Human OLA1 Defines an ATPase Subfamily in the Obg Family of GTP-binding Proteins

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Purine nucleotide-binding proteins build the large family of P-loop GTPases and related ATPases, which perform essential functions in all kingdoms of life. The Obg family comprises a group of ancient GTPases belonging to the TRAFAC (for translation factors) class and can be subdivided into several distinct protein subfamilies. The founding member of one of these subfamilies is the bacterial P-loop NTase YchF, which had so far been assumed to act as GTPase. We have biochemically characterized the human homologue of YchF, for human Obg-like ATPase 1. Further biochemical characterization of YchF proteins from different species revealed that ATPase activity is a general but previously missed feature of the YchF subfamily of Obg-like GTPases. To explain ATP specificity of hOLA1, we have solved the x-ray structure of hOLA1 bound to the nonhydrolyzable ATP analogue AMPPCP. Our structural data help to explain the altered nucleotide specificity of YchF homologues and identify the Ola1/YchF subfamily of the Obg-related NTases as an exceptional example of a single protein subfamily, which has evolved altered nucleotide specificity within a distinct protein family of GTPases.

In all organisms, P-loop NTases play a pivotal role in the regulation of diverse cellular processes such as protein translation, intracellular transport, signal transduction, cell motility, cell division, growth, and others. Most P-loop GTPases act as molecular switches such that the GTP-bound form constitutes the active state and triggers the biological output whereas the GDP-bound form is inactive (for review see Refs. 1–3). The guanine nucleotide-binding domain (G domain) is about 20 kDa in size and structurally conserved. The G domain adopts an αβ fold, typical for many nucleotide-binding proteins, formed by six central β-strands surrounded by five α-helices. At the sequence level, the G domain contains five characteristic sequence motifs, termed G1–G5, involved in nucleotide binding and hydrolysis (2). The G1/Walker A motif (GXXGGK(S/T)), also referred to as P-loop (4), helps to position the phosphate moiety of the bound nucleotide. The G2 (X(T/S)X) and G3/Walker B (hhhDXXG) motifs are involved in the coordination of a Mg2+ ion that is required for nucleotide binding and hydrolysis. Specificity in nucleotide binding is conferred by the G4 motif, which has a (N/T)KXD signature in guanine nucleotide binding P-loop NTases. The G5 motif ((T/G)(C/S)A) supports guanine base recognition (2).

The conformational switch between the GDP- and GTP-bound forms of P-loop GTPases manifests itself in two peptide segments defined as switch I and switch II. The switch I region, a loop preceding strand β2, has also been termed the effector loop because it exerts downstream functions in many GTPases by binding to effector molecules. The switch I and II regions interact with the γ-phosphate of the bound GTP, resulting in a protein conformation that is highly responsive to GTP hydrolysis and loss of the γ-phosphate. Upon GTP hydrolysis, the molecule undergoes a conformational change, and the switch domains move into a different position and conformation, a mechanism that has been compared with the relaxation of a loaded spring (3).

P-loop GTPases can be classified into TRAFAC (translation factor-related) and SIMIBI (signal recognition particle, MinD, and BioD) NTases (5). Based on conserved sequence motifs and structural features, these two classes are further split into more than 20 distinct families and about 60 subfamilies (5).

Bacteria contain fewer GTPases than eukaryotes, with less diverse functions (6–8). Apart from those involved in translation, bacteria possess very few GTPases, and archaea have even fewer. Bacteria with small genomes (e.g. Mycoplasma genitalium) contain as few as 11 GTPases (6, 9). Strikingly, most of these GTPases are universally conserved and can be assigned to four main ancestral groups: elongation factors (EF-G, EF-Tu, and IF2), protein secretion factors (FtsY and Ffh), Era-related GTPases (Era, EngA, and ThdF/TrmE), and Obg-re-

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The atomic coordinates and structure factors (code 2OHF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org/) contains supplemental Tables S1 and S2 and supplemental Figs. S1–S4.

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lated proteins (Obg and YchF) (5, 6). Mutational analyses in many organisms have shown that most of these GTPases are essential (7, 10).

YchF is one of the highly conserved GTPases (Escherichia coli to Homo sapiens: 45% identity, 62% similarity) belonging to the Obg-related GTPase family. The Obg protein family is thought to comprise five ancient subfamilies, namely Obg/CgtA, YchF/YyaF, Drg/Rbg, Nog1, and Ygr210 (5). Functionally, the Obg-like GTPases are poorly characterized. The best-studied member is the essential Obg/CgtA (SpoOB-associated GTPase). Similarly, the Obg-like GTPases are poorly characterized. The best-studied member is the essential Obg/CgtA (SpoOB-associated GTPase) from Bacillus subtilis (11). Obg and its homologues have been implicated to function in cellular processes as diverse as sporulation, stress response, control of DNA replication, and ribosome assembly (for review see 12).

In contrast to Obg, YchF/YyaF has been hardly investigated, and functional information on YchF homologues is scarce. In Brucella melitensis, the YchF homologue DugA has been suggested to be involved in iron metabolism and may be a regulator of the Ton system (13). Structural analysis of yeast and bacterial YchF revealed a modular organization formed by three domains, a central G domain, flanked by a coiled-coil domain and a TGS (ThrRS, GTPase, SpoT) domain of unknown function (14).

One anomaly in YchF and its homologues is the unusual G4 motif, which strongly diverges from the (N/T)KXD consensus, suggesting that guanine nucleotide binding specificity of YchF homologues might be either governed by other means or altered. We have biochemically characterized the human homologue of YchF and found that it binds and hydrolyzes ATP more efficiently than GTP. For this reason, we have termed the hOLA1, for human Obg-like ATPase 1. To explain ATP specificity of hOLA1, we have solved the x-ray structure of hOLA1 bound to the nonhydrolyzable ATP analogue AMP-PCP. Our structural data help to explain the altered nucleotide specificity of YchF homologues. Biochemical characterization of YchF proteins from different species revealed that ATPase activity is a general but previously missed feature of the YchF subfamily of Obg-like GTPase.

**EXPERIMENTAL PROCEDURES**

**Phylogenetic Tree Construction and Sequence Alignment**—Phylogenetic distribution and multiple sequence alignment of Obg-like proteins were calculated with ClustalW (www.ebi.ac.uk/clustalw) and displayed using TreeView (15) or JalView.

**Cloning and Site-directed Mutagenesis**—For hOLA1 cloning, the human gene PTD004 coding region was amplified by PCR using cDNA from HeLa cells. The PCR product was digested with NcoI and BamHI and inserted into the pQE60 protein expression vector (Qiagen) to allow for the expression of a C-terminally hexahistidine-tagged protein. The coding regions of S. cerevisiae Yhr025c (yOla1p) was amplified by PCR from yeast genomic DNA. The PCR product was digested with NcoI/BamHI and inserted into pQE60.

Point mutations were generated using the QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. DNA oligonucleotides used for PCRs and mutagenesis are listed in supplemental Table S1.

**Protein Expression and Purification**—Expression and purifi-
cation of B. subtilis Obg and Hemophilus influenzae YchF was performed as described elsewhere (14, 16). Expression of all other proteins was done in E. coli BLR (pRep4). Protein expression was induced by the addition of 1 mM isopropyl β-D-thio-
galactopyranoside and performed overnight at 19 °C. The cells were lysed by cell cracking (EmulsiFlex-SC, Avestin) in 50 mM Tris, pH 7.6, 700 mM NaCl, 5 mM MgCl₂, 2 mM 2-mercaptoethanol, 5% glycerol (w/v). The bacterial cell lysate was cleared by ultracentrifugation (90 min, 48,000 rpm, Ti70; Beckman) and was passed over nickel-nitrilotriacetic acid-agarose (Qiagen). After intense washing with lysis buffer, bound protein was subjected to buffer exchange (hOLA1 elution buffer: 50 mM Tris, pH 7.0, 100 mM NaCl, 5 mM MgCl₂, 2 mM 2-mercaptoethanol, 5% glycerol (w/v); yOla1 elution buffer: 50 mM Tris, pH 6.5, 100 mM NaCl, 5 mM MgCl₂, 2 mM 2-mercaptoethanol, 5% glycerol (w/v)) and then eluted with 400 mM imidazole in the corres-
ponding elution buffer. The eluted protein was subsequently bound to a Hi Trap SP Sepharose HP column (GE Healthcare) and eluted in a gradient (buffer A: 50 mM Tris, 50 mM NaCl, 5 mM MgCl₂, 5% glycerol (w/v); buffer B: 50 mM Tris, 1 mM NaCl, 5 mM MgCl₂, 5% glycerol (w/v) at the respective pH). Peak fractions were pooled and concentrated by Centricon centrifuga-
tion (Milian) prior to gel filtration on a Superdex 200 10/30 column (GE Healthcare) in 50 mM Tris, pH 7.6, 100 mM NaCl, 5 mM MgCl₂. Peak fraction were pooled and supple-
mented with 2 mM dithiothreitol and 250 mM sucrose. Purified proteins were 98–99% pure and 95–98% nucleotide-free, as analyzed by SDS-PAGE and HPLC analysis, respectively.

**Fluorescence Spectrometry**—Nucleotide binding of 2’/3’-O-
(N-methylanthraniloyl)-labeled nucleotides (mant-nucleo-
tides; Jena Bioscience) was examined by fluorescence spec-
trometry (Cary Eclipse, Varian). All of the assays were performed at 25 °C, and mant-nucleotide fluorescence was monitored at an excitation wavelength of 355 nm (slit width, 5 nm) and an emission wavelength of 448 nm (slit width, 10 nm). The experiments were carried out in 50 mM Tris, pH 7.6, 100 mM NaCl, 5 mM MgCl₂. hOLA1 was titrated to a 0.5 μM mant-nucleotide solution up to a final concentration of 15 μM. Alternatively, nucleotide binding was followed by polarization meas-
urement with a Fluoromax 2 spectrofluorometer (Spex Industries). Mant-nucleotides were used at a concentration of 0.2 μM.

**Isothermal Titration Calorimetry**—Nucleotide binding affini-
ty was determined by isothermal titration calorimeter (MCS-ITC, MicroCal, Inc.). Calorimetric experiments were per-
formed at 25 °C in 50 mM Tris, pH 7.6, 200 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol. The concentration of protein in the sample cell was 58 μM, and the titrated nucleotide were used at 600 μM. The data were analyzed using the manufacturer’s soft-
ware. Curve fitting were done according to single ligand bind-
ing equations.
Nucleotide Hydrolysis Assay—To quantify nucleotide hydrolysis, 5 μM protein was incubated with an excess of either ATP or GTP (125 μM) in 50 mM Tris, pH 7.6, 200 mM NaCl, 5 mM MgCl₂ at 25 °C. At various time points, nucleotide diphosphate production was analyzed by HPLC (Shimadzu). The nucleotides were separated on a hydrophobic C18-column (Macherey-Nagel) with 50 mM potassium-phosphate, pH 6.5, 10 mM tetrabutylammonium bromide, 5% acetonitrile as polar, mobile phase. Protein was denatured on a C-18 precolumn (Bischoff-Chromatography). For Fig. 7B, the nucleotides were separated by anion exchange chromatography on a Nucleosil 4000–7 PEI column (Macherey-Nagel) using a linear salt gradient (buffer A: 10 mM Tris/ HCl, pH 8.5; buffer B: 10 mM Tris/ HCl, pH 8.5, 1.5 mM NaCl).

Alternatively, nucleotide hydrolysis was quantified using [γ-32P]ATP/GTP. In brief, the proteins were incubated with [γ-32P]ATP/GTP (50 μCi, 0.05 μCi), and nucleotide hydrolysis reactions were stopped by the addition of 1 ml of a charcoal suspension. After centrifugation at 10,000 × g, release of [32P]phosphate was determined by scintillation counting of the supernatant. Competition was done with 50 μM of unlabeled nucleotides.

Crystallization and Data Collection—hOLA1 was incubated with 0.5 mg/ml trypsin to remove flexible parts of the protein and subsequently purified by gel filtration. Rod-like crystals of hOLA1 approximately 0.4 × 0.1 × 0.1 mm in size were obtained at 4 °C by vapor diffusion using the hanging drop method and 12% polyethylene glycol 3350, 50 mM Tris/HCl, pH 8, 10 mM MgCl₂ as crystallization buffer. The nonhydrolyzable ATP analogue AMPPCP was added to the protein drop to a final concentration of 1 mM. The crystals were soaked with 30% glycerol as cryo-protectant, mounted using a nylon-fiber loop (Hampton Research), and flash-cooled to 100 K in a nitrogen stream. The data were collected at Beamline X06SA (SLS, Villigen) as consecutive series of 0.5° rotation images on a MAR-CCD detector. The data sets were indexed and integrated with XDS (17), scaled with XSHELL, and converted to CCP4, XPLOR, or SHELX format with XDS/NDV. Data set statistics are given in Table 1. Molecular Replacement, Model Building, and Refinement—Molecular replacement was performed with the programs MOLREP (18) and PHASER (19) and homologous YchF proteins (Protein Data Bank codes 1JAL and 1NI3) as search models. The search models were modified with CHAINSAW (20) to X-ray structures, namely an YchF homodimer (Protein Data Bank code 1LNZ). The quality of the superposition statistics are given in Table 1. The figures were prepared with PyMOL (pymol.sourceforge.net).

Superpositions—Superpositions were performed with the SSM superpose function within Coot. Briefly, hOLA1 full protein or the individual domains (G domain: residues 16–44, 58–65, 85–90, 107–126, and 198–305; coiled-coil: residues 139–171 and 180–195; TGS domain: residues 66–87 and 107–126) were superposed on full-length Thermus thermophilus YchF (Protein Data Bank code 2DBY), Schizosaccharomyces pombe YchF (Protein Data Bank code 1NI3), H. influenzae YchF (Protein Data Bank code 1AL), and B. subtilis Obg (Protein Data Bank code 1LNZ). The quality of the superposition was judged on the sequence and optical alignment. Core root mean square deviation (RMSD) values are in Angstroms.

RESULTS

hOLA1 is closely related to the bacterial protein YchF, a member of the TRAFAC class of P-loop NTPases (5) that constitute one of four evolutionary conserved superfamilies belonging to the family of Obg-related GTPases (Fig. 1). The other three superfamilies comprise the Obg-, Nogl-, and Drg-like GTPases. Previously, the Obg family had been divided into five subfamilies, with the group of Ygr210-like proteins defining an independent fifth subfamily. Our phylogenetic comparison revealed that the Ygr210 branch and the Ola1/YchF branch are closely related and group together into one subfamily (Fig. 1A and supplemental Fig. S1). In general, Obg-related proteins share two conserved sequence features, namely an YXXF(T/C)XXXXX segment in the switch I region and a glycine-rich motif in the switch II region (GAXXGXXGXXX(I/L/V)). A distinguishing feature of the Ola1/YchF subfamily is a noncanonical G4 motif, distinct from the (N/T)KXD consensus (Fig. 1B). The G4 motif in the Ola1/YchF subfamily is as variable as NVNE in bacteria (YchF), NMSE in yeast (Ybr025cp/ yOla1p), and NLSE in human (hOLA1). Both, the conserved lysine and aspartate are replaced. In contrast, the G1, G2, G3, and G5 motifs match the signature found in other P-loop GTPases (Fig. 1B).

The variation of the G4 motif may lead to an altered nucleotide binding specificity. Indeed, when we analyzed nucleotide binding of recombinant, highly purified hOLA1 using fluorescent, mant-labeled nucleotides by polarization analysis, we noticed that hOLA1 bound ATP. In contrast, we hardly observed GTP binding at a micromolar concentration of hOLA1 (Fig. 2A). Even binding of a nonhydrolyzable GTP analogue such as mant-GMPNP was not saturable as shown by the linear dependence on hOLA1 concentration and failed to reach the level of ATP binding (see Fig. 6B). Further evidence that ATP is the preferred nucleotide-binding partner of hOLA1 stems from the observation that only ATP but not GTP could efficiently displace mant-ATP from hOLA1 (Fig. 2B). Together, this analysis shows that purified hOLA1 is an ATP-binding protein rather than a GTP-binding protein.
isothermal titration calorimetry was used to determine the relative binding affinities for ATP and GTP. The dissociation constant (K_D) for ATP was calculated to be 8 μM (Fig. 2C), whereas the K_D for GTP could not be determined exactly because of its low affinity for hOLA1 (data not shown). Curiously, when the G4 motif of hOLA1 was reverted to the G4 consensus NKXD by site-directed mutagenesis, the hOLA1-NKXD mutant retained ATP-binding specificity (Fig. 2D), suggesting that the overall fold of the nucleotide-binding pocket in hOLA1 might define nucleotide specificity (see below).

To test whether hOLA1 hydrolyzes ATP, we used HPLC analysis to assess the intrinsic ATP hydrolysis rate of hOLA1. Recombinant hOLA1 was incubated with an excess of ATP, and the amount of ADP production was measured at different time points (Fig. 3). The hydrolysis rate of hOLA1 was calculated to 0.050 ± 0.002 min⁻¹. The affinity of hOLA1 for ATP and the intrinsic ATP hydrolysis rate are comparable with the GTP binding and hydrolysis properties of other Obg family members (28–30). hOLA1 might therefore constitute a novel group of ATPases, with biochemical features resembling those of related Obg-like low affinity GTPases (16, 28, 29).

To obtain structural insight into ATP binding of hOLA1, we determined the crystal structure of hOLA1 complexed with AMPPCP, a nonhydrolyzable ATP analogue, at a resolution of 2.7 Å. Table 1 summarizes the data set and refinement statistics. Alternative names or corresponding gene loci are listed in supplemental Table S2.

| TABLE 1 | Data set and refinement statistics |
|-----------------|-----------------|
| **Wavelength**  | 0.976 Å         |
| **Resolution**  | 50.2-2.7 Å      |
| **Completeness**| 99.8% (100.0%)  |
| **Rfree**       | 16.3 (2.8)      |
| **Rcryst**      | 0.084 (0.80)    |
| **Space group** | C222            |
| **Unit cell parameters** |          |
| a               | 141.3 Å         |
| b               | 159.8 Å         |
| c               | 55.1 Å          |
| **Monomers per asymmetric unit** | 1          |
| **Matthews coefficient** | 3.1 Å³      |
| **Wilson temperature factor** | 0.29       |
| **RMSD bond angle** | 1.4°       |
| **RMSD bond length** | 0.050 Å       |
| **Rcryst**      | 0.23            |
| **Rsym**        | 0.29            |
| **Ramachandran plot** | 92.1%       |
| **Favored**     | 92.1%           |
| **Allowed**     | 7.9%            |
| **Outliers**    | 0%              |

a The values in parentheses are for the high resolution shell.

b \( R_{\text{free}} = \sum_{i} I_i(h) - (\langle h \rangle) / \sum_{i} I_i(h), \) where \( I_i(h) \) and \( \langle h \rangle \) are the ith and mean measurement of the intensity of reflection h.

c \( R_{\text{cryst}} = \sum_{h} |F(h) - F(h)| / \sum_{h} F(h), \) where \( F(h) \) are the observed and calculated structure factor amplitudes, respectively.

d Phasing power \( = \sum_{h} F(h) / \sum_{h} F(h), \) where \( F(h) \) is the calculated heavy atom amplitude and \( F(h) \) and \( F(h) \) are the observed and calculated heavy atom derivative structure factor amplitudes, respectively (5% test set).

e RMSD, root mean square deviation from the parameter set for ideal stereochemistry.

FIGURE 1. hOLA1 belongs to the Obg family of GTPases. A, phylogenetic tree of the Obg family of GTPases. Tree topology of full-length Obg-related proteins was generated by ClustalW and displayed using TreeView. Obg-related proteins assemble into four main branches, namely Obg (red), Nog1 (purple), Drg (blue), and Ola1/YchF (yellow). Note that Ygr210 homologues are only found in fungi and archaea and build a distinct subclass in the Ola1/YchF branch. In contrast, hOLA1 homologues are not present in archaea. Interestingly, the so far uncharacterized human protein GTPBP10/LOC85865 and its homologues from Xenopus laevis, Drosophila melanogaster, and Caenorhabditis elegans form a novel, independent branch close to the Obg branch (supplemental Fig. S2). Note that Obg-like proteins of higher eukaryotes were named after their closely related human homologues GTPBP5 and GTPBP10. Proteins from S. pombe were named after the homologous protein from S. cerevisiae. Alternative names or corresponding gene loci are listed in supplemental Table S2. B, sequence alignment of conserved elements in the G domain of selected Ola1/YchF subfamily members in comparison with members of the Ygr210 and Obg subfamilies. Conserved G1 to G5 motifs and the highly conserved Gly-rich domain following G3 are indicated. The species abbreviations are hs, H. sapiens; xl, X. laevis; xt, Xenopus tropicalis; dm, D. melanogaster; ce, C. elegans; at, Arabidopsis thaliana; sc, S. cerevisiae; sp, S. pombe; ec, E. coli; bs, B. subtilis; hi, H. influenzae; ss, Sulfurobacter solfataricus; af, Archaeoglobus fulgidus. For accession numbers, see Table S2.
2.7 Å. The protein crystallized in space group C222₁ (No. 20) with one monomer in the asymmetric unit and no obvious formation of higher oligomeric states in the crystal packing, consistent with the observation that the protein is a monomer in solution (static light scattering; data not shown). We could build 327 of 396 amino acids into the electron density map. Missing are the N-terminal residues 1–15, the switch I and switch II regions with residues 45–57 and 96–106, respectively, some disordered loop regions with residues 127–138, 172–179, 196–197, and the C-terminal residues 389–396. The model was completed by building AMP-PCP and 26 water molecules into the electron density maps. The final model has a crystallographic R factor of 0.23 and a free R factor of 0.29 with very good stereochemistry (Table 1).

hOLA1 consists of an N-terminal G domain, flanked on either side by an inserted coiled-coil and a C-terminal TGS domain (Fig. 4), as was reported for the homologous bacterial and yeast YchF proteins (Ref. 14 and Protein Data Bank codes 2DBY and 1NI3). The structures of hOLA1 and of the homologous H. influenzae YchF protein are quite similar with an RMSD value of 1.9 Å for all Cα atoms. The Cα atoms of the individual domains can be superimposed with RMSD values of 1.8 Å (G domain), 2.2 Å (coiled-coil domain), and 1.1 Å (TGS domain). Differences are more pronounced between hOLA1 and the GDP bound T. thermophilus YchF (Protein Data Bank code 2DBY, overall RMSD value 2.3 Å), the largest changes being observed for the coiled-coil domain (RMSD 2.5 Å), as well as helices α8b and α10 (for secondary structure assignment; see supplemental Fig. S3). The coiled-coil domain is tilted toward helix α7 in the latter structure, making it more compact. Helices α8b and α10, but not the coiled-coil domain, show similar changes in the superposition of the yeast and bacterial YchF structures, indicating a general variability in these structural elements.

The cofactor AMP-PCP is bound in-between the P-loop (residues 28–36), helix α1, the adjacent helix α9, and the short loop following strand β6, which carries the unique Ola1 G4 motif N230LSE (Fig. 5A).

The triphosphate moiety appears tightly bound through a network of H-bonds of main and side chain donors to the phosphate oxygens (Fig. 5B). The conserved interaction of the P-loop Lys²⁵ with the β-phosphates (via main chain NH) and γ-phosphates (via NH₂) is also observed in hOLA1. We observe electron density at the potential binding site of the essential magnesium between Ser³⁶ in the G1 P-loop, Thr⁵⁵ in the G2-loop (switch I region), and the β and γ-phosphates, but the resolution is too low to identify the ion or its position unambiguously.
The cofactor triphosphate moiety forms hydrogen bonds to residues from the P-loop, whereas its sugar moiety has no interaction with hOLA1, and its adenine base has only a few weak interactions with hOLA1. N-7 of the pyrimidine ring in the adenine base can form a hydrogen bond with NH$_2$ of Asn$_{230}$ (distance 3.3 Å). The N-6 of the base can engage into a weak potential hydrogen bond to the main chain carbonyl oxygen of Leu$_{231}$ with an N-O distance of 2.7 Å but a suboptimal C-O-N-angle of 103°. An interaction between the side chain carboxylate group of Glu$_{233}$ and the nucleotide as proposed for GTP bound to YchF (14) appears not to be possible, because Glu$_{233}$ is too far away.

In comparison with the G4 motif of the GTPase Ras (116NKCD), the conformation of the hOLA1 G4 motif N$_{230}$LSE differs dramatically (Fig. 5C). Although the G4 motif possesses an extended β-strand conformation in hOLA1, it shows a zigzag-like conformation in Ras (31). Consistently, Ras Lys$_{117}$ can stack with the cofactor, and Asp$_{119}$ interacts with the N-1 and N-2 group of the guanine. In hOLA1, the individual positions of the G4 motif are shifted such that only position 1 (Asn$_{116}$) matches with position 1 of Ras (Asp$_{119}$). The Glu$_{233}$ of the hOLA1 G4 motif lies at the beginning of a small helix α$_8$a preceding helix α$_8$b, and intramolecular H-bonds force the entire motif into an extended conformation. This places Glu$_{233}$ too far away to interact with the nucleotide. Apparently, the intramolecular forces that define the position of helices α$_8$a and α$_8$b do not allow the hOLA1 G4 motif to adopt a Ras-like conformation, explaining why the ATP specificity of hOLA1 could not be switched to GTP via mutations of the G4 motif to NKXD (Fig. 2D).

To validate the role of key amino acids in the nucleotide-binding pocket of hOLA1, we mutated selected residues and tested these mutants for their ability to bind to mant-labeled ATP (Fig. 6A). As expected from the structural analysis, mutation of Asn$_{230}$ to alanine (N$_{230}$A) resulted in loss of ATP binding without perturbing solubility or other biochemical characteristics of the protein. Additionally, we mutated the conserved Phe$_{127}$, which had been proposed to contribute to nucleotide binding in H. influenzae YchF through stacking of the aromatic side chain with the base (14). Indeed, when we replaced Phe$_{127}$ with Ala (F$_{127}$A), the mutant protein no longer bound mant-labeled ATP. Like wild-type hOLA1, both mutants also failed to bind GMPNP or GTP (Fig. 6B and data not shown). This supports that Asn$_{230}$ and Phe$_{127}$ are essential for ATP binding. It seemed plausible that the conserved Phe$_{127}$, that we could not...
see in the electron density map abolished proper conformation of the G4 motif and, together with the unconventional sequence in G4, would prevent efficient binding of GTP. However, combining the F127A and hOLA1-NKXD mutations did not result in a version of hOLA1 that could bind GTP (Fig. 6B), indicating that the overall fold of the nucleotide-binding pocket in hOLA1 must in addition be determined by other parameters.

Because the unconventional G4 motif is present in all members of the Ola1/YchF subfamily, we next tested whether ATPase activity is a general feature of Ola1-like proteins. Analysis of ATP hydrolysis revealed that indeed two other Ola1/YchF subfamily members, namely YchF from *H. influenzae* and Ola1p (Ybr025c) from *S. cerevisiae* preferentially hydrolyzed ATP when compared with GTP (Fig. 7). In contrast, and as reported before, *B. subtilis* Obg preferred GTP as a substrate for nucleotide hydrolysis. Together, these data uncover an ATPase subfamily in the family of Obg-like GTPases.

**DISCUSSION**

Our biochemical characterization of hOLA1 reveals a new subclass of ATPases in the Obg family of GTPases. Hence we have named this subclass Ola1, for Obg-like ATPases.

It is assumed that P-loop ATPases and GTPases have a common P-loop-containing ancestor. Several ATPases have been derived secondarily form the GTPase superclass through loss of GTPase specificity or activity (5). The kinesin-myosin superfamily of motor ATPases, for instance, may have evolved from the TRAFAC class by loss of the NKXD G4 motif, accompanied by the gain of ATPase activity. Another example is the subclass of MinD-like ATPases, which derived within the SIMIBI class of the GTPase superclass (5). The Ola1/YchF subfamily of the Obg family of GTPases, however, presents a unique exception of a sin-
gle protein subfamily, which shows altered nucleotide specificity within a distinct protein family of GTPases, whereas in the aforementioned examples whole protein families of distinct nucleotide specificity evolved. This implies that in the case of the Ola1/YchF subfamily the change in nucleotide specificity occurred relatively late in the evolution of the Obg family.

Biochemically, Ola1-like proteins hOLA1, yeast Ola1p (Ybr025c), and YchF behave similarly to the related GTPase Obg, apart from different nucleotide specificity. Both subfamilies of enzymes display a slow rate of nucleotide hydrolysis in vitro and are characterized by a high rate of nucleotide dissociation (our data and Refs. 16, 28, and 29).

The biochemical characterization of the ATPase activity of hOLA1 was supported by structural analysis of hOLA1 complexed with AMPPCP. The interactions between the bound adenine nucleotide and hOLA1 mainly involve the phosphate groups of the bound AMPPCP. The α-phosphate contacts the main chain amide of Thr37, the β-phosphate the main chain amides of Ser36 and Val33, and the γ-phosphate forms a hydrogen bond with Asn230. Nucleotide specificity is conferred by Leu231 main chain, which forms a hydrogen bond with the exocyclic amino group in adenine, whereas guanine would be repelled.

FIGURE 5. The nucleotide-binding site of hOLA1 bound to AMPPCP. A, the cofactor is bound in between the P-loop (magenta), helices α1, α9, and the G4 motif bearing the loop following strand β6. B, close-up view of interactions between AMPPCP and hOLA1. C, comparison of the cofactor binding site in hOLA1 (white) and p21-H-Ras (orange). Note that the G4 motif of hOLA1 (123NLSE) adopts an extended β-strand conformation, whereas the G4 motif of Ras (123NKCD) possesses a zigzag-like conformation. The position of Glu127 at the beginning of the small helix α8a preceding helix α8b likely prevents hOLA1 from adopting a Ras-like conformation of the G4 motif (see text). D, model of interactions between hOLA1 and ATP (left) or GTP (right). The purine base is stabilized (blue) by hydrogen bonds to Asn230 and stacking with the aromatic ring of Phe127. Note that the contribution of Phe127 is based on our biochemical data but not visible in the structure. Nucleotide specificity (red) is conferred by Leu231 main chain (mc), which forms a hydrogen bond with the exocyclic amino group in adenine, whereas guanine would be repelled.

FIGURE 6. Mutations in the nucleotide-binding pocket of hOLA1 impair ATP binding. A, binding of mant-labeled ATP to hOLA1 (wild type (wt)), hOLA1 (F127A), and hOLA1 (N230A) was analyzed by fluorescence measurement as in Fig. 2. Both mutations impair binding of mant-ATP. B, combining the F127A and hOLA1-NKXD mutations prevents binding of both ATP and GTP. Binding of the indicated mant-labeled nucleotides to hOLA1 wild-type and hOLA1 F127A-NKXD was analyzed by fluorescence measurements. Note that the hOLA1 F127A-NKXD is impaired in both ATP and GMPPNP binding.

FIGURE 7. ATPase activity is a general feature of the YchF/OLA1 subfamily of Obg-like proteins. A, ATP and GTP hydrolysis activity of YchF, yOla1p, hOLA1, and B. subtilis Obg was analyzed by HPLC. Like hOLA1, the two homologues from bacteria and yeast showed higher ATPase than GTPase activity. In contrast, B. subtilis Obg clearly favored GTP hydrolysis. Analysis of nucleotide hydrolysis was performed in the presence of both ATP and GTP (100 μM each). The nucleotides were separated by anion exchange HPLC in a linear gradient. Although Obg prefers GTP, OLA1-like proteins clearly favor ATP as substrate. The N230A mutation in hOLA1 abolishes ATP hydrolysis. C, specificity of nucleotide hydrolysis activity of YchF, yOla1p, hOLA1, and B. subtilis Obg was examined by competition experiments using γ-32P-labeled NTPs in combination with unlabeled ATP or GTP. ATP hydrolysis by OLA1-like proteins was efficiently competed by ATP but not GTP, whereas B. subtilis Obg preferentially hydrolyzed GTP.
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bond to the main chain amide of Asn\textsuperscript{32} in hOLA1. In our structure, we cannot observe any contacts of the ribose to the protein.

The specificity of nucleotide interactions must be determined by contacts of the protein with the adenine base. The (unprotonated) N-7 of the adenine base forms a hydrogen bond with NH\textsubscript{2} of Asn\textsuperscript{230}. The N-6 group of the base might engage into a hydrogen bond with the main chain CO of Leu\textsuperscript{231}, which is part of the unconventional G4 motif of hOLA1. The distance of 2.7 Å falls well within acceptable H-bond range, although the geometry is not optimal in the refined model. Therefore, we cannot exclude an energetically more favorable orientation of the peptide carbonyl group. Our biochemical data, however, support an important contribution of Asn\textsuperscript{230} to nucleotide binding, because its mutation to alanine abolished nucleotide binding. Another contact from the protein contributing to base recognition might be established by Phe\textsuperscript{127}. Mutating this residue to Ala diminishes ATP binding drastically, indicating an important involvement of this residue in nucleotide binding.

The electron density for Phe\textsuperscript{127} is too poorly defined for the residue to be modeled. Phe\textsuperscript{127} may contribute to the fixation of hOLA1, an Obg-like ATPase.

The G4 motifs of the GTPase Ras (\textsuperscript{116}NKCD) and the ATPase hOLA1 (\textsuperscript{230}NLSE) show different conformations (Fig. 5B). Although the G4 motif in Ras adopts a zigzag-like conformation (31), the G4 motif in hOLA1 possesses an extended β-strand conformation. In the G4 motif of hOLA1, Ser\textsuperscript{232} and Glu\textsuperscript{233} cannot interact with the nucleotide because of the altered conformation of the G4 loop. Our attempts to convert hOLA1 back into a GTPase by conversion of the G4 motif to NKXD did not result in a version of hOLA1 that could bind GTP but retained ATP specificity (Fig. 2D). This indicates that the overall fold of the nucleotide-binding pocket in hOLA1 is determined by additional parameters.

In Ras-like GTPases, a Gln residue is involved in (GAP-induced) GTP hydrolysis. This conserved Gln residue is replaced by a hydrophobic amino acid in all Obg family members (e.g. Leu\textsuperscript{96} in hOLA1), a substitution that inactivates Ras-like GTPases (32). This indicates a different NTP hydrolysis mechanism used by Obg-like NTPases. P-loop NTPases undergo conformational changes upon NTP hydrolysis. The structural differences between AMPPPCP-bound hOLA1 and nucleotide-free YchF are minimal, and it remains to be seen which structural differences might exist between ATP and ADP-bound hOLA1.

Ola1-like proteins display a low intrinsic nucleotide hydrolysis and a high nucleotide dissociation rate, as observed for Obg, the founding member of the Obg family. It remains to be seen whether these features are important for hOLA1 function in vivo or whether yet to be identified binding partners of Ola1-like proteins influence their nucleotide binding and hydrolysis properties in the cellular context. This awaits the identification of potential binding partners and their functional characterization.

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