FRAT1, a Substrate-specific Regulator of Glycogen Synthase Kinase-3 Activity, Is a Cellular Substrate of Protein Kinase A

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FRAT1, like its Xenopus homolog glycogen synthase kinase-3 (GSK-3)-binding protein, is known to inhibit GSK-3-mediated phosphorylation of β-catenin. It is currently unknown how FRAT-GSK-3-binding protein activity toward GSK-3 is regulated. FRAT1 has recently been shown to be a phosphoprotein in vivo; however, the responsible kinase(s) have not been determined. In this study, we identified Ser188 as a phosphorylated residue in FRAT1. The identity of the kinase that catalyzes Ser188 phosphorylation and the significance of this phosphorylation to FRAT1 function were investigated. Protein kinase A (PKA) was found to phosphorylate Ser188 in vitro as well as in intact cells. Importantly, activation of endogenous cAMP-coupled β-adrenergic receptors with norepinephrine stimulated the phosphorylation of FRAT1 at Ser188. GSK-3 was also able to phosphorylate FRAT1 at Ser188 and other residues in vitro or when overexpressed in intact cells. In contrast, endogenous GSK-3 did not lead to significant FRAT1 phosphorylation in cells, suggesting that GSK-3 is not a major FRAT1 kinase in vivo. Phosphorylation of Ser188 by PKA inhibited the ability of FRAT1 to activate β-catenin-dependent transcription. In conclusion, PKA phosphorylates FRAT1 in vitro as well as in intact cells and may play a role in regulating the inhibitory activity of FRAT1 toward GSK-3.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that phosphorylates multiple substrates in the cell and is involved in distinct cellular signaling pathways, including insulin/growth factor and Wnt signaling (1–3). GSK-3 is a constitutively active kinase. Upon stimulation of both insulin/growth factor and Wnt signaling (1–3), GSK-3 is inactivated. Insulin-dependent GSK-3 inhibition involves activation of phosphatidylinositol 3-kinase and Akt/protein kinase B, which then phosphorylates GSK-3 at an N-terminal serine residue (Ser21 in GSK-3α and Ser9 in GSK-3β) (4). The phosphorylated N terminus is believed to act as a pseudosubstrate and compete for substrate binding, thus leading to autoinhibition of the catalytic activity of GSK-3 (5, 6). Inactivation of GSK-3 in response to insulin stimulation leads to dephosphorylation and activation of the GSK-3 substrates glycogen synthase and eukaryotic protein synthesis initiation factor 2B, ultimately contributing to the stimulation of glycogen and protein synthesis.

In the Wnt signaling pathway, GSK-3 is known to phosphorylate β-catenin at defined residues at the N terminus, thus targeting the protein for ubiquitin/proteasome-mediated degradation (1, 7). β-Catenin phosphorylation occurs in a complex that includes the tumor suppressor protein APC and the scaffold protein Axin. Activation of Wnt signaling through binding of Wnt-secreted glycoproteins to their receptors leads to inhibition of the Axin-APC-GSK-3 complex. Consequently, β-catenin becomes stabilized and translocates into the nucleus, where it acts as a transcriptional coactivator of transcription factors of the TCF/LEF family, activating target genes such as c-myc and cyclin D1 (8–10). The mechanism of Wnt-dependent GSK-3 inactivation is distinct from the insulin pathway and does not involve phosphorylation of the N-terminal serine (11, 12).

The protein GSK-3-binding protein (GBP) was identified in Xenopus (13). GBP inhibits GSK-3, leading to stabilization of β-catenin in Xenopus embryos and induction of a secondary body axis (13). GBP is homologous to the mammalian T cell protooncogene FRAT1 (frequently rearranged in advanced T cell lymphomas) 1 (14). Two homologs of FRAT1 have been cloned, FRAT2 (15) and Frat3 (16). However, no GBP/FRAT homologs appear to be present in the genomes of Drosophila and Caenorhabditis elegans (2).

All GBP/FRAT homologs have been shown to induce a secondary axis in Xenopus embryos, indicating that they all inhibit GSK-3 activity toward β-catenin (15, 16). The mechanism by which GBP/FRAT inhibits GSK-3 activity toward β-catenin appears to involve preventing Axin from binding to GSK-3, probably by competition for a common (or closely overlapping) binding site on GSK-3. GBP, FRAT, and FRATtide, a 39-residue peptide derived from FRAT1 that is sufficient to bind GSK-3,
have all been shown to dissociate GSK-3 from the Axin complex (17–21). Interestingly, although FRATtide inhibits GSK-3-mediated phosphorylation of β-catenin and Axin in vitro, it does not inhibit GSK-3 activity toward peptides derived from glycogen synthase or eukaryotic protein synthesis initiation factor 2B (19). In addition, using adenoviral expression of FRAT1 in PC12 cells, we have shown that FRAT1 overexpression results in β-catenin stabilization but does not alter glycogen synthase activity (22). These results indicate that FRAT1-mediated GSK-3 inhibition is also selective in vivo.

It is not known how FRAT activity toward GSK-3 is regulated. A recent report demonstrated that murine Frat1 and Frat2 are phosphoproteins (23); however, the responsible kinases have not been identified. We observed that FRAT1 immunoprecipitated from cells is phosphorylated by one or more endogenous kinases. We identified protein kinase A (PKA) as a kinase that phosphorylates FRAT1 in vitro as well as in cells. We also provide evidence that GSK-3 is not a major FRAT1 kinase in vivo.

**MATERIALS AND METHODS**

**Plasmids and Cell Culture**—A full-length human FRAT1 clone was tagged with the Myc epitope at the N terminus and inserted into the mammalian pcDNA3 expression vector to generate Myc-FRAT1-pcDNA3. C-terminally 2×FLAG- or V5-tagged or untagged expression plasmids for GSK-3β or FRAT1 were generated by PCR amplification of the human GSK-3β and FRAT1 open reading frame from HEK293 or fetal brain cDNA and insertion into pcDNA3. pFC-PKA, encoding the mouse PKAα catalytic subunit, was obtained from Stratagene. Subconfluent HEK293T cells were transfected using Fugen reagent (Roche Applied Science) according to the manufacturer’s instructions. 48 h after transfection, the cells were washed with ice-cold PBS, lysed in 2.5 ml of lysis buffer (25 mM Tris/HCl, 0.1 mM EGTA, 0.1% (v/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol, Roche Applied Science “Complete” protease inhibitors, pH 7.5), and the lysates were snap frozen in liquid nitrogen and then stored at −80°C until required. Lysates were precleared by centrifugation before use.

**Immunoprecipitation of Myc-FRAT1—10 μl of protein G-Sepharose was coupled to 5 μg of monoclonal anti-Myc antibody (clone 9E10; Autogen Bioclear), and the pellet was used to immunoprecipitate Myc-FRAT1 from 1 ml of precleared lysate. The pellets were then washed three times in 1 ml of Buffer A (50 mM Tris/HCl, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, pH 7.5) containing 0.5 mM NaCl and then twice with 1 ml of Buffer A. The washed immunoprecipitates were used for in vitro kinase reactions or denatured in SDS-sample buffer and subjected to SDS-PAGE.

**Immunoblotting**—Cells were lysed as described above. Equal amounts of protein lysate or immunoprecipitated protein were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and immunoblotted. The following antibodies were used: monoclonal anti-Myc (clone 9E10; Autogen Bioclear), monoclonal anti-V5 (Seraotec), monoclonal anti-FLAG (M2; Sigma), monoclonal anti-GSK-3β (BD Biosciences), rabbit polyclonal anti-PKAα catalytic subunit (sc-903 (C-20); Santa Cruz Biotechnology), and polyclonal FRAT1 phospho-Ser198-specific antibody, raised in rabbit and generated against a peptide representing residues 182–194 in human FRAT1 (LQQRRGpSQPETRT; where pS represents phosphoserine), which was conjugated to keyhole limpet hemocyanin. Blots were developed using the Amersham Biosciences Enhanced Chemiluminescence kit. The Western blots shown to detect protein expression and phosphorylation in intact cells and in vitro phosphorylation of FRAT1 are representative of three independent experiments.

**In Vitro Phosphorylation Assays**—FRAT1 immunoprecipitates were incubated on a shaking platform for 10 min with 35 μl of Buffer A in the presence of the different protein kinase inhibitors or recombinant kinases. The kinase reaction was then initiated by the addition of 10 μl of 50 mM MgCl₂, 0.5 mM ATP (standard ATP for mass spectrometry analysis or [γ-32P]ATP (200–1000 cpm/pmole) for autoradiography) and incubated on a shaking platform for 30 min at 30°C. LiCl and the selective GSK-3 inhibitors SB216763 and SB415286 were used as described previously (22, 24–26). Following the reaction, the samples were denatured in SDS-sample buffer and subjected to SDS-PAGE. The gels were dried, and phosphorylation of Myc-FRAT1 was analyzed using autoradiography, where the reactions contained [γ-32P]ATP. Alternatively, the gels were stained with Coomassie and Myc-FRAT1 bands excised for phosphorylation site mapping using mass spectrometry.

**Mass Spectrometry Analysis**—The Myc-FRAT1 bands were excised from the SDS-polyacrylamide gel, and the protein was then digested in gel with trypsin (modified sequencing grade; Promega Corp.). Following digestion, the resulting peptides were desalted using C18 ZipTips (Millipore Corp.) and analyzed using electrospray ionization on an ion trap mass spectrometer (Thermo Finnigan). The mass spectrometer was set to acquire an ion map by fragmenting each ion within a given range. In this way, each peptide in the sample was fragmented within the mass spectrometer to generate a tandem mass spectrum (MS/MS). The ion map, a representation of the tandem mass spectra of all components in a given sample, was then examined to see if any peptides had potentially lost a phosphate group. Identification of phosphorylated peptides was then confirmed by interpretation of the corresponding MS/MS spectra.

**In Vivo Labeling**—HEK293T cells were transiently transfected with Myc-FRAT1-pcDNA3. 16 h after transfection, each dish of cells was labeled with 5 mCi of [32P]orthophosphate and treated without or with the selective GSK-3 inhibitors SB216763 (10 μM) or SB415286 (40 μM). After 4 h of compound treatment, the cells were washed with phosphate-buffered saline and then lysed in lysis buffer, as described above. Myc-FRAT was immunoprecipitated from lysates using monoclonal 9E10 antibody, and immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose. Phosphate incorporation into Myc-FRAT was determined by autoradiography.

**β-Catenin-TCF/LEC-regulated Gene Reporter Assay**—Luciferase activity in cells transiently transfected with a TCF/LEF
intact cells, HEK293T cells transfected with Myc-FRAT1 were
absence (performed as described under “Materials and Methods” in the presence (HEK293T cells and immunoprecipitated. The Myc-FRAT1 kinase assay was as described under “Materials and Methods.”

When Myc-FRAT1 immunoprecipitated from transfected HEK293T cell lysates was incubated with Mg\(^{2+}/[^{32}P]ATP\) in vitro, the protein became phosphorylated (Fig. 1b). Since FRAT1 has no intrinsic kinase catalytic activity, it was concluded that the observed FRAT1 phosphorylation reflected the in vitro activity of one or more endogenous kinases that had co-immunoprecipitated with Myc-FRAT1. In keeping with previous reports of a FRAT-GSK3 complex, the presence of the GSK-3 inhibitors LiCl or SB415286 (Fig. 1b) suppressed the phosphorylation of Myc-FRAT1 markedly. However, inhibition of GSK-3 activity did not completely abolish Myc-FRAT1 phosphorylation, raising the possibility of additional co-immunoprecipitating kinase activities within the Myc-FRAT1 complex.

Mass spectrometry (MS) was used to identify the sites in Myc-FRAT1 that were phosphorylated. To this end, Myc-FRAT1 was immunoprecipitated from cells and subjected to in vitro phosphorylation with standard ATP, followed by SDS-PAGE and mass spectrometry analysis of the excised Myc-FRAT1 band. This approach is expected to detect residues that are phosphorylated in intact cells or in vitro by endogenous coimmunoprecipitating kinases. Data from a series of MS/MS ion maps indicated that the tryptic peptide containing Ser188 is phosphorylated. This peptide also contains a threonine residue (Thr192), which, unlike Ser188, is not conserved in FRAT2 (see Fig. 2a). It was, therefore, assumed that the serine was the phosphorylated residue (see below). However, since not all tryptic peptides were analyzable by mass spectrometry, it is possible that there are additional phosphorylation sites.

**RESULTS**

Ser\(^{188}\) in FRAT1 Is Phosphorylated in Vitro and in Vivo—To determine whether FRAT was modified by phosphorylation in intact cells, HEK293T cells transfected with Myc-FRAT1 were \(^{32}P\)-labeled. Autoradiography showed that isolated Myc-FRAT1 was indeed phosphorylated, indicating that Myc-FRAT1 is phosphorylated in intact cells (Fig. 1a). Moreover, treatment of cells with selective GSK-3 inhibitors resulted in no significant reduction in FRAT phosphorylation in the case of SB216763 and a moderate reduction with SB415286, suggesting the presence of compound-insensitive FRAT kinase activities in intact cells (Fig. 1a).

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Ser\(^{188}\) in FRAT1 Is Phosphorylated by PKA and PKC in Vitro—Analysis of residues around Ser\(^{188}\) in FRAT1 suggested that this residue lay within a consensus sequence for phosphorylation by PKA (RX(S/T)) (Fig. 2a). Ser\(^{188}\) also conforms to consensus motifs for phosphorylation by protein kinase C (PKC), which are (S/T)(K/R), (K/R)(S/T), or (K/R)(S/T) (27). To evaluate further the role of PKA and/or PKC in FRAT phosphorylation, a FRAT1 mutant was generated in which Ser\(^{188}\) was mutated to Ala, and in vitro phosphorylation of wild type and mutant Myc-FRAT1 by recombinant PKA and PKC was determined in the presence of the GSK-3 inhibitor SB415286. As shown in Fig. 2, both PKA and PKC phosphorylated wild type Myc-FRAT1. In contrast, no \(^{32}P\) incorporation into the S188A
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mutant could be observed with PKA, indicating that PKA exclusively phosphorylates FRAT1 at Ser\(^{188}\) \textit{in vitro} (Fig. 2b). In comparison, phosphorylation of Myc-FRAT1 by PKC was only slightly reduced in the S188A mutant compared with wild type FRAT1 (Fig. 2c), indicating that PKC does not exclusively phosphorylate Ser\(^{188}\) \textit{in vitro}.

To characterize further the phosphorylation of Ser\(^{188}\) in FRAT1, a polyclonal phosphosite-specific pS188 antibody was generated. Incubation of immunoprecipitated wild type Myc-FRAT1 with either recombinant PKA or PKC increased the immunoreactivity of the phospho-Ser\(^{188}\)-specific antibody, and this was abolished in the S188A mutant (Fig. 3). This result verified that the antibody only recognizes phosphorylation of Ser\(^{188}\), and confirmed that both PKA and PKC are able to phosphorylate this site in FRAT1 \textit{in vitro}.

These findings indicated that recombinant PKA and PKC could phosphorylate Ser\(^{188}\) \textit{in vitro}. We next wished to determine whether Ser\(^{188}\) is phosphorylated by a co-immunoprecipitating protein kinase. Interestingly, the addition of 8-bromo-cAMP to the Myc-FRAT1 protein complex significantly increased the amount of phosphate incorporated into Ser\(^{188}\) \textit{in vitro} (Fig. 4a). Furthermore, consistent with this idea, the phosphorylation of Ser\(^{188}\) resulting from treatment with 8-bromo-cAMP was strongly inhibited by PKA inhibitor, as determined by autoradiography and Ser\(^{188}\)-phosphospecific immunoblotting (Fig. 4, a and b). In contrast, 8-bromo-cAMP did not induce phosphorylation of the S188A mutant Myc-FRAT1 (Fig. 4b). In addition, PKA was observed by immunoblotting to co-immunoprecipitate with FRAT1 (Fig. 4b). We did not observe stimulation of Ser\(^{188}\) phosphorylation when adding PKC activators phosphatidoserine and diacylglycerol plus calcium to FRAT1 immunoprecipitates in the presence of MgATP (data not shown). Together, these data indicated that PKA, but not PKC, is a co-immunoprecipitating kinase that mediates phosphorylation of FRAT1 at Ser\(^{188}\).

PKA Phosphorylates Ser\(^{188}\) in FRAT1 in Intact Cells—To assess whether PKA and/or PKC can phosphorylate Ser\(^{188}\) in intact cells, HEK293T cells were transfected with Myc-FRAT1 and treated with activators of PKA (forskolin and 8-bromo-cAMP) or PKC (TPA and bryostatin-1) for 1 h. Phosphorylation of Ser\(^{188}\) was then measured in immunoprecipitates of Myc-FRAT1 using the phospho-Ser\(^{188}\)-specific antibody. Fig. 5a shows that the PKA activators forskolin and 8-bromo-cAMP, but not the PKC activators TPA and bryostatin-1, were able to induce phosphorylation of Ser\(^{188}\). To confirm phosphorylation of Ser\(^{188}\) by PKA in cells, PKA was cotransfected with wild type and S188A mutant Myc-FRAT1. As shown in Fig. 5b, cotransfection of PKA resulted in a strong signal in wild type but not in S188A mutant Myc-FRAT1. Taken together, these results indicated that PKA can phosphorylate Ser\(^{188}\) in intact cells as well as \textit{in vitro} and that PKA is more important to the regulation of phosphorylation of Ser\(^{188}\) in a cellular context than PKC.

Activation of Endogenous cAMP-coupled \(\beta\)-Adrenergic Receptors Induces the Phosphorylation of FRAT1 at Ser\(^{188}\)—HEK293 cells express endogenous \(\beta\)-adrenergic receptors (28). To determine whether elevation of the cellular cAMP concentration through activation of an endogenous cAMP-coupled receptor induces Ser\(^{188}\) phosphorylation, HEK293 cells were transfected with FRAT1-FLAG followed by treatment with norepinephrine for 2 h. As shown in Fig. 5c, norepinephrine treatment led to marked Ser\(^{188}\) phosphorylation of FRAT1.
Phosphorylation of Ser\textsuperscript{188} by GSK-3 in Vitro and in Intact Cells—Given that GSK-3 is known to interact with FRAT1 and that we found that the kinase could induce marked phosphorylation of Myc-FRAT1 in vitro, we determined whether this kinase can also phosphorylate Ser\textsuperscript{188}. As shown in Fig. 6a, Ser\textsuperscript{188} phosphorylation of immunoprecipitated FRAT1 in vitro, as measured using the phosphosite-specific antibody, was inhibited in the presence of LiCl and SB415286, indicating that co-immunoprecipitating GSK-3 can phosphorylate Ser\textsuperscript{188}. To determine GSK-3-mediated phosphorylation of FRAT1 at Ser\textsuperscript{188} in cells, cells transfected with FRAT1 were treated with GSK-3 inhibitors or cotransfected with an expression plasmid for GSK-3β, followed by immunoblotting of cell lysates with Ser\textsuperscript{188} phosphospecific antibody. Cotransfection of GSK-3β increased Ser\textsuperscript{188} phosphorylation, but to a much smaller degree compared with the effect observed when PKA was cotransfected (Fig. 6b). GSK-3 is a constitutively active kinase; thus, if GSK-3 phosphorylates Ser\textsuperscript{188} in vivo, GSK-3 inhibitors would be expected to reduce Ser\textsuperscript{188} phosphorylation. However, treatment of cells with SB415286 or LiCl had no effect on Ser\textsuperscript{188} phosphorylation (Fig. 6b). When cells transfected with FRAT1 were treated with phosphatase inhibitor okadaic acid, a marked increase in Ser\textsuperscript{188} phosphorylation was detected (Fig. 6c). Again, no inhibition of okadaic acid-induced Ser\textsuperscript{188} phosphorylation was observed in the presence of SB415286. Taken together, these results indicate that whereas GSK-3 can phosphorylate Ser\textsuperscript{188} in vitro or in cells when overexpressed, endogenous GSK-3 does not contribute to FRAT1 phosphorylation at Ser\textsuperscript{188} in intact cells.

GSK-3-mediated Phosphorylation of FRAT1 at Ser\textsuperscript{188} Does Not Require Priming—The majority of physiological GSK-3 substrates require prior priming through phosphorylation at a Ser or Thr residue at the N\textsuperscript{1101} position (where N is the site of GSK-3-mediated phosphorylation). Interestingly, the residue localized four amino acids downstream of Ser\textsuperscript{188} is a Thr. In order to gain further insight into the possible significance of Ser\textsuperscript{188} phosphorylation by GSK-3, we investigated whether a priming event is necessary. Mutation of Arg\textsuperscript{96} to Ala in GSK-3 prevented phosphorylation of primed substrates but does not...
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The results presented in Fig. 1 suggest that although GSK-3 can phosphorylate FRAT1 in vitro, endogenous GSK-3 does not significantly contribute to FRAT1 phosphorylation in intact cells. To further study the phosphorylation of FRAT1 in cells, HEK293 cells were co-transfected with FRAT1 and wild type or R96A mutant GSK-3β. Co-transfection with both GSK-3β plasmids resulted in a number of FRAT1 bands that migrated more slowly in the SDS gel, which is probably due to phosphorylation events (Fig. 8a). Interestingly, the R96A mutant induced more phosphorylation of FRAT1 compared with wild type GSK-3β. This is also apparent in Figs. 6b and 7a, where the R96A mutant induced slower, Ser188-phosphorylated FRAT1 bands compared with wild type GSK-3. This mobility shift was not due to Ser188 phosphorylation, since co-transfected GSK-3β induced a similar mobility shift in both wild type and S188A mutant FRAT1 (Fig. 8b).

Having observed a GSK-3-dependent slower migration of FRAT1, we then determined whether phosphatase inhibitor okadaic acid and GSK-3 inhibitor SB415286 affected FRAT1 mobility. As shown in Fig. 8c, treatment with okadaic acid had only a marginal effect. No obvious difference in FRAT1 mobility was observed in the absence or presence of SB415286 except upon longer exposure of the Western blot, when a faint slow band in the control (indicated by an arrow) disappeared when SB415286 was added. These results, combined with the findings in Fig. 1, suggest that endogenous GSK-3 is not a major FRAT1 kinase.

Phosphorylation of Ser188 by PKA Inhibits the Ability of FRAT1 to Activate β-Catenin-dependent Transcription—The presented data indicate that PKA, but not GSK-3, is a FRAT1 kinase in vitro and in intact cells and that PKA-mediated phosphorylation of FRAT1 occurs exclusively at Ser188. We therefore wished to investigate the functional relevance of PKA-induced phosphorylation of Ser188 in FRAT1. It is well known that FRAT1 binds to GSK-3, resulting in increased stability of cytoplasmic β-catenin and its translocation into the nucleus, where it activates transcription in conjunction with transcription factors of the TCF/LEF family. Thus, to measure FRAT1 activity we utilized a TCF/LEF-dependent luciferase reporter assay (24). Transfection of either wild type or S188A mutant Myc-FRAT1 increased luciferase reporter activity to a similar extent (Fig. 9a). In order to examine the effect of Ser188 phosphorylation, PKA was cotransfected with wild type or S188A
FRAT1. Co-expression of PKA resulted in a much lower stimulation of luciferase reporter activity by wild type FRAT1 compared with S188A FRAT1 (Fig. 9b). We noted that cotransfection of PKA by itself led to a significant increase in luciferase activity. This is probably due to direct stimulation of gene expression from transfected expression plasmids by PKA, as has been previously been observed (29). We also noted significantly increased expression from other transfected plasmids when PKA was cotransfected (data not shown). Because of this nonspecific effect of PKA, we could not directly compare the results in Fig. 8, a and b. Nevertheless, when comparing the effects of wild type and S188A mutant FRAT1 in the absence versus presence of PKA, the results clearly indicate that Ser\textsuperscript{188}-phosphorylated FRAT1 had a markedly reduced ability to stimulate TCF/LEF-dependent reporter activity. These findings suggest that PKA-mediated phosphorylation of Ser\textsuperscript{188} in FRAT1 inhibits its activity toward GSK-3. This was not due to reduced binding of Ser\textsuperscript{188}-phosphorylated FRAT1 to GSK-3, since wild type and S188A mutant FRAT1 from cells co-transfected with PKA immunoprecipitated GSK-3\textbeta{} equally well (Fig. 9c). FRAT1 has been reported to mediate nuclear export of GSK-3 and require interaction with LRP5/6 to activate \beta{}-catenin-dependent transcription (30, 31). However, PKA-dependent Ser\textsuperscript{188} phosphorylation did not affect FRAT1 subcellular localization or its interaction with LRP6 (data not shown). On the other hand, we observed that Ser\textsuperscript{188} phosphorylation of wild type FRAT1 by cotransfected PKA reduced its half-life compared with S188A FRAT1, as determined by following FRAT1 degradation in the presence of cycloheximide (Fig. 9d). In contrast, in the absence of PKA, no difference in the half-life of wild type and S188A FRAT1 was observed (data not shown). The shorter half-life of Ser\textsuperscript{188}-phosphorylated FRAT1 may contribute to its reduced ability to activate \beta{}-catenin-TCF/LEF-dependent transcription.

**DISCUSSION**

In this study, we investigated the mechanism of FRAT1 phosphorylation. We identified two kinases, GSK-3 and PKA, that coimmunoprecipitate with FRAT1 and phosphorylate FRAT1 \textit{in vivo}. Furthermore, mass spectrometry studies identified Ser\textsuperscript{188} as a phosphorylated residue in FRAT1. Ser\textsuperscript{188} conforms to consensus motifs for phosphorylation by GSK-3, PKA, and PKC, and all of these kinases phosphorylated Ser\textsuperscript{188} \textit{in vitro}.

Recently, both FRAT1 and FRAT2 were shown to be phosphorylated in cells (23), and GSK-3 was found to phosphorylate FRAT2 \textit{in vitro} (32). Our results indicate that GSK-3 phospho-
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FRAT1 functions as an inhibitor of glycogen synthase kinase 3 (GSK-3) in vivo as well as in cells when overexpressed. However, the effect of transfected GSK-3β on FRAT1 Ser188 phosphorylation was much smaller compared with that of transfected PKA. Although most physiological GSK-3 substrates require priming through phosphorylation of a Ser or Thr at the +4-position, we have shown that phosphorylation of Thr199 is not necessary for GSK-3-mediated Ser188 phosphorylation. Furthermore, inhibition of endogenous GSK-3 with specific inhibitors had no effect on Ser188 phosphorylation. Given that GSK-3 is a constitutively active kinase, these results suggest that it does not contribute to Ser188 phosphorylation in vivo. Transfection of GSK-3β also induced a slower migration of FRAT1 that was not due to Ser188 phosphorylation, indicating that the kinase can phosphorylate other residues in FRAT1 when overexpressed. However, our in vivo labeling experiments (Fig. 1a) showed that the presence of GSK-3 inhibitors had no effect or only a small effect on FRAT1 phosphorylation. This suggests that GSK-3 is not a major FRAT1 kinase in vivo.

Given that phospho-Ser188 lies within a consensus sequence for PKA and for PKC (see above), we studied their role in mediating Ser188 phosphorylation in vitro and in cells. Although both kinases phosphorylated Ser188 in vitro, activation of endogenous PKA with forskolin and 8-bromo-cAMP, but not of endogenous PKC, resulted in FRAT1 phosphorylation at Ser188 in intact cells. Furthermore, activation of endogenous cAMP-coupled β-adrenergic receptors with norepinephrine stimulated the phosphorylation of FRAT1 at Ser188. These findings clearly indicate that endogenous PKA, when activated, is a FRAT1 kinase that phosphorylates Ser188. We also noted that although basal levels of phosphorylated Ser188 were very low to undetectable, the addition of phosphatase inhibitor okadaic acid increased Ser188 phosphorylation markedly. Okadaic acid-induced phosphorylation was not reduced by the GSK-3 inhibitor SB415286 (Fig. 6c) or by inhibitors of PKA (H-89 and cell-permeable protein kinase A inhibitor 14-22) (data not shown). These results confirm that constitutively active GSK-3 is not a physiological Ser188 kinase and that Ser188 phosphorylation by PKA, which is inactive under basal conditions, requires the cAMP-dependent dissociation of the catalytic from the inhibitory regulatory subunits. The okadaic acid-induced Ser188 phosphorylation also indicates that in addition to PKA, a second unidentified kinase may mediate Ser188 phosphorylation under basal conditions in vivo. Given that the basal phosphorylation of FRAT1 at Ser188 was very low, the significance of this kinase for the regulation of FRAT1 is not clear. However, it is also possible that this unidentified kinase(s) becomes activated under certain conditions and then contributes significantly to Ser188 phosphorylation of FRAT1.

Functionally, phosphorylation of FRAT1 at Ser188 by PKA inhibited the ability of FRAT1 to activate β-catenin-dependent transcription, suggesting that PKA-mediated phosphorylation of FRAT1 reduces its inhibitory activity toward GSK-3. This is not a result of reduced binding of Ser188-phosphorylated FRAT1 to GSK-3 but may at least partially be due to decreased FRAT1 protein stability.

The FRAT homolog GBP is required for maternal Wnt signaling in Xenopus (13). Mechanistically, FRAT/GBP prevents binding of Axin to GSK-3 (17–21) and can also induce dephosphorylation of GSK-3 on the dorsal side of the embryo (33). It has been proposed that activation of the Wnt signaling cascade causes Dvl to recruit FRAT/GBP into the β-catenin degradation complex, leading to dissociation of GSK-3 from Axin and consequently to stabilization of β-catenin (17, 34). More recently, it was reported that the activation of Wnt signaling by FRAT1 is mediated through its interaction with the Wnt co-receptor LR5 (31). However, a study by van Amerongen et al. (35), which used triple-knock-out mice lacking all three murine Frat homologs, demonstrated that Frat is dispensable for Wnt/β-catenin signaling in mammals. PKA has not been implicated directly in the transduction of the Wnt signal. We also could not detect any change in FRAT1 phosphorylation at Ser188 after stimulation of the canonical Wnt pathway by means of cotransfection of Wnt3a or Dvl2 (data not shown). Thus, PKA-mediated FRAT phosphorylation is unlikely to play a role in transducing the Wnt signal but may regulate GSK-3 activity toward β-catenin in a Wnt-independent manner. Alternatively, FRAT1 phosphorylation may also regulate GSK-3 activity toward other cellular substrates.

Ser188 as well as the PKA consensus at this site are conserved in the mammalian FRAT1 homologs, FRAT2 (Thr199 in human FRAT2) and Frat3 (Ser181 in mouse Frat3), suggesting that PKA-mediated phosphorylation of FRAT and modulation of its activity is a general mechanism in mammals. However, a homologous residue to Ser188 is absent in Xenopus and zebrafish GBP. Thus, regulation of FRAT activity by PKA would be a mechanism that is specific to mammals and may reflect the evolution of additional levels of regulation of FRAT1 function necessary in mammalian cells.

PKA is also known to phosphorylate GSK-3β at Ser9, resulting in inhibition of its catalytic activity (36, 37). Thus, PKA may regulate GSK-3 activity at multiple levels. PKA-mediated phosphorylation of GSK-3β at Ser9 would have the opposite consequence compared with PKA-dependent FRAT1 phosphorylation. However, GSK-3 is known to exist in different pools in the cell. For instance, insulin, which inhibits GSK-3β via Ser9 phosphorylation, leading to activation of glycogen synthase, does not stabilize β-catenin, whereas Wnt-dependent GSK-3β inhibition is not mediated through Ser9 phosphorylation and does not activate glycogen synthase activity (11, 12). FRAT/GBP has also been shown to only inhibit the activity of GSK-3 toward specific substrates (19, 20, 22), suggesting that the phosphorylation of FRAT1 described in this study affects only specific GSK-3-directed phosphorylation events. A GSK-3-binding protein, p24, which is unrelated to FRAT/GBP but also inhibits the catalytic activity of GSK-3, has recently been identified (38). Interestingly, p24 is also a substrate for PKA, and similar to what we have observed with FRAT1, phosphorylation of p24 by PKA reduces its inhibitory activity toward GSK-3 (38). In addition, PKA-mediated phosphorylation may also regulate other functions of FRAT, such as regulation of GSK-3 stability and nuclear export or its binding to kinesin light chains (30, 33, 39).

In summary, we identified PKA as a FRAT1 kinase in vitro as well as in intact cells. Phosphorylation of FRAT1 by PKA may be a mechanism by which FRAT activity toward GSK-3 is regulated in a Wnt-independent manner.
