Phosphorylation of Supernatant Protein Factor Enhances Its Ability to Stimulate Microsomal Squalene Monooxygenase*

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Supernatant protein factor is a 46-kDa cytosolic protein that stimulates squalene monooxygenase, a downstream enzyme in the cholesterol biosynthetic pathway. The mechanism of stimulation is poorly understood, although supernatant protein factor belongs to a family of lipid-binding proteins that includes Sec14p and α-tocopherol transfer protein. Because recombinant human supernatant protein factor purified from Escherichia coli exhibited a relatively weak ability to activate microsomal squalene monooxygenase, we investigated the possibility that cofactors or post-translational modifications were necessary for full activity. Addition of ATP to rat liver cytosol increased supernatant protein factor activity by more than 2-fold and could be prevented by the addition of inhibitors of protein kinases A and C. Incubation of purified recombinant supernatant protein factor with ATP and protein kinases A or C8 similarly increased activity by more than 2-fold. Addition of protein phosphatase 1α, a serine/threonine phosphatase, to rat liver cytosol reduced activity by 50%, suggesting that supernatant protein factor is partially phosphorylated in vivo. To determine whether dietary cholesterol influenced the phosphorylation state, cytosols were prepared from livers of rats fed a high fat diet. Although supernatant protein factor activity was reduced by more than one-half, it could not be restored by the addition of ATP or protein kinase C8 with ATP, suggesting that dietary cholesterol reduced the expression of this protein. Supernatant protein factor thus appears to be regulated both post-translationally through phosphorylation and at the level of expression. Phosphorylation may provide a means for the rapid short term modulation of cholesterol synthesis.

The regulation of cholesterol biosynthesis beyond HMG-CoA reductase is relatively poorly characterized despite its importance in mammalian physiology. The steps that follow the formation of squalene in mammalian somatic cells typically yield only cholesterol, and squalene monooxygenase catalyzes the second and potentially rate-limiting step in this downstream or committed pathway for cholesterol synthesis. In addition to being regulated at the transcriptional level by sterols (1, 2), squalene monooxygenase activity is stimulated by supernatant protein factor (SPF), a cytosolic 46-kDa protein first characterized in Konrad Bloch's laboratory (3). SPF belongs to a family of lipid-binding proteins that includes Sec14p, α-tocopherol transfer protein, and cellular retinol-binding protein (4). The recently reported crystal structure of human SPF confirms this relationship (5). The mechanism by which SPF stimulates squalene monooxygenase has not been established; most evidence suggests that SPF facilitates the transfer of squalene into and between membrane compartments in the cell (4, 6, 7), although SPF has not been shown to bind squalene. SPF in vitro is only effective with membrane-bound (microsomal) squalene monooxygenase consistent with a lipid transfer function, and has no effect on the purified enzyme (8).

Recent studies from Shibata et al. (4) revealed that the overexpression of SPF in hepatoma cells increases cholesterol synthesis by 2-fold. These findings suggest that SPF may have a role in regulating cholesterol synthesis in vivo. The regulation of SPF itself has not been described, although two groups (9, 10) have reported that a cholesterol-supplemented diet did not alter SPF activity in rat liver cytosol. Unexpectedly, SPF is identical to tocopherol-associated protein (13), a recently identified protein that is thought to be involved in the intracellular processing of α-tocopherol (14). Tocopherol-associated protein binds α-tocopherol with high affinity and appears to act as a tocopherol-dependent transcription factor (15). The gene targets of this protein have not been identified, but it is intriguing that α-tocopherol down-regulates the expression of the cholesterol scavenger receptors SR-A (16) and CD36 (17). It should also be noted that the rat contains a second SPF-like gene of unknown function that is highly expressed in epithelial tissue (most notably the olfactory epithelium) and that the encoded protein exhibits high affinity for GTP (11, 12).

The present studies were undertaken to better understand the role SPF plays in influencing cholesterol synthesis and squalene monooxygenase activity. A DNA clone to human SPF was generated by polymerase chain amplification, and the protein was expressed in Escherichia coli. Because the purified protein demonstrated unexpectedly weak activation of microsomal squalene monooxygenase, we explored the possibility that post-translational modifications to SPF might be necessary for full activity.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Human SPF and Associated Proteins—The SPF cDNA was amplified with Pfu polymerase (Stratagene) from a human liver cdNA library (Quick-Clone, Clontech) with primers based on the reported human SPF sequence (4) and that incorporated an NcoI restriction site at the translation start codon and an XhoI restriction site immediately following the stop codon. The amplification product

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was cloned into the PCR-BLUNT II TOPO vector (Invitrogen), plasmid DNA was isolated, and the sequence of the insert was determined at the Molecular Structure and Analysis Facility, University of Kentucky, to confirm identity with SPF. The SPF cDNA insert was released with NcoI and XhoI, purified by agarose-gel electrophoresis, and cloned into the pTBY4 expression vector (New England Biolabs). SPF protein was purified following the protocol for expression of intact fusion proteins with the IMPACT T7 system as follows. SPF expression was induced in E. coli ER2566 cells overnight with 1 mM isopropyl-β-D-thiogalactosidase at 30 °C with slow shaking. All subsequent steps were carried out at 4 °C. Cells were broken in a French pressure cell in buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 0.1 mM EDTA, and the lysate was cleared by centrifugation for 30 min followed by digestion with 1 µg of DNA nuclelease for 1 h. The cleared lysate was loaded onto a chitin affinity column (2-ml bed volume), washed with 40 ml of lysis buffer, and incubated overnight at 4 °C in 4 ml of buffer containing 30 mM β-mercaptoethanol to promote cleavage of the intein-SFP bond. SPF was eluted with 10–20 ml of the same buffer, which was then replaced by centrifugal dialysis with 20 mM Tris-HCl, pH 7.4, and the sample was stored at -80 °C. The purified protein retains four amino acids (Leu-Glu-Pro-Gly) at the C terminus that are derived from the intein fusion sequence. Recombinant human squa-lene monooxygenase, foreshortened at the N terminus to facilitate expression, and rat cytochrome P450 reductase were expressed in E. coli and purified as described (18), excluding Triton X-100 from the lysis and purification buffers. Protein was quantified with the Coomassie Plus assay reagent kit (Pierce).

**SF/SPF Monooxygenase Activity Assays**—The micromolar frac- tion (100,000 × g pellet, ~15 mg of protein/ml) and cytosolic fraction (100,000 × g supernatant, ~20 mg of protein/ml) were prepared from the livers of untreated male Harlan Sprague-Dawley rats (~200 g) by standard procedures. Animals were maintained on a normal light-dark cycle with free access to rat chow and were killed in late morning by decapitation. Squalene monooxygenase activity in rat liver microsomes was determined on the basis of the procedure described by Wagner et al. (19) with 200 µg of microsomal protein, 30 µM FAD, 40 µM [14C]squalene, 10 µg of phosphatidylycerol, and 0.3 mM AMO 1618 (Calbiochem) to test for radiomicrosomal squalene cyclase in 200 µl of 200 mM Tris-HCl buffer, pH 7.4, with 1 mM EDTA. Reactions were started by the addition of NADPH to 1 mM, incubated in a 37 °C water bath for 1 h, and were stopped by the addition of 0.5 ml of 10% KOH in methanol after the incubation volume was brought to 1 ml with water. The tubes were capped, and after saponification at 80 °C for 1 h the neutral lipids were extracted with 3 ml of petroleum ether. The solvent was removed under evaporative centrifugation, and the lipids were resuspended in 50 µl of the same and spotted onto silica thin-layer plates. Lipids were fractionated in 5% ethyl acetate in hexane and visualized and quantified by electronic autoradiography (Packard Instant Imager). Radiolabeled squalene (55 Ci mmol⁻¹) was synthesized by the Chemical Synthesis Facility, Department of Medicinal Chemistry, University of Utah, at 7 µCi/mmole.

SPF activity (the ability to increase microsomal squalene monooxygenase activity) was determined by adding either rat liver cytosol to the above reactions at a ratio of 5 to 1 (cytosol:microsomes) on a per µg of protein basis unless otherwise indicated or with 0.8 µg of purified recombinant human SPF (unless otherwise indicated). Maximal activa-tion of microsomal squalene monooxygenase was determined by adding Triton X-100 to 0.1% final concentration and was used as a reference for the cytosolic and recombinant SPF assays. Activity assays with purified squalene monooxygenase and cytochrome P450 reductase were carried out as described (18).

**Activation of SPF by ATP**—The ability of ATP and other nucleotides to activate SPF was determined by preincubating cytosol (2 mg of protein) or 5 µg of recombinant SPF with 3 mM ATP or other nucleotide in the presence of 4.5 mM MgCl₂ for 30 min at 37 °C on the basis of the procedure of Senjo et al. (20). Unbound nucleotide was then removed by centrifugal filtration, and the SPF preparation was resuspended in Tris buffer. The ability to stimulate squalene monooxygenase was deter-mined as described above. When included, protein kinase inhibitors were added before the addition of ATP at the following concentrations: staurosporine, 50 nM; 4-cyano-3-methylisooquinoline (CMI), 30 nM; protein kinase A inhibitor–(6–22)–amide (PRAI), 2 mM; bisindolylmaleimide I (BIM-I), 20 mM; rottlerin, 10 µM; H-8, 500 nM; KT5823, 300 nM; AG 213, 5 µM. All inhibitors were obtained from Calbiochem.

**Stimulation of SPF Activity by Protein Kinases**—The ability of various protein kinases to activate SPF was determined by adding human protein kinase 1, γ isoform (0.1 unit), λ protein phosphatase (APF, 200 units), or Verrinia enterococlitica protein-tyrosine phosphatase (100 units), to cytosol (2 mg of protein) and incubating at 37 °C for 30 min in 50 mM Tris, HCl, pH 7.5. 0.1 mM EDTA, 5 mM β-glycerophosphate, and 2 µl of 10 mM MnCl₂ in accord with the instructions for use provided by the supplier (Calbiochem). The ability of each mixture to stimulate squalene monooxygenase was determined as described above.

**Dietary Manipulation of SPF Activity**—Five male Harlan Sprague-Dawley rats (250–300 g, final weight) were fed a high fat diet (15.6% fat, Research Diets Inc., D12266B) containing 0.1% cholesterol for 10 weeks. A second group of five rats was fed the same diet for 16 weeks, followed by 1 week on standard rodent chow (Teklad Global 2018, containing 5% fat). Livers were removed after decapsulation, and microsomes and cytosol were prepared for the determination of squalene monooxygenase and SPF activities.

**RESULTS**

**Cloning and Purification of Human SPF**—The published sequence of human SPF (4) was used to design primers for the amplification of SPF from a human liver cDNA library. The resulting cDNA exhibited two differences from the published sequence: at codon 2, where incorporation of an NcoI site into the primer to facilitate cloning replaced a serine with glycine; and at codon 11, where a lysine was encoded instead of arginine. A lysine is also found at this latter position in the rat sequence (4). Expression and purification of SPF from E. coli yielded a largely homogeneous protein that migrated at 45 kDa, consistent with the predicted molecular mass of 46 kDa (Fig. 1).

**Stimulation of Squalene Monooxygenase**—Triton X-100 (0.1%) activates microsomal squalene monooxygenase by ~10-fold and is required for activity with the purified enzyme in a reconstituted system (8, 18, 21). Consistent with these find-ings, purified recombinant SPF was unable to replace Triton X-100 in assays with the purified recombinant proteins squalene monooxygenase and cytochrome P450 reductase. Recombinant SPF did stimulate rat liver squalene monooxygenase in microsomes, although the extent of stimulation (~2-fold) was significantly less than that obtained with Triton X-100 (Fig. 2). The addition of phosphatidylycerol increased the stimulation by recombinant SPF as has been shown with purified native AMP-kinase, 0.1 unit (Upstate USA, Inc.) according to the supplier’s instructions. Each kinase was incubated with either purified recombinant SPF (5 µg) or cytosol (2 mg of protein) for 30 min at 30 °C. The ability of each mixture to stimulate squalene monooxygenase was determined as described above.
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SPF (22), but the stimulation was still less than that obtained with rat liver cytosol.

The inability of recombinant SPF to match the stimulation obtained with cytosol raised the possibility that the bacterial protein was not fully active and that native SPF contained bound cofactors or was post-translationally modified. The rat expresses a second protein closely related to SPF, which has been reported to bind GTP (11), and both SPF and this related protein contain a putative nucleotide binding domain at the C terminus (13). Moreover, Senjo et al. (20) reported that the preincubation of rat liver cytosol or partially purified SPF with 3 mM ATP for 30 min prevented the stimulation of microsomal squalene monooxygenase by these preparations. However, the addition of 3 mM GTP, GDP, or ATP to our purified recombinant human SPF had no effect on its ability to stimulate squalene monooxygenase (data not shown). Tocopherol-associated protein is identical to SPF and has been shown to bind α-tocopherol with high affinity (14, 15). The addition of 50 μM α-tocopherol to recombinant SPF similarly had no effect on its ability to stimulate squalene monooxygenase either in the presence or absence of GTP, GDP, or ATP. Although these studies do not rule out the possibility that SPF binds a nucleotide or α-tocopherol, it appears that they do not influence the ability of the recombinant protein to stimulate squalene monooxygenase.

Activation of SPF—To address the possibility that nucleotide binding by SPF required additional cytosolic components, we added various nucleotides to a rat liver cytosol preparation. In contrast to the inhibition obtained by Senjo et al. (20), the addition of ATP increased the ability of cytosolic SPF to stimulate squalene monooxygenase by more than 2-fold (Fig. 3). Other nucleotides, including GTP, were ineffective. The inability of ADP and ATPγS, a nonhydrolyzable form of ATP, to activate SPF suggested that SPF was a substrate for an ATP-dependent protein kinase and that phosphorylation activated SPF. This ATP-dependent activation was not affected by the presence of GTP or α-tocopherol, and the addition of ATP to microsomes in the absence of cytosol had no effect on squalene monooxygenase activity (data not shown).

To determine whether phosphorylation was occurring, we tested the ability of a variety of protein kinase inhibitors to block the activation of SPF by ATP (Fig. 4). Staurosporine, a broad spectrum inhibitor of protein kinases A, C, and G, greatly reduced the activation of SPF by ATP. Two inhibitors that are relatively specific for PKA (CMI and PKAI) and BIM-I (which is relatively specific for PKC isoforms) also were effective inhibitors of activation. Inhibitors of PKG (H-8 and KT5823) and AG 213, a broad range tyrphostin inhibitor of protein-tyrosine kinases, had no effect on the activation of SPF. These results suggest that phosphorylation by protein kinases A or C may be responsible for the activation of SPF in rat liver cytosol.

To determine whether recombinant SPF could be activated by phosphorylation, the purified protein was incubated with various protein kinases in the presence of ATP (Fig. 5). PKA and PKCδ increased SPF activity by more than 2-fold, similar to the activation obtained by the addition of ATP to cytosol. PKC isoforms α, β1, and β2 were also able to activate recombinant SPF, whereas PKG and AMP-activated protein kinase, which has been shown to down-regulate the activity of HMG-CoA reductase (23), were ineffective. The addition of GTP, GDP, or α-tocopherol to incubations with PKCδ did not affect the activation of SPF, indicating that these potential ligands do not impair or augment phosphorylation by this protein kinase. The addition of protein kinases with ATP to microsomes alone had no effect on squalene monooxygenase activity. These results support a role for protein kinase A or C in the activation of SPF in vitro and possibly in vivo.

To determine whether SPF was phosphorylated in isolated liver preparations, cytosol was incubated with several protein phosphatases, and the ability of the treated preparations to stimulate squalene monooxygenase was determined. Human
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Effect of Dietary Fats on SPF Activity—To determine whether SPF activity was regulated by dietary cholesterol, rats were fed a high fat diet (15.6% fat, 0.1% cholesterol) for 10 weeks, and SPF activity in liver cytosol was compared with that of rats on a normal chow diet. The high fat diet reduced SPF activity by half and could be restored by returning the rats to a chow diet for 1 week (Fig. 7). Similarly, squalene monooxygenase activity was reduced by half and could be restored by refeeding a chow diet. SPF activity from the fat-fed animals could not be increased by incubation with ATP or by adding ATP with PKC isoforms to the cytosol, suggesting that the lower activity was caused by a decrease in the expression of SPF. The expression of squalene monooxygenase has previously been shown to be regulated by dietary lipids via a transcriptional mechanism (1, 10).

DISCUSSION

Supernatant protein factor has been a puzzle for more than 25 years, and this mystery has deepened with recent observations that overexpression of SPF in hepatoma cells increases cholesterol synthesis (4) and that SPF is identical to a vitamin E-binding protein of unknown function (13, 14). In the present studies, the expression of human supernatant protein factor in bacteria yielded a purified protein with weaker than expected biological activity that could be blocked by the prior addition of inhibitors of protein kinases A and C, which argues that cytosolic SPF is a substrate for one or more serines or threonines. These results are consistent with the protein kinase experiments in which the activation of SPF was inhibited by serine/threonine kinase inhibitors (staurosporine) and catalyzed by serine/threonine kinases (PKA and PKC).

Fig. 5. Activation of recombinant SPF by phosphorylation. Purified recombinant SPF was incubated in the absence (−) or presence of the indicated protein kinases and ATP, and the ability of the preparations to stimulate squalene monooxygenase was determined as described under “Experimental Procedures.” PKC isoforms are indicated by their Greek letter assignments; AMPK, AMP-activated protein kinase. Each value represents the mean ± S.E. of two experiments carried out in duplicate.

Fig. 6. Deactivation of SPF by protein phosphatases. Rat liver cytosol was incubated with human protein phosphatase 1γ (PP1), λ protein phosphatase (λPP), or protein-tyrosine phosphatase (PTP) and the ability of the treated preparations to stimulate squalene monooxygenase was determined as described under “Experimental Procedures.” Each value represents the mean ± S.E. of three experiments carried out in duplicate.

Fig. 7. Down-regulation of SPF and squalene monooxygenase by a high fat diet. The effect of dietary fat on SPF and squalene monooxygenase activities was determined by feeding rats a high fat diet as described under “Experimental Procedures”; refed animals were returned to a standard chow diet for 1 week before assay. SPF activity in the cytosol of treated animals was determined with micosomes from untreated animals (left axis, gray bars); squalene monooxygenase activity in microsomes from treated animals was determined with 0.1% Triton X-100 for activation (right axis, solid bars). Each value represents the mean ± S.E. of five animals analyzed in duplicate.
Protein kinase A was also very effective in activating recombinant SPF, and the PKA inhibitors CMI (30) and PKAI, an inhibitory peptide specific to this kinase, were effective in preventing SPF activation in cytosol. PKA has not previously been implicated in the regulation of cholesterol synthesis, although it plays a prominent role in the activation of cholesterol transport into mitochondria for steroligenesis (31). PKA has also been implicated in the up-regulation of expression of the low density lipoprotein receptor (32). PKA is a cAMP-dependent kinase and thus can be activated by the many signaling pathways that activate adenylyl cyclase. The present studies do not allow us to determine which protein kinase (A or C) is active in isolated rat liver cytosol, because the kinase inhibitors CMI and PKAI, which block PKA, and BIM-I, which blocks PKC, all blocked SPF activation despite being used at concentrations reported to allow selective inhibition (30, 33, 34). The limited specificity of protein kinase inhibitors has previously been discussed (35). As noted above, the results with purified recombinant SPF indicate that full activation requires phosphorylation by both kinases (Fig. 8).

Protein phosphatase 1, the principal serine/threonine phosphatase in liver, is activated by a variety of regulatory proteins that respond to glucagon, insulin, and glucocorticoids and has been implicated in cholesterol homeostasis (36) and steroligenesis (37). Protein phosphatase 2A is also present in liver and is responsible for the dephosphorylation of HMG-CoA reductase (38). In the present study protein phosphatase 1 reduced SPF activity in cytosol by ~50%, indicating that SPF is partially phosphorylated in this preparation. Although this finding suggests that SPF is partially phosphorylated in vivo, predicting that the actual phosphorylation state of SPF in the intact liver by this approach is limited by the possible activation of kinases and phosphatases during isolation of the subcellular fractions. A similar problem was encountered in the analysis of HMG-CoA reductase inactivation by AMP-dependent protein kinase and was solved by rapid cooling of the liver during preparation (39). Although this technique was not employed in the present studies, these results nonetheless strongly suggest that phosphorylation of SPF is physiologically relevant and occurs in the intact liver of normal animals. Partial phosphorylation under normal conditions would allow rapid up-regulation and down-regulation of squalene monoxygenase activity in response to changing sterol levels or other stimuli.

In an effort to identify physiologic conditions that alter the phosphorylation state of SPF, we obtained livers from rats that had been maintained on a high fat diet. This diet decreased squalene monoxygenase activity by approximately 50% as compared with animals on a standard chow diet, consistent with earlier reports (9, 10). This diet also decreased SPF activity in contrast with earlier results from these two groups (9, 10); this may reflect differences in the dietary treatment regimens. However, the decrease in SPF activity was not caused by a decrease in the phosphorylation state of SPF; in fact, SPF appeared to be fully phosphorylated in these preparations because the cause of the decrease with ATP treatment still remains to be elucidated.

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