 (81.3) |
| Completeness (%) | 99.5 (99.9) | 99.9 (99.6) |
| Multiplicity    | 4.05 (4.02) | 4.24 (3.44) |

**Refinement**

| Resolution (Å) | 43.4-1.50 |
| No. reflections | 50,251 |
| R$_{cryst}$/R$_{free}$ (%) | 15.6/18.3 |
| No. atoms | |
| Protein | 2,083 |
| Other | 5 |
| Water | 293 |
| B-factor (Å$^2$) | |
| Protein | 18.7 |
| Other | 32.1 |
| Water | 12.9 |
| RMSD | |
| Bond lengths (Å) | 0.01 |
| Bond angles (°) | 1.248 |
FIGURE 1. Structures of τ55-HPD and Huf. A, structure of the τ55-HPD proteins, with helices shown in green and β-strands shown in yellow. The helices are numbered, and the N and C termini are marked. The topology diagram of τ55-HPD is shown as an inset.

B, structure of the Huf protein, with helices shown in brown and β-strands shown in yellow. The topology diagram of Huf is shown in the inset.

C, structural alignment of τ55-HPD, Huf, and the HPDs of mouse Sts-1 (Protein Data Bank code 2IKQ), human BPGM (Protein Data Bank code 1K6M), human F26BPase (Protein Data Bank code 2H4Z), human BPGM (Protein Data Bank code 5PGM), Bacillus stearothermophilus PhoE (Protein Data Bank code 1H2F), and E. coli SixA (Protein Data Bank code 1UJB). Secondary structure elements for τ55-HPD and Huf are marked above the structure. As in A and B, all sheets are colored yellow, and helices are colored green for τ55-HPD, brown for Huf, and gray for all other proteins. Conserved helices that are absent in τ55-HPD and Huf are shown as dotted lines. Conserved active site residues are shaded in red. Residues corresponding to τ55-HPD and Huf R14 are shaded in light blue. Sequences were aligned using structural information with STRAP (42).
Structure-Function Analysis of \(\tau 55\)-HPD and Huf

| TABLE 2 |
|---|
| **Crystalllographic statistics for Huf** |

| Data collection |
|---|
| Space group | P3\(_1\)21 |
| Cell dimensions | \(a = b, c (\text{Å})\) 86.7, 99.0 |
| Wavelength (Å) | 0.97633 |
| Resolution (Å) | 50.0-2.05 (2.10-2.05) |
| \(R_{\text{free}}\) (%) | 7.0 (99.2) |
| \(<\|I/I(\|)\>\>) | 18.2 (2.54) |
| Completeness (%) | 99.9 (99.9) |
| Multiplicity | 11.2 (11.4) |

Refinement

| Resolution (Å) | 40.0-2.05 |
| No. reflections | 27,399 |
| \(R_{\text{work}}/R_{\text{free}}\) (%) | 20.0/23.6 |
| No. atoms |
| Protein | 2,040 |
| Other | 3 |
| Water | 110 |
| B-factor (Å\(^2\)) |
| Protein | 56.9 |
| Other | 43.9 |
| Water | 52.4 |
| RMSD |
| Bond lengths (Å) | 0.007 |
| Bond angles (\(\text{°}\)) | 0.913 |

resulted in a stable domain corresponding to the predicted histidine phosphatase domain of \(\tau 55\) (residues 1–272). After obtaining crystals of \(\tau 55\)-HPD, prepared by preparative limited proteolysis, its structure could be solved using single isomorphous replacement with anomalous scattering and refined at a resolution of 1.5 Å to \(R_{\text{work}}/R_{\text{free}}\) values of 15.6/18.3% (Table 1). The structure shows a canonical histidine phosphatase fold of the branch 1 subfamily. It consists of six \(B\)-strands, forming a mixed parallel/antiparallel \(B\)-sheet that makes up the core of the domain, surrounded by nine \(\alpha\)-helices (Fig. 1A).

Huf could also be crystallized, and its structure was solved by molecular replacement, using the \(\tau 55\)-HPD structure as search model. The structure was subsequently refined at a resolution of 2.05 Å to \(R_{\text{work}}/R_{\text{free}}\) values of 20.0/23.6% (Table 2). Huf and \(\tau 55\)-HPD possess 64% sequence identity and show almost identical overall structures that can be superimposed with a RMSD of 0.65 Å over 237 residues. Differences are limited to the C-terminal region, where helix \(\alpha 9\) is absent in Huf, while present in \(\tau 55\)-HPD. In contrast, Huf possesses an additional \(\alpha\)-helix located more toward the C terminus, in a region that is disordered in the \(\tau 55\)-HPD structure (Fig. 1B). This latter helix in Huf has been termed \(\alpha 10\) to discriminate it from helix \(\alpha 9\) in \(\tau 55\)-HPD (Fig. 1B). In summary, the spatial organization of Huf is very similar to that of the \(\tau 55\)-HPD and other branch 1 histidine phosphatases.

Structural Analysis and Comparison of the Active Sites of \(\tau 55\)-HPD and Huf—The catalytic residues in the active sites (Arg-10, His-11, Arg-68, His-169 (\(\tau 55\))/His-168 (Huf)) are highly conserved between \(\tau 55\)-HPD, Huf, and all other known histidine phosphatases (Fig. 1C). The putative proton donor (Glu-92) is conserved among branch 1 histidine phosphatases. The catalytic cavities of \(\tau 55\)-HPD and Huf are positively charged, and the spatial orientation of the catalytic residues is almost identical in both enzymes (Fig. 2, A and B). Interestingly, we could identify two different substrate/product mimics in the putative catalytic sites of \(\tau 55\)-HPD and Huf. We could locate a phosphate ion from the crystallization buffer in the \(\tau 55\)-HPD active site, whereas a formate molecule is present in the active site of Huf that was crystallized in formate containing buffer (Fig. 2, A and B). In addition to side chain-specific contacts, \(\tau 55\)-HPD Ala-170 makes a contact with the bound phosphate through its main chain NH group, and the corresponding Huf Ala-169 makes an equivalent contact with the formate molecule.

Structural comparison reveals the highest structural similarity of \(\tau 55\)-HPD and Huf with Sts-1 and Sts-2 proteins (\(\tau 55\)-HPD RMSD Sts-1 = 2.10 Å over 197 residues and RMSD Sts-2 = 1.86 Å over 188 residues; Huf RMSD Sts-1 = 2.03 Å over 187 residues and RMSD Sts-2 = 1.93 Å over 186 residues), which both show phosphotyrosine phosphatase activity (20, 21). Interestingly, residue Arg-14 of \(\tau 55\)-HPD and Huf, which contacts the respective phosphate and formate ions in the active sites, is conserved in Sts-1 and Sts-2 but not in other members of the branch 1 histidine phosphatase family (Fig. 1C). In addition, \(\tau 55\)-HPD, Huf, Sts-1, and Sts-2 lack two \(\alpha\)-helices, which are located between \(\alpha 4\) and \(\alpha 5\) in the other branch 1 histidine phosphatases. These four enzymes also share a specific subset of nonconserved residues that were shown to regulate the specific activity in Sts-1 and Sts-2 (22). These pieces of evidence indicate that \(\tau 55\)-HPD, Huf, Sts-1, and Sts-2 might act on similar substrates and might well represent a specific subbranch within the branch 1 histidine phosphatase family.

\(\tau 55\)-HPD Is a Functional Phosphatase—As the catalytic residues of \(\tau 55\)-HPD and Huf are highly conserved and harbor all necessary active site residues, we wondered whether these proteins have catalytic activities, which were missed in previous studies (10). We tested purified \(\tau 55\), \(\tau 55\)-HPD, and Huf proteins (Fig. 2C) for their ability to dephosphorylate 3-O-methyl fluorescein phosphate, an unspecific substrate of phosphatases (23, 24). All three tested proteins show phosphatase activity. Importantly, \(\tau 55\) and \(\tau 55\)-HPD show similar enzymatic activities and kinetic parameters (Fig. 2E), indicating that the C terminus of \(\tau 55\) does not influence the phosphatase activity of \(\tau 55\)-HPD and that the two domains act independently of each other. Huf shows higher phosphatase activity, although substrate binding constants are similar for both enzymes.

To test the specificity and conserved enzymatic reaction mechanism, we generated mutated versions of \(\tau 55\)-HPD and Huf lacking signature catalytic residues known to be essential for the phosphatase activity in other family members (8). Although the stability of the mutated proteins is almost identical to the wild type proteins or even higher for R68A mutants (Fig. 2D), we could observe a drastic drop of activity for all mutated versions of the proteins (Fig. 2E). Most members of the large histidine phosphatase superfamily have been shown to be active and have been assigned distinct enzymatic activities, whereas this was not the case for \(\tau 55\)-HPD and Huf. Our results address this gap, and we now show that both proteins possess phosphatase activity in vitro. In addition, we observed that the enzyme kinetics of \(\tau 55\)-HPD, Huf, and Sts-1 for OMFP are in a comparable range (Fig. 2F), which again reflects the strong similarities between this subclass of histidine phosphatases.

Docking to \(\tau 55\)-HPD—Because no information was available on suitable in vivo substrates of \(\tau 55\)-HPD, we performed docking studies using the crystal structure of \(\tau 55\)-HPD. The first,
unbiased round of docking used 15,402 molecules of the Kyoto Encyclopedia of Genes and Genomes database (11, 12). The top-ranked 500 molecules were inspected visually for the feasibility of their binding pose, namely, satisfied hydrogen bond interactions, lack of strain in the molecules, and correctness of the protonation state. The vast majority of the highest ranking 500 molecules (even those that did not pass the visual inspection) contained a phosphate group (Fig. 3C). Among the 31 molecules with reasonable binding modes, mainly phosphorylated sugars and nucleotides were found, likely a tribute to the polar nature of the binding site. We tested two of these hits that were commercially available, namely AMP and Ser(P), as potential substrates for 55-HPD and Huf in vitro phosphatase assays. Although we detected some residual activity, the enzymatic activities are much lower than those of known highly active phosphatases for these small compounds, like alkaline phosphatase (calf intestinal) and phage protein phosphatase (Fig. 3D).
Because ϑ55-HPD and Huf showed the highest similarity to Sts-1 and Sts-2, two known protein phosphatases, calculations were extended to phosphorylated di- and tripeptides with the aim to explain the preferences of ϑ55-HPD toward certain peptide sequences. Of the five tripeptides corresponding to the three central amino acids of the tested Pol II peptides (below), only two, the SpYS and YpSP peptides, docked with highly favorable scores and with the phosphate positioned between Arg-10, Arg-14, Arg-68, and His-169. However, the rather narrow nature of the binding site seemed to be more compatible with the dephosphorylation of a tyrosine residue, because it allows a more relaxed geometry of the peptide backbone.

In Vitro Analysis of the Substrate Specificity of ϑ55-HPD and Huf—ϑ55 is an integral part of the general transcription factor TFIIC, which in addition to Pol III-dependent transcription, has been recently shown to be involved in Pol III-independent cellular processes (25, 26). In detail, TFIIC was found to localize to genomic extra TFIIC sites, which are often in close proximity of Pol II genes (27) and to regulate the expression of one of its own subunits from a Pol II specific promoter (26). Furthermore, Pol III-transcribed genes are often co-occupied by Pol II and are situated in regions with epigenetic marks resembling functional Pol II promoters (28, 29). Therefore, we tested the phosphatase activity of ϑ55-HPD, Huf with Sts-1 as control toward a series of candidate peptides known to be phosphorylated in the Pol III transcription system and a set of known monophosphorylated Pol II C-terminal domain (CTD) peptides (Fig. 4, A and B) (30–33). Strikingly, we observed specific phosphatase activity for all three enzymes toward the Tyr(P)-Y1 peptide, which recently has been shown to be important for regulating the recruitment of transcription termination factors within the Pol II transcription cycle (34). In contrast, Ser(P)-2, Thr(P)-T4, Ser(P)-5, and Ser(P)-7 peptides are not hydrolyzed by ϑ55-HPD or Huf. Not only ϑ55-HPD by itself, but also full-length ϑ55 and the ϑ55/ϑ95 heterodimer are also able to specifically dephosphorylate Tyr(P)-1 (Fig. 4, C and D). Although with lower activity, Huf is also able to specifically dephosphorylate a phosphoserine peptide derived from C53, a
specific subunit of Pol III (Fig. 4B, inset) known to be contacted by TFIIIC (35). Neither r55-HPD nor Sts-1 showed any detectable phosphatase activity toward the Pol III-related phosphopeptides tested. To relate the phosphatase activity of r55-HPD, Huf, and Sts-1 with other well characterized phosphatases, we also compared their relative activities with the activities of λ protein phosphatase and calf intestinal phosphatase. A protein phosphatase and calf intestinal phosphatase hydrolyze a phosphotyrosine containing peptide (but also AMP and phosphorylase) with ~50- to ~500-fold higher activity, whereas r55-HPD, Huf, and Sts-1 show very comparable activities (Fig. 3D). We therefore conclude that r55-HPD and Huf show similar activities and preferences toward phosphotyrosine containing peptides like the well characterized Sts-1/2 proteins (20, 21). For Huf, we could also demonstrate that it dephosphorylates phosphoserine-containing peptides, whereas for r55-HPD, no phosphoserine-containing peptide was identified as a possible substrate.

In Vivo Identification of Potential r55-HPD and Huf-regulated Phosphosites—Although r55-HPD and Huf are conserved in the Saccharomyces clade and r55-HPD is conserved in all hemiascomycetes, there are no obvious phenotypes connected to the deletion of the two described phosphatase domains under normal growth conditions in S. cerevisiae (10, 36). Considering the above described highly specific in vitro peptide phosphatase activities, we investigated the effects of deleting these two potentially redundant phosphatase domains on the yeast phosphoproteome. Because the C terminus of r55 is essential, we generated a knock-out strain that specifically lacks the r55-HPD by rescuing a full-length r55 deletion strain with a plasmid expressing only the C-terminal part of r55 (hereafter called r55-HPDΔ). We further knocked out Huf in the r55-HPDΔ strain to obtain a strain lacking both phosphatase domains (hereafter called r55-HPDΔ hufΔ). In addition, we rescued the r55 deletion strain with a plasmid expressing full-length r55, which served as the wild type control strain. We used this control wild type strain to guarantee the comparison of strains with the most similar genetic background possible. Consistent with the literature, we could not observe any obvious growth phenotypes between r55-HPDΔ hufΔ and the used wild type strain (10, 36). In a subsequent step we compared the phosphoproteomes of both strains by mass spectrometry using differential isotopic dimethyl labeling. In a second round, we also analyzed the heparin-bound fraction of the yeast phosphoproteome enriched in DNA-binding proteins (Fig. 5A). By focusing on the DNA-binding proteome, we attempted to facilitate the identification and analyses of regulated phosphosites present in transcriptional complexes. Each of these experiments was performed in duplicate combined with swapping the labels on the individual replicates.

In the combined data set, we identified 1706 protein groups in both replicates (2261 and 1787, respectively), of which we could quantify 1281 containing at least three unique peptides. None of the proteins showed a more than 2-fold increase of their relative abundance in the r55-HPDΔ hufΔ strain compared with the wild type strain. Only for seven proteins and one protein group containing two proteins, we observed a more than 2-fold decrease of relative abundance in the r55-HPDΔ hufΔ strain compared with the wild type (Fig. 5, B and C, and supplemental Table S1). We subsequently focused on the identification of phosphopeptides following an optimized TiO₂ phosphopeptide enrichment step. Of the pool of over 1900 identified phosphopeptides, 200 phosphopeptides were more than 2-fold up- or down-regulated in at least one of the two replicates. To focus on phosphopeptides with the highest reliability, we only kept the ones identified in both replicates. In the r55-HPDΔ hufΔ strain, 14 of those were down-regulated (less phosphorylated) in both replicates by at least 2-fold, suggesting indirect effects (see discussion). Our special interest was raised by the protein UTP-glucose-1-phosphate

FIGURE 4. Investigation of the substrate specificities of r55-HPD and Huf. A, description of all phosphopeptides used in the assays. B, dephosphorylation activities of r55-HPD, Huf, Sts-1, and the respective active site mutants using Pol II CTD and Pol III-related phosphopeptides. C, schematic model of the TFIIIC complex, highlighting subunits r55 and r95. D, dephosphorylation activities of r55/r55, r55, r55-HPD, and Huf using CTD phosphopeptides.
uridylyltransferase (Ugp1) that contains the only phosphopeptide that was strongly up-regulated (more phosphorylated) in the \( \text{HEK293} \times 10 \) 55-HPD/H9004 strain in both replicates. Of all regulated phosphopeptides, this is the only peptide where the phosphorylation state was found more than 5-fold changed in both replicates, suggesting that Ugp1 is a direct target of 55-HPD and/or Huf as discussed below (Fig. 5D and supplemental Table S2).

**DISCUSSION**

Our *in vitro* results identify 55 and Huf as bona fide phosphatases. Using the high resolution crystal structure of 55-HPD, we docked a large number of small molecules and a variety of di- and tripeptides into the crystal structure. Possible substrates included phosphorylated sugars and nucleotides, as well as different phosphopeptides, some of which were subsequently experimentally tested in *vitro*. Remarkably, the *in silico*
results were well in line with subsequent experimental measurements, which demonstrates the strong preorganization of the binding pocket for recognizing phosphate moieties, as manifested by the vastly enriched phosphate-bearing molecules in the top hits. Protein phosphorylation events play important roles in regulating all known cellular functions, including the general transcription machinery. It therefore appears plausible to speculate that the general transcription factor IIIC uses its "built-in" τ55-HPD protein phosphatase activity to regulate its transcription machinery, thereby contributing to the orchestrated balance between phosphorylation and dephosphorylation events. In addition, the heterodimeric τ55/τ55 subcomplex that exists independently of TFIIIC and the paralogue Huf could dephosphorylate additional components of the transcription machinery in vitro, thereby complementing the activity of the TFIIIC-bound τ55 protein. Consistent with an involvement of τ55 and Huf in transcription regulation, both proteins are localization to the nucleus (37). Despite the plausibility of our hypothesis, using a broad in vivo phosphoproteomic approach, we were not able to reliably detect any of the phosphopeptides used in our in vitro dephosphorylation assay that are present in components of the transcription machinery. Further studies will have to clarify whether these peptides are not detected for technical reasons (e.g., proteome coverage, sampling rate, strict filtering criteria), if they are not phosphorylated under the tested environmental conditions, whereas they are genuine in vivo targets under different growth conditions, or whether they are simply not targets of τ55-HPD and/or Huf. We also observed lower phosphorylation states for 14 phosphopeptides in the τ55-HPDΔHuf strain lacking the two phosphatases. This result appears counterintuitive but suggests that these peptides are downstream targets of τ55-HPD and/or Huf and are indirectly influenced by them. Strong indirect responses have been also observed in earlier system-wide studies of protein kinases and phosphatases in yeast (38). Finally, the in vivo tyrosine phosphatase activity of τ55-HPD and Huf also needs to be analyzed in greater detail. The abundance of phosphotyrosine containing peptides is known to be very low in yeast, and it is difficult to investigate their status in a global phosphoproteome analysis. A more specific proteomic approach especially targeting phosphotyrosine peptides will have to be applied in future studies to clarify the contribution of τ55-HPD and Huf in the in vivo dephosphorylation of phosphotyrosine sites. In this study, we identified several targets with most of them showing no direct connection to transcription regulation, but we have identified effects of τ55-HPD and Huf on a set of proteins involved in metabolism. Interestingly, we observed lower abundance of several mitochondrial proteins in the τ55-HPDΔHuf strain that are mainly associated with metabolic functions. This finding might explain the earlier described observation that τ55-HPD and Huf have an effect on mitochondrial protein synthesis (39). Another strong effect includes the regulation of the major metabolic enzyme Ugp1. We show that the phosphorylation of an N-terminal peptide of Ugp1 is strongly up-regulated after the depletion of τ55-HPD and Huf, which suggests that Ugp1 is a direct and specific target of these phosphatases. It was shown earlier that the phosphorylation of serine 11 of Ugp1 is important for changing the metabolic network from glycogen synthesis toward intensive glucan production, whereas the inability to phosphorylate Ugp1 leads to a weak cell wall and increased glycogen content in the cell (40). These observations strongly support our results that Ugp1 is a direct target of τ55-HPD and Huf. It is also interesting to note that a partial τ55-HPD deletion leads to increased sensitivity of yeast cells to low concentrations of calcifluor white and caffeine, which are two cell wall-interfering drugs (41).

In conclusion, at present our in vivo results demonstrate that τ55-HPD as an integral part of the general transcription factor TFIIIC and its paralogue Huf mainly affect metabolic processes. Further analysis might identify additional proteins that are directly or indirectly affected by both phosphatases and will further clarify the link between the transcriptional regulator TFIIIC and metabolic signaling networks.

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