Characterization of the nucleotide-binding capacity and the ATPase activity of the PIP_3-binding protein JFC1

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In this work, we demonstrate that the phosphatidylinositol 3,4,5-trisphosphate-binding protein JFC1 is an ATP-binding protein with magnesium-dependent ATPase activity. We show that JFC1 specifically binds to the ATP analog 8-azido-[α-^{32}P]ATP. The affinity of JFC1 for [α-^{32}P]ATP was 10× greater than its affinity for [α-^{32}P]ADP; the protein did not appear to bind to [α-^{32}P]GTP. JFC1 hydrolyzed [α-^{32}P]ATP in a Mg^{2+}-dependent manner. JFC1, which also hydrolyzed dATP, has a relatively high affinity for ATP, with a K_M value of 58 μM, and a k_{cat} value of 2.27 per min. The predicted amino acid sequence of JFC1 denotes a putative nucleotide-binding site similar to those in the GHKL ATPase/kinase superfamily. However, a truncation of JFC1 that contains boxes G2 and G3 but not boxes N and G1 of the Bergerat-binding site showed residual ATPase activity. Secondly, the antitumor ATP-mimetic agent geldanamycin, which inhibits the ATPase activity of Hsp-90, did not affect JFC1 ATPase. Therefore, the characteristics of the ATP-binding site of JFC1 are unique. Phosphatidylinositol 3,4,5-trisphosphate, a high-affinity ligand of JFC1 did not affect its ATPase kinetics parameters, suggesting that the phosphoinositide have a different role in JFC1 function.

JFC1 is a tandem C2 domain-containing protein that have been recently identified in our laboratory by using a yeast-two hybrid system (1) with the C-terminal half of the leukocyte NADPH oxidase cytosolic factor $p67^{PHOX}$ as bait. In the same work, the JFC1-$p67^{PHOX}$ interaction was confirmed *in vitro*, supporting the idea that JFC1 could play a regulatory role over the NADPH oxidase. However, although the NADPH oxidase is restricted to leukocytes, JFC1 mRNA was detected in several tissues (1), leading to the idea that JFC1 could play a more general role. Moreover, that the C-terminal half of $p67^{PHOX}$ contains two SH3 domains suggests that JFC1 could interact with other proteins containing similar domains.

Several tandem C2 domain-containing proteins have been described in the literature and, although their functions are not necessarily related, all of them seems to have the common characteristic of binding to phospholipids. An example of this group is the synaptic vesicle membrane protein synaptotagmin I, considered to be essential for calcium-dependent neurotransmitter release (2). Several partners have been described for synaptotagmins, including proteins associated with regulated secretion like syntaxin (3), the plasma membrane attachment protein t-SNARE, SNAP-25 (4), and inositol phosphates (5). Other examples of tandem C2 domain-containing proteins are the members of the GTPase-activating protein (GAP)1 family of proteins. $GAP1^{IP4BP}$ not only binds to phospholipids through its C2 domains (6) but also interacts with the second messenger inositol-tetrakisphosphate (IP_4) through its pleckstrin homology domain (7). It is now clear that $GAP1^{IP4BP}$ possesses an IP_4-dependent Ras GAP activity mediated by its GAP-related domain (6). Again, although C2 domains are essential in these proteins for normal docking and phospholipid-mediated regulation, domains different from the tandem C2 seem to characterize the function of these proteins. Rabphilin, effector of Rab3a (8) and Doc2α (9), are other examples of tandem C2 domain-containing proteins whose functions are also defined by adjacent domains.

JFC1 possesses tandem C2 domains and shows the ability to bind to phosphoinositides (PPI) phosphorylated in position 3′ (1). There is general consensus that PI 3-kinase products are generated mainly in response to adequate stimuli, therefore implying that JFC1 would be involved in cell signaling. Moreover, although it is possible that JFC1 could interact with other SH3 domain-containing proteins, acting as an adaptor protein downstream in the PI 3-kinase pathway, the function of JFC1 remains to be clarified. The analysis of the amino acid sequence of JFC1 denotes the presence of a putative ATP-binding site constituted by four structures that resemble those found in the GHKL ATPase/kinase superfamily (10). This emergent superfamily, whose members are a group of functionally unrelated proteins, is characterized by the presence of a novel ATP-binding site referred to as Bergerat fold (11).

The presence of a putative ATP-binding site in JFC1 is an important observation, because phenomena that involve tandem C2 domain-containing proteins like membrane fusion, exocytosis, cellular trafficking, and cell signaling are energy-dependent mechanisms. Several proteins that are essential in these processes bind and/or hydrolyze ATP or GTP. This is the case for *N*-ethylmaleimide-sensitive factor (12), the small GTP-binding protein Rab3a (13), synapsins I and II (14), and a long list of regulatory proteins including protein kinases and lipid kinases, to cite some examples. Clearly, the question whether JFC1 utilizes ATP is of importance in understanding the molecular mechanism of this protein and eventually determining its function *in vivo*. Presented in this work is the characterization of the ATP-binding and hydrolysis properties of JFC1.

Experimental Procedures

Materials. [α-^{32}P]ATP, [α-^{32}P]dATP, [γ-^{35}S]ATP, and [α-^{32}P]GTP were purchased from New England Nuclear/Life Science (Boston, MA). 8-Azido-[α-^{32}P]ATP was obtained from ICN.

Methods

Plasmid Construction. JFC1 or JFC1 truncation constructs were engineered by standard techniques (15). JFC1 full length or truncation 256–377 containing the C2A domain (C2A) and 256–562 containing both C2 domains (C2AB-COOH) were amplified from wild-type JFC1 cDNA with *pfu* polymerase (Promega) and by use of 5′ primers that contained a *Sal*I site (underlined) and 3′-antisense primers that contained a *Not*I site (underlined). The 5′ primers used for amplification were

Abbreviations: PIP_3, phosphatidylinositol 3,4,5-trisphosphate; GST, glutathione *S*-transferase; PPI, phosphoinositide.

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(GGGGTCGACCCATGCCCCAGAGGGGCCACCCATCGCAA) for wild-type JFC1 and (GGGGTCGACCCAGCCTGTCAGGCGACGCGGAGGCGGTG) for truncations 256–377 and 256–562, whereas the 3′ primers were (GGGGCGGCCGCGCGCCCCAGTCCCACGTGTCCAGGGG) for the truncation 256–377 and (GGGGCGGCCGCCGTCCTGGGGGCCAGGTTGGTTCT) for wild-type and truncation 256–562. PCR products were cloned into pGEX-6P-1 (Amersham Pharmacia Pharmacia Biotech). Constructs were sequenced by using an automated fluorescent dye-terminator sequencer.

Preparation of Recombinant Fusion Protein. Recombinant fusion proteins composed of an upstream glutathione *S*-transferase (GST) linked to a downstream JFC1 (GST-JFC1), truncations C2A (GST-C2A), or C2AB-COOH (GST-C2AB-COOH) were purified by affinity chromatography on glutathione-agarose beads (Amersham Pharmacia Pharmacia Biotech). In some cases, recombinant proteins were cleaved from GST by using PreScission protease (Amersham Pharmacia Pharmacia Biotech), as described by the manufacturer. Concentrations of all proteins (95–99% pure) were determined by the method of Bradford by using a Bio-Rad. assay kit and BSA as standard.

Crosslinking of JFC1 with 8-Azido-[α-^{32}P]ATP. To analyze the ability of JFC1 to crosslink to 8-azido ATP, 3 μg of JFC1 was incubated in the presence of 3.5 μM (2 μCi) of 8-azido-[α-^{32}P]ATP in Hepes/K^+ buffer (50 mM), pH 8, containing 2 mM EDTA, 1 mM DTT, and 0.1 mM Mg^{2+} (buffer A). In some cases, the indicated concentration of unlabeled ATP was included in the reactions. Incubations were performed for 30 min at 30°C in 50 μl of final volume. To induce crosslinking, samples were then exposed to UV irradiation (1,500 W/cm^2 in a FB UBXL-1000 UV cross linker (Fisher) at a distance of 10 cm. After addition of trichloroacetic acid (10% vol/vol), samples were placed on ice for 1 h, spun down, and washed with cold acetone. Samples were resolved by SDS/PAGE in 12% polyacrylamide gel, transferred to nitrocellulose, and JFC1 was immunodetected with a previously described anti-JFC1 antibody (1). The membranes were dried and detected by radioautography. Alcohol dehydrogenase and creatine kinase were used as negative and positive controls, respectively.

Nucleotide Binding to JFC1. Binding of [α-^{32}P]ATP to JFC1 was determined by the rapid filtration technique, as described previously (16). In brief, JFC1 or GST-JFC1 (3 μg) was incubated in buffer A (0.0128 μM free Mg^{2+} concentration as calculated by using the MAXCHELATOR software by C. Patton, Stanford University, Stanford, CA). Reactions were started by the addition of 2.5 μM (0.5–2 μCi)[α-^{32}P]ATP and were carried out for 30 min at 30°C in 96-well plates in a final volume of 50 μl. Where indicated, [α-^{32}P]GTP was used instead of [α-^{32}P]ATP. For competitive assays, reactions were carried out in the presence of the indicated concentrations of various unlabeled nucleotides added at the same time as 0.25 μM [α-^{32}P]ATP. Standard and competitive reactions were terminated by the addition of 300 μl of ice-cold reaction buffer containing 25 mM $MgCl_2$ (stop buffer). Samples were immediately vacuum filtered on 0.45 μm pore size-cellulose nitrate membrane filters (Whatman). The filters were immediately washed four times with 2 ml of ice-cold stop buffer, the four washes requiring a total of less than 20 s. Filters were counted in a scintillation counter (Beckman LS 6000SC). Nonspecific binding was evaluated by filtration of the reaction mixture in the excess of nonradiolabeled nucleotide and was always less than 5% of total binding. Nonspecific binding values were subtracted from the values of mixtures containing JFC1 to calculate JFC1-dependent binding. Negative controls in the absence of protein were always run in parallel. In some experiments, thrombin protease (Amersham Pharmacia Pharmacia Biotech) was used as a negative control.

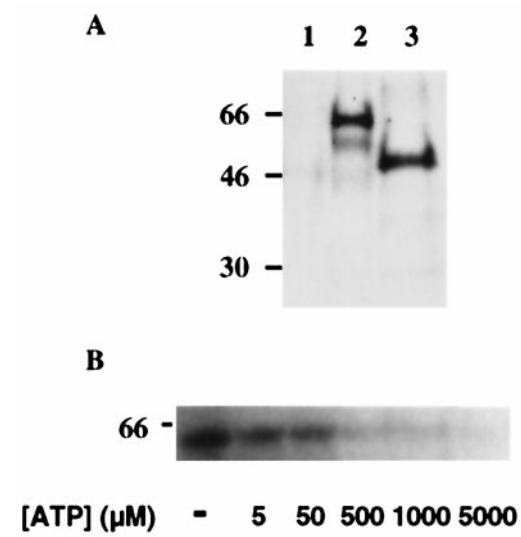

Fig. 1. Crosslinking of JFC1 with 8-azido-[α-^{32}P]ATP. (*A*) Three micrograms of alcohol dehydrogenase (lane 1), JFC1 (lane 2), or creatine kinase (lane 3) was incubated with 3.5 μM (2 μCi) of 8-azido-[α-^{32}P]ATP, and samples were processed as described in *Experimental Procedures.* The autoradiography is representative of two separate experiments. The band at 50 kDa that is noticeable in the autoradiography in lane 2 was also detected by Western blot analysis, indicating that is a degradation product of JFC1. (*B*) Three micrograms of JFC1 was incubated with 3.5 μM (2 μCi) of 8-azido-[α-^{32}P]ATP in the presence of ATP at the indicated concentrations for 30 min. Samples were then processed as described in *Experimental Procedures*. These results are representative of three separate experiments.

ATPase Assay. ATPase activity of JFC1 was determined by the hydrolysis of [α-^{32}P]ATP to [α-^{32}P]ADP followed by the resolution of labeled nucleotides by ion exchange TLC. Briefly, JFC1 (3 μg) or, when indicated, equimolar concentration of GST-JFC1, GST-C2A, or GST-C2AB-COOH fusion proteins were incubated in buffer A containing varying concentrations of $MgCl_2$ (0.1, 0.8, 1.8, 2, and 5 mM), to reach the indicated free Mg^{2+} concentration. In some experiments, JFC1 was incubated in the presence of 3 nmol of PIP_3 (Matreya, Pleasant Gap, PA) at 30°C for 15 min before the addition of [α-^{32}P]ATP. Reactions were started by the addition of 1 mM [α-^{32}P]ATP (0.5–2 μCi) and were carried out for the indicated time at 30°C in 96-well plates in a final volume of 50 μl. Reactions were terminated by the addition of 3 μl of a mixture containing 36 mM of unlabeled ATP, 36 mM ADP, and 36 mM EDTA to 3 μl of the reaction mixture. Samples were spotted on a polyethylimine cellulose TLC and resolved by using 0.5 M LiCl, 1 M formic acid as the carrier solvent. ATP and ADP *Rf* were 0.08 and 0.6, respectively. Nucleotides were visualized by radioautography and spots scraped off and counted in a scintillation counter.

Results

Azido-ATP Crosslinking to JFC1. Crosslinking of the radiolabeled ATP analog 8-azido[α-^{32}P]ATP has been successfully used to show protein affinity for the nucleotide in several studies (17, 18). In this work, we show that JFC1 crosslinks to 8-azido-ATP after UV irradiation (Fig. 1 *A* and *B*). The radiolabeled band detected with JFC1 in the photoaffinity experiment was similar in intensity to that obtained with the positive control, creatine kinase (Fig. 1*A*), a protein known to bind ATP and crosslink to the radioactive analog (19). On the other hand, alcohol dehydrogenase, a protein that is not known to bind ATP and that does

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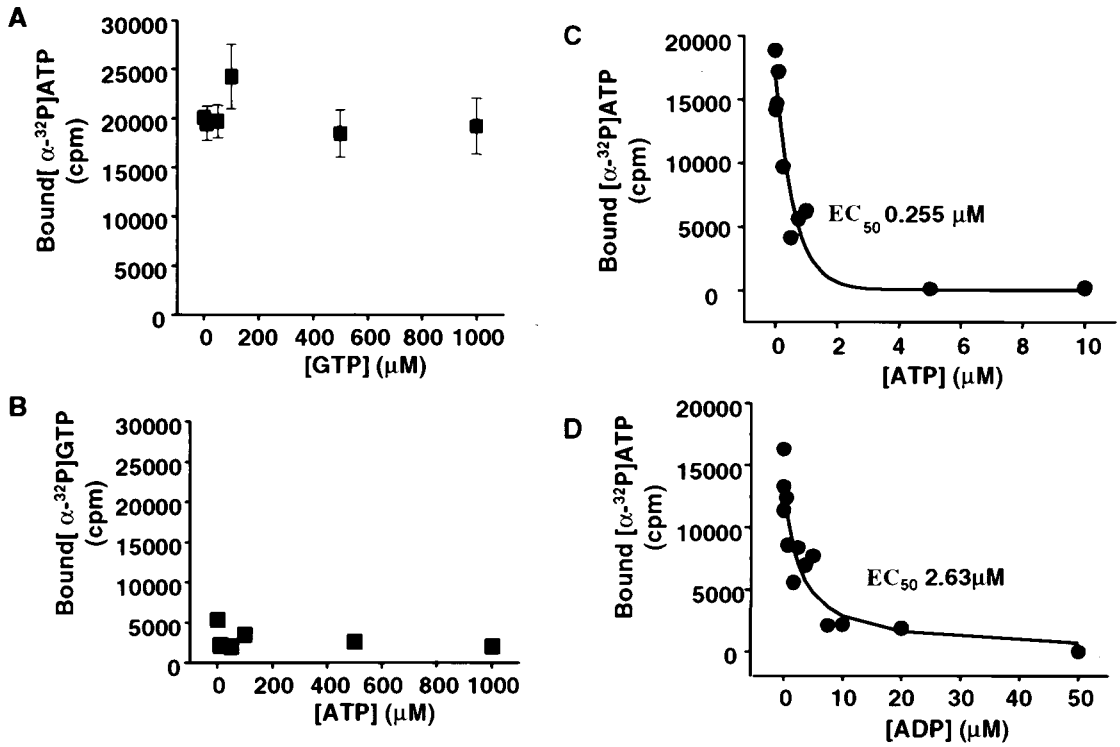

Fig. 2. Binding of ATP and GTP by JFC1 Binding of [α-^{32}P]ATP or [α-^{32}P]GTP to JFC1 was determined by a rapid filtration technique, as described in *Experimental Procedures*. JFC1 (3 μg) was incubated in buffer A (0.1 mM $MgCl_2$), and reactions were started by the addition of a mix containing 2.5 μM (1 μCi) [α-^{32}P]ATP (*A*) or [α-^{32}P]GTP (*B*) and the indicated concentration of nonradiolabeled competitor. Reactions were carried out for 30 min at 30°C and terminated by the addition ice-cold stop buffer. Samples were vacuum filtered, and filters were washed and counted in a scintillation counter. Results are means (±SE) of three independent experiments. In these experiments, thrombin was used as a negative control. (*C* and *D*) ATP-binding reactions were performed with 0.25 μM [α-^{32}P]ATP in the presence of the indicated concentration of unlabeled competitor. Reactions were conducted as described above. The data were fitted to a binding curve predicted for a single binding site; half-maximal inhibition concentrations are shown next to the curve. The results are representative of four independent experiments.

not contain a known putative nucleotide-binding site, did not show affinity for the ATP analog, suggesting that the binding to JFC1 was specific under the conditions used. It has been previously reported that some non-ATP-binding proteins (for example, BSA) could bind 8-azido-ATP in a nonspecific way. However, in these cases, the bound analog could not be displaced by the presence of cold nucleotide (17). In this work, photoaffinity labeling of JFC1 with 8-azido-[α-^{32}P]ATP was decreased by the presence of unlabeled ATP with an IC_{50} of 5 μM (Fig. 1*B*), suggesting that ATP and the analog 8-azido-ATP recognize the same binding site.

Specificity of ATP Binding to JFC1. Because a high degree of similarity exists between nucleotide-binding domains present in ATP-binding proteins and guanine nucleotide-binding proteins, competition experiments were done to determine the specificity of JFC1 for the binding of ATP. In these experiments, JFC1 was incubated with [α-^{32}P]ATP (Fig. 2*A*) or [α-^{32}P]GTP (Fig. 2*B*) in the presence of an increasing concentration of the indicated nonradiolabeled nucleotide. The capacity of JFC1 to bind [α-^{32}P]ATP was not affected by GTP at concentrations up to 400 times larger than that of the radiolabeled ATP (Fig. 2*A*). On the other hand, JFC1 showed a relative low binding capacity for [α-^{32}P]GTP (Fig. 2*B*), as evaluated in the absence of competitor at a nucleotide-specific activity equal to that used for [α-^{32}P]ATP in the experiment shown in Fig. 2*A*. It is noteworthy that at all concentrations examined, nonradiolabeled ATP displaced the low bound GTP to the background level, indicating that JFC1 specifically binds ATP.

To estimate the relative affinity of JFC1 for ATP, competition experiments by using [α-^{32}P]ATP as the radiolabeled ligand and either unlabeled ATP or ADP as the competitive agent were performed (Fig. 2 *C* and *D*, respectively). The curves represent the calculated best fit to the equation for binding equilibrium in the presence of a competitive inhibitor as previously described (20). Under the conditions evaluated, the relative affinity of JFC1 for ATP was 10.3 times larger than that observed for ADP, the EC_{50} for ATP and ADP being 0.255 μM and 2.63 μM, respectively.

ATPase Activity of JFC1. We evaluated the ATPase activity of JFC1 by monitoring the hydrolysis of [α-^{32}P]ATP and the resulting increase of [α-^{32}P]ADP. The rate of ATP hydrolysis increased as a function of the concentration of Mg^{2+}, indicating that the presence of Mg^{2+} is essential for enzyme activity (Fig. 3*A*). Maximal activity was detected under experimental conditions at which 97% of the ATP present is complexed with the ion. JFC1 ATPase activity was not affected by the presence of GTP, confirming that the enzyme specifically recognize the adenine nucleotide (Fig. 3*B*). In the same way, although residual hydrolysis was detected when JFC1 was incubated in the presence of [α-^{32}P]GTP, no significant hydrolysis of [α-^{32}P]GTP was detected when ATP was present in the same reaction (Fig. 3*B*). On the other hand, JFC1 recognized and hydrolyzed dATP (Fig. 3*B*); the physiological meaning of this finding is being explored in our laboratory.

The kinetic analysis of the ATPase activity of JFC1 indicates that under the conditions used, the initial rate is maintained for

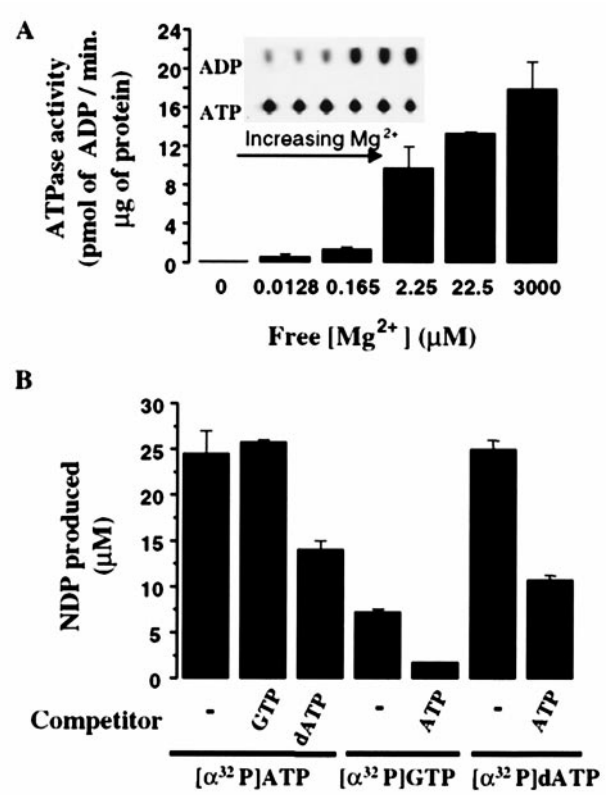

Fig. 3. JFC1 hydrolyzes ATP and dATP but not GTP-A; effect of Mg^{2+} on JFC1 ATPase activity. The ATPase activity of JFC1 was determined by measuring the hydrolysis of [α-^{32}P]ATP to [α-^{32}P]ADP followed by the resolution of labeled nucleotides by ion exchange TLC, as described in *Experimental Procedures*. JFC1 (3 μg) was incubated in the presence of 2.5 μM [α-^{32}P]ATP at varying concentrations of $MgCl_2$ (0.1, 0.8, 1.8, 2, and 5 mM), to reach the indicated free Mg^{2+} concentration. Results are mean ($\pm$SE) of three independent experiments. *Inset* shows the autoradiography of a single representative experiment. (*B*) The ability of JFC1 to hydrolyze [α-^{32}P]ATP (lanes 1–3), [α-^{32}P]GTP (lanes 4 and 5), or [α-^{32}P]dATP (lanes 6 and 7) was evaluated as described in above, except that 500 μM of the indicated [α-^{32}P]NTP and 5 mM Mg^{2+} were used in the reactions. In some experiments, 500 μM of the indicated competitor was added in the reaction at the same time as the radiolabeled nucleotide. Samples were processed and resolved as described in *Experimental Procedures.* Results are mean ($\pm$SE) of two different experiments.

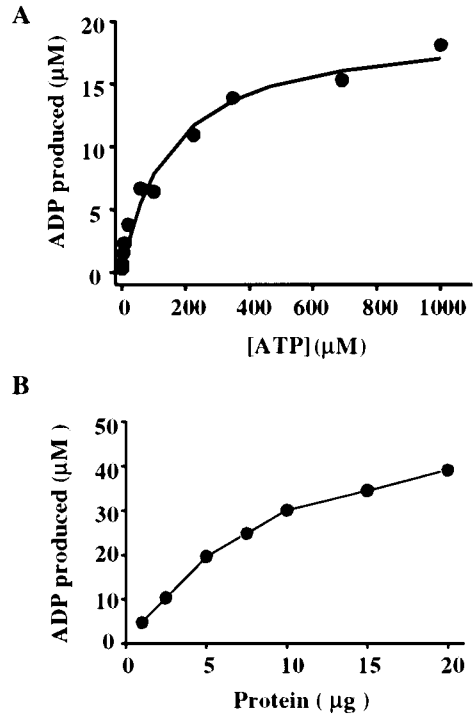

Fig. 4. Determination of the kinetic parameters of JFC1 ATPase activity. (*A*) The ATPase activity of JFC1 was determined as described in *Experimental Procedures*, except that the concentration of [α-^{32}P]ATP was varied at a fixed concentration of JFC1 (3 μg). Reactions were carried out for 10 min at 30°C at a final Mg^{2+} concentration of 5 mM. The kinetic analysis of the ATPase activity of JFC1 indicates that under the conditions used, the initial rate was maintained for 12 min (not shown). The curve represents the calculated best fit to the Michaelis–Menten equation. The results are representative of three independent experiments. The calculated K_M was 58 μM, whereas the extrapolated V_{max} was 33.3 pmol of ATP hydrolyzed per minute per microgram of protein. (*B*) ATPase activity as a function of protein concentration. ATPase assays were performed as described in *Experimental Procedures*. The final concentrations of [α-^{32}P]ATP and Mg^{2+} were 1 mM and 5 mM, respectively. Reactions were carried out for 10 min. The results are representative of two different experiments.

at least 10 min (not shown). Only at saturating Mg^{2+} concentrations (5 mM) was the maximum rate achieved. Lower Mg^{2+} concentrations resulted in a slower rate of hydrolysis, and maximum product formation was not reached even at 90 min (not shown).

To determine the kinetic parameters of ATP hydrolysis by JFC1, we evaluated the ATPase activity of the protein as a function of the concentration of ATP at a high Mg^{2+} concentration (5 mM) (Fig. 4*A*). The data were fit directly to the Michaelis–Menten equation. JFC1 has a relatively high affinity for ATP, with a K_M value of 58 μM, whereas the extrapolated V_{max} was 33.3 pmol of ATP hydrolyzed per minute per microgram of protein, which corresponds to a k_{cat} value of 2.27 per min and a k_{cat}/K_M of 0.0391 $min^{-1}\cdot\mu M^{-1}$. Therefore, JFC1 catalytic efficiency is 20 times larger than that of MutL [k_{cat}/K_M 2.09 $\times$ $10^{-3}\cdot min^{-1}\cdot\mu M^{-1}$ (21) and 60 times larger than that of Hsp-90 ($6.38 \times 10^{-4}\cdot min^{-1}\cdot\mu M^{-1}$ (22)] to compare with some of the GHKL ATPase/kinase superfamily members. In the experiment shown in Fig. 4*B*, ATPase activity was evaluated as a function of JFC1 concentration. The nonsigmoidal characteristic of the curve obtained implied that no cooperativity is exerted by ATP over the ATPase activity, suggesting that JFC1 possesses only one ATP-binding site.

As discussed above and presented in Fig. 5, JFC1 possesses four domains whose primary structures are highly homologous to those described in the ATPase/kinase superfamily ATP-binding domain, referred to as the Bergerat fold (11). In an attempt to determine the localization of the ATPase catalytic domain of JFC1, we evaluated the enzymatic activity of JFC1 and truncations. Truncation GST-C2A, which does not contain any of those domains involved in the putative ATPase catalytic site, did not show any detectable ATPase activity (Fig. 6). On the other hand, truncation GST-C2AB-COOH, which possesses two of the four domains, showed residual activity [26% of that observed with the GST-JFC1 full length-fusion protein (Fig. 6.)]. However, the antitumor agent geldanamycin, known to inhibit the ATPase activity of the 90-kDa heat-shock protein family chaperone (Hsp-90) by binding to the Bergerat ATP-binding site (23), did not affect the ATPase activity of JFC1 (Fig. 6).

Finally, we have previously described that JFC1 binds to the second messenger, PI 3-kinase product PIP_3 (1). We further investigated whether the kinetic parameters of the ATPase activity of JFC1 were affected by PIP_3. It is noticeable that, despite its high affinity for the PPI, JFC1 ATPase kinetics

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Fig. 5. Schematic presentation of the putative ATP-binding site in JFC1. The amino acid number is indicated running from the N to the C terminus. Shaded areas represent putative nucleotide-binding regions corresponding to the N box, G1 box, G2 box, and G3 box of the Bergerat ATP-binding fold, according to refs. 10 and 11. The secondary structure of the JFC1 putative ATP-binding site was predicted by OMIGA 2.0 software (Genetics Computer Group, Madison, WI) and the Chou and Fasman method. Arrows represent the tendency to form β sheet, zigzags represent the tendency to form α helices, and square zigzags represent the tendency to form β turns.

parameters were not altered by the presence of the mediator in the reaction medium (data not shown).

Discussion

In this work, we demonstrate that the protein JFC1 is an ATP-binding protein with magnesium-dependent ATPase activity. First, we showed that JFC1 binds specifically to the ATP analog 8-azido ATP. Furthermore, we characterized the relative nucleotide-binding affinity by competitive experiments. The results indicate that JFC1 binds ATP with 10 times higher affinity than ADP, implying that after the ATP is hydrolyzed, the ADP will be exchanged. It is also significant that JFC1 showed specificity for ATP as opposed to GTP. Although GTP-binding proteins show, in general, high homology with the consensus sequence for the phosphate-binding site present in various ATP-binding proteins and contain an equivalent structure for the Walker B domain (24), the guanine nucleotide-binding proteins also possess a characteristically conserved NKXD sequence that is considered to be the site for the recognition of the guanine ring (24), and this, not surprisingly, is not present in JFC1 (1). On the other hand, JFC1 used dATP as a substrate, suggesting that the 2′ OH of the ribose does not take part in the recognition of the nucleotide. Furthermore, the hydrolysis of dATP could have physiological connotations. It has been recently described that the apoptotic enzyme Apaf-1 recognizes dATP as well as ATP as a substrate (25); in that case, binding to the nucleotides is necessary for Apaf-1 to form a stable complex with cytochrome *c* (25). Although the characteristics of JFC1 suggest that it could be involved in forming ATP/dATP complexes with different partners, further studies are necessary to establish whether this takes place.

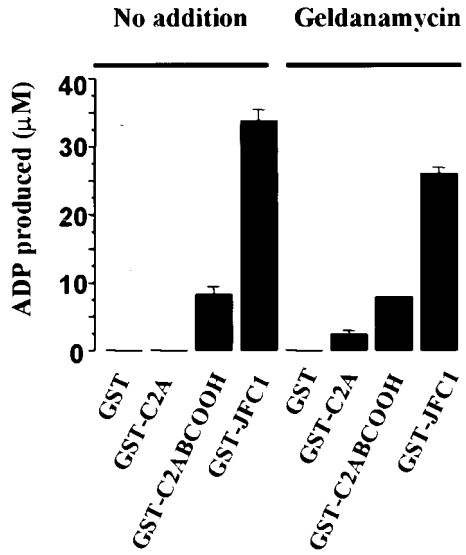

Fig. 6. Effect of geldanamycin on ATPase activity of JFC1 and JFC1 truncations. Hydrolysis [α-^{32}P]ATP by GST-JFC1, by the truncation GST-C2A, or by the truncation GST-C2AB-COOH, was evaluated in reactions that contained 55 pmol of GST-JFC1 or its truncations, in buffer A (5 mM Mg^{2+}). Reactions were started by the addition of 1 mM [α-^{32}P]ATP and were carried out during 10 min at 30°C. Where indicated, the reaction mixtures were incubated in the presence of 90 μM geldanamycin during 5 min before the addition of [α-^{32}P]ATP. Incubation in the presence of geldanamycin for up to 30 min did not alter the results (not shown). Samples were processed as described in *Experimental Procedures*. Results are mean (±SE) of two different experiments.

Interestingly, JFC1 showed an absolute requirement for Mg^{2+} to manifest ATP hydrolysis, reaching its maximum ATPase activity at Mg^{2+} concentrations that saturate all of the nucleotide present in the medium. Considering that this event would occur in an environment with a constant Mg^{2+} concentration of 0.5–2 mM *in vivo*, most of the ATP would be complexed to the ion, and therefore JFC1 ATPase activity would be constitutively active, unless a still unknown factor keeps the ATP hydrolase in an inactive form.

The predicted amino acid sequence of JFC1 denotes the presence of a Bergerat fold, a putative nucleotide-binding site similar to those present in a group of functionally unrelated enzymes that constitute the GHKL ATPase/kinase superfamily (10). Although the primary sequence homology between the various members is very poor, the three-dimensional structure of their ATP-binding domains is considered almost superimposable (10). Members of this family are enzymes as diverse as Hsp-90 (23), the type II topoisomerase like DNA gyrase (11), the DNA repair protein MutL (26), and prokaryotic histidine kinases (27). The Bergerat fold is characterized by the presence of four structures named boxes N, G1, G2, and G3 (10). As described in Fig. 5, JFC1 possesses exact matches to the boxes G1, G2, and to the recently described box G3. However, some differences between the ATP putative binding site in JFC1 and the Bergerat fold can be noticed. The EXXXN box (N box) is characteristic in every member of the ATPase/kinase superfamily. The conserved glutamate in the N box has been described as involved in the hydrolysis of ATP (26, 28). However, the N box is not present as such in JFC1, which instead has an E^{86}XXXQXXXN^{94} box, with Gln-90 replacing the usual Asn.

Moreover, the most significant difference within the Bergerat ATP-binding sites has been described as the domain located between boxes G1 and G2. JFC1 does not seem to be an exception, because the C2A domain of JFC1 is located in that position. This C2 domain has been suggested to be responsible for the JFC1 PIP_3-binding capacity (1). Nevertheless, no other member of the GHKL superfamily has been shown to possess this kind of membrane-binding property. Whether the Bergerat core is able to properly fold despite the presence of the C2 domain can be answered only by three-dimension-structure analysis of JFC1. Nevertheless, the data presented in this work suggest that not all structures mentioned above may take part in the binding of ATP by JFC1. First, a truncation of JFC1 that contains boxes G2 and G3, but not boxes N and G1, showed residual ATPase activity (Fig. 6), suggesting that, although the intact protein is essential for the ATPase to develop maximal activity, ATP binding and hydrolysis depend on the carboxyl terminal half of JFC1. Second, the antitumor agent geldanamycin, which has been recently demonstrated to inhibit the ATPase activity of the chaperone Hsp-90 by binding to the Bergerat fold, acting as an ATP mimetic agent (23), did not affect JFC1 ATPase activity (Fig. 6). This piece of data indicates that even if boxes G2 and G3 were involved in the recognition of ATP by JFC1, the characteristics of this binding site are unique. Several experiments, including directed mutagenesis, are being performed in our laboratory to elucidate the ATP-binding site of JFC1. Meanwhile, the involvement of a Walker B domain that is located in the carboxyl terminal end of JFC1 (R^{488}RXXXXXLXLWD^{498}) should not be disregarded, although no recognizable Walker A domains are present in this protein.

It is known that a glycine-rich loop in the ATP-binding site is one of the most highly conserved sequence motifs in protein kinases (29), the conserved feature being GXGXXG. The loop is known to exclude H_2O from the catalytic site, reducing the ATPase activity of these enzymes (30). JFC1 possesses a glycine-rich sequence G^{412}XXGXG ^{417}, with a mismatch at the middle glycine. To exclude the possibility that JFC1 undergoes autophosphorylation *in vitro*, as has been described for various proteins kinases (31, 32), we incubated the protein in the presence of [γ-^{32}P]-ATP for up to 60 min under conditions similar to those used for the ATPase assays. No evidence that JFC1 could be a self-phosphotransfer enzyme was detected (data not shown). Moreover, the cytosolic factors $p67^{phox}$ and $p47^{phox}$, both known to undergo phosphorylation (33, 34) and to interact with JFC1 (1), are not phosphorylated when incubated with JFC1 in the presence of [γ-^{32}P]ATP (data not shown). These results indicate that JFC1 does not act as a phosphoryl donor *in vitro* under the assay conditions evaluated in this work, at least for those two proteins, although its potential as such depends on finding a specific substrate.

We demonstrated that JFC1 binds selectively to the second messenger PIP_3 (1). Because PIP_3 does not affect the ATPase kinetics parameters of JFC1, the PPI could act just as a docking point in the inner membrane once the PI 3-kinase pathway is activated. It is unlikely that JFC1 constitutively hydrolyzes ATP without coupling this energy to work. Therefore, although PIP_3 does not directly affect ATPase activity, it may control the circumstances under which ATP is hydrolyzed. The PI 3-kinase pathway has been extensively involved in membrane trafficking pathways (35) as well as in cell proliferation, survival, and cytoskeletal reorganization (36) and, as discussed before, JFC1 could act as an accessory protein between PIP_3 and SH3 domain-containing proteins in an ATP-dependent manner, thus possibly regulating some of these processes.

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