Identification of a New Acute Phase Protein*

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We have previously reported mouse SIP24 protein as a secreted inducible protein produced by quiescent Balb/c 3T3 cells. SIP24 can be produced in response to many factors, including serum, basic fibroblast growth factor, prostaglandin F2α, phorbol ester, and dexamethasone. Here we present evidence to show that SIP24 is the product of mouse 24P3 mRNA. The 24P3 cDNA was originally cloned from an SV40-transformed quiescent mouse primary kidney cell culture, and it has been classified as a new member of the lipocalin protein family. We show that the SIP24/24P3 protein and mRNA increase dramatically in mouse serum and liver during the acute phase response induced by turpentine injection. Tissue sectioning studies revealed that SIP24/24P3 is mainly expressed in liver during the acute phase response. SIP24/24P3 was also detected in the brain and the uterus. In mouse BNL (Balb/c normal liver) cells, the production of SIP24/24P3 is stimulated by tumor necrosis factor α, which is a major regulator of the expression of other acute phase proteins. From its pattern of regulation, we conclude that SIP24/24P3 is a new type 1 acute phase protein.

The acute phase response (APR) is a complex reaction to various inflammatory or stressful stimuli such as surgery, wounding, bacterial or virus infection, or elevated levels of stressful and tissue-damaging agents. During this mammalian stress response, the plasma levels of a group of proteins change rapidly. These proteins are called the acute phase proteins (APPs; Refs. 1–3). Those proteins whose plasma levels increase are called positive APPs; examples include C-reactive protein, α1-acid glycoprotein (AGP), α2-macroglobulin, and haptoglobin. The plasma levels of negative APPs decrease in response to inflammation or other invasive stress; examples include retinol-binding protein and albumin. The APPs are synthesized mainly in the liver and are secreted into the bloodstream (4). The precise functions of many APPs are still largely unknown. It is generally believed that the APPs play an anti-inflammatory role to prevent ongoing tissue damage and to return the organism to normal function. The known functions of APPs can be classified into three categories, including the maintenance of homeostasis, the transport of a variety of factors, and defense against infection (3).

The regulation of hepatic APP production is mediated by several classes of factors (2, 3, 5). First, the interleukin-1-type cytokines, which include interleukin 1α, interleukin 1β, tumor necrosis factor α, and tumor necrosis factor β, induce type-1 APPs such as human C-reactive protein and rat haptoglobin. The interleukin-1-like cytokines also decrease the expression of type 2 APPs. Second, the interleukin 6-type cytokines, which include interleukin 6 (IL-6), interleukin 11, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor induce primarily type 2 APPs such as rat α2-macroglobulin. Third, glucocorticoids are believed to play a permissive role during the APR by themselves they cause small increases in the production of most APPs but strongly enhance the effect of cytokines on most APPs. Fourth, growth factors such as FGF, insulin, transforming growth factor β, and hepatocyte growth factor regulate APPs in a way similar to glucocorticoids. Experimentally, the APR can be induced by injection of animals with inflammatory agents such as turpentine (1, 6).

We have reported previously the induction, characterization, and partial peptide sequencing of a superinducible protein, SIP24, produced by quiescent Balb/c 3T3 mouse fibroblast cells (7–9). SIP24 is a 24-kDa secreted glycoprotein induced by serum, basic fibroblast growth factor (bFGF), prostaglandin F2α (PGF2α), phorbol ester, and dexamethasone. It is superinducible because it can be further induced in a synergistic manner with growth factors if the cells are pretreated with the protein synthesis inhibitor, cycloheximide. We have identified SIP24 as the protein product of the mouse 24P3 mRNA, whose cDNA was originally cloned from SV40-infected mouse kidney primary cell cultures (10). The 24P3 protein is also a major secretory protein of cultured mouse P5.1.8 macrophage cells which have been stimulated by lipopolysaccharide (11).

Based on the amino acid sequence deduced from its cDNA, Flower et al. (12) identified 24P3 as a new member of the lipocalin protein family. Proteins closely related in sequence to 24P3 are rat α2-microglobulin-related protein and human neutrophil gelatinase-associated lipocalin (12, 13). The lipocalin family is mainly composed of extracellular ligand binding proteins with high specificity for small hydrophobic molecules. Examples of lipocalin family members include α1-acid glycoprotein (AGP), α2-microglobulin, plasma retinol binding protein, and β-lactoglobulin. Some lipocalin proteins, including AGP, α2-microglobulin, and retinol-binding protein, are also APPs, and they can be regulated by glucocorticoids in vitro and in vivo (2, 3). Here we present evidence to show that SIP24/24P3 is highly induced during the APR in vivo, and it is mainly expressed in liver during the APR. SIP24/24P3 is also expressed in the brain and the uterus. Moreover, the expression of SIP24/24P3 can be regulated in cultured cells by TNF-α which is a
Identification of a New Acute Phase Protein

EXPERIMENTAL PROCEDURES

Materials—Dexamethasone and cycloheximide were purchased from Sigma. For studies with cultured cells, the dexamethasone was dissolved in ethanol as a 1,000-fold concentrated stock. The dexamethasone stocks used for injection into animals were prepared from Sigma. For studies with cultured cells, the dexamethasone was dissolved in ethanol as a 1,000-fold concentrated stock. For injection into animals, the stock was further diluted 1,000 fold in water and given subcutaneously. The stock for dexamethasone used for inducing the APR in mice was purchased from E. E. Zimmer.

Cell Culture and Preparation of Medium Containing SIP24—Balb/c 3T3 cells or BNL cells were plated on plastic dishes in DME containing 10% calf serum, 100 units/ml of penicillin and 100 units/ml of streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 10% CO2 in air.

Cell Culture—Stock cultures of mouse Balb/c 3T3 and BNL cells were grown in Dulbecco-Vogt's modified Eagle's (DME) medium, calf serum, penicillin, and streptomycin were from Life Technologies, Inc. Dulbecco-Vogt's modified Eagle's (DME) medium, calf serum, penicillin, and streptomycin were from Life Technologies, Inc.

Induction of the Acute Phase Response in Mice—Turpentine or 0.9% sterile saline (5 ml/kg body weight) was injected intramuscularly into 5-week-old to 6-month-old male and female CF-1 mice. Dexamethasone or the vehicle alone (5 mg/kg body weight) were injected intraperitoneally. Twelve hours after injection, blood samples from each mouse were taken by tail-bleeding. Twenty-four hours after injection, the mice were sacrificed by cervical dislocation. Blood samples were taken immediately by heart puncture and incubated at 37°C for 1 h. A spatula was used to detach the clots from the walls of the tube every 15 min. The samples were then centrifuged in a microcentrifuge for 5 min at room temperature. The supernatants (sera) were collected for later Western blot analysis. From the same animal, the liver and other organs were quickly collected, frozen in liquid nitrogen, and stored at −70°C prior to RNA extraction.

RNA Analysis—For extraction of total RNA, organs were homogenized in 4°C glycine-isoosmotic bovine serum, and centrifuged through CsCl (17). Northern blot analysis was carried out by separation of 15 µg of total RNA on 0.9% agarose gel containing formaldehyde. The contents of the gel were transferred to nylon membranes and hybridized to a 32P-labeled 24P3 5′ region cDNA probe (440 base pairs), a mAGP coding region cDNA probe (700 base pairs), and a rat APP coding region cDNA probe (750-base pair EcoR1/PstI fragment). The membranes were stripped between hybridization with different probes. The RNA band was visualized by autoradiography. The results were quantitated by using a PhosphorImager (Molecular Dynamics). Tests of the quantitative nature of the data obtained from the PhosphorImager showed that the amount of radioactivity reported by the instrument was linear with respect to that determined by scintillation spectrometry over a range of 0 to 2 x 106 cpm per band.

Peptide Sequence Analysis—SIP24 was purified and sequenced as described previously (9).

Metabolic Labeling of SIP24/24P3—BNL cells were grown to quiescent confluence with either 10% or 0.2% calf serum and the serum was replaced with fresh DME. The cells were incubated for 4 h by incubation with 100 mCi/ml Tran35S-label in DME (with 10% of the normal methionine concentration), 0.2% calf serum, and the same additions as present during the previous 24-h incubation. Details of this procedure have been described (7–9, 15). The samples of medium were resolved by SDS-PAGE, and the gels were impregnated with 2,5-diphenyloxazole and exposed to film. The relative amounts of SIP24/24P3 produced under each test condition were quantitated by densitometric analysis of the resulting fluorograms. The results from densitometry which were proportional to the relative amount of radioactive label associated with each protein band were normalized to the trichloroacetic acid-precipitable counts/min in the postnuclear supernatant of the cell population corresponding to the sample of conditioned medium. In this way, the rate of incorporation into each secreted protein was normalized to the overall rate of protein synthesis.

RESULTS

SIP24 Shares Extensive Identity with the Deduced Amino Acid Sequence of 24P3—We have previously reported the purification and partial sequencing of SIP24 (9). In those studies, four peptides from a clostripain-digested purified SIP24 were chosen to sequence and to compare with GenBank and EMBL data bases. No identical sequence was found. The longest peptide (peptide B) showed 94% identity with mouse cyclophilin, and other peptides showed some identity with cyclophilin. From these results, SIP24 was thought to be a cyclophilin-like protein.

The sequences of seven peptides obtained from a clostripain digestion of the purified SIP24 protein were compared with the sequences found in an updated SwissProt data base. All peptide sequences except peptide B were found to share identity with the deduced amino acid sequence of 24P3 cDNA (Fig. 1). The six SIP24 peptides that are identical with 24P3 cover 25% of the complete 24P3 sequence and are spread over the entire 24P3 coding region. The 24P3 cDNA was cloned by Hraba-Renevey et al. (10) from quiescent mouse kidney primary cell cultures infected by SV40. 24P3 mRNA can also be induced by serum in 3T3 fibroblast cells as can the SIP24 protein (7, 10). These findings suggested that SIP24 is the product of the 24P3 mRNA, and that peptide B containing the cyclophilin-like sequence came from a contaminating protein in the SIP24 preparation. As judged by the area under the peak B peak after major mediator of the APR. Our results also show that SIP24/24P3 is a type 1 APP.
Identification of a New Acute Phase Protein

**Fig. 2. Recognition of SIP24 by antisera raised against 24P3 by Western blot analysis.** Quiescent Balb/c 3T3 cells were incubated with or without 1 mg/ml cycloheximide and 400 ng/ml dexamethasone (DEX/CHX) in serum-free DME medium for 18 h. The media were concentrated and resolved by SDS-PAGE, and the Western blots were stained with preimmune serum (PI), anti-SIP24 (α-SIP24), or two preparations of anti-24P3 sera (37f, 37t) either alone or as a 1:1 mixture. The Western blot was visualized by using the Stratagene picoBlue alkaline phosphatase kit. Arrows on the left indicate the positions of the molecular weight markers expressed in thousands, from top to bottom: bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), myoglobin (18,000), and cytochrome c (12,500).

**Fig. 3. SIP24/24P3 is an acute phase protein.** A. Northern blot of saline- and turpentine-injected mouse liver total RNA. Male and female mice were injected with 0.9% sterile saline (Saline) or turpentine (Turpentine). Twenty-four hours after injection, total RNAs were extracted from the livers and resolved by agarose gel electrophoresis. The resolved RNAs were transferred to nylon membranes and hybridized sequentially with 32P-labeled 24P3, mAGP, and rat 18S rRNA cDNA probes. The same experiment was performed twice, and a total of 12 mice (6 male and 6 female) were treated under each condition. Numbers on the right show the positions of the 18S and 28S rRNA markers and the estimated molecular weight of the 24P3 mRNA. B. Western blot analysis of sera from saline- and turpentine-injected mice. The same male and female mice were used as for A. Serum samples were prepared 12 and 24 h after injection of saline or turpentine. Samples (30 μl) of 2.5% sera were resolved by SDS-PAGE and blotted against preimmune serum (PI) and anti-SIP24 serum (α-SIP24) as described under "Experimental Procedures." A sample of SIP24-enriched cell culture medium as used in Fig. 2 was included as positive control (C). Numbers on the left indicate molecular weight markers in thousands.

Reverse-phase high performance liquid chromatography, the molar ratio of the cyclophilin-like peptide to the other peptides in the clostripain digests was less than 0.4 (the sequencing of this peptide did not go to completion). Although such a molar ratio could have arisen because a larger proportion of peptide B was lost during the procedure, it is also consistent with peptide B having been derived from a contaminating protein.

Anti-SIP24 and Anti-24P3 Immune Sera Recognize the Same Protein—To test the hypothesis that SIP24 and 24P3 are the same protein, we performed Western blot analyses with anti-SIP24 and two preparations of anti-24P3 antisera raised against the 24P3 protein expressed in Escherichia coli. The concentrated control and SIP24-containing media were blotted with either anti-SIP24 or anti-24P3 sera. The antibodies raised against 24P3 detected the same protein band in the SIP24-enriched medium as did anti-SIP24 serum (Fig. 2). To rule out the possibility that anti-SIP24 and anti-24P3 sera recognized two different proteins of similar apparent size, a mixture of anti-SIP24 and anti-24P3 sera was also tested, and the result showed a single stained band (Fig. 2). Therefore, the results of Western blot analysis support the prediction from the analysis of SIP24-derived peptide sequences that SIP24 protein and 24P3 mRNA are derived from the same or from two very closely related genes.

24P3 mRNA and SIP24 Protein Both Increase Dramatically during the Acute Phase Response—To test the hypothesis that SIP24 and 24P3 are an acute phase protein, we induced the APR in male and female mice by injection with turpentine. Twenty-four hours after injection, total RNA was extracted from mouse liver and analyzed by Northern blot. The 24P3 cDNA probe recognized a uniform band corresponding to an mRNA of about 1.0 kilobases, which was in perfect agreement with the previously reported size of 24P3 mRNA (10). The 24P3 mRNA level was very low or undetectable in the livers of un.injected (data not shown) or of saline-injected mice, whereas the livers of both male and female mice injected with turpentine had increased levels of this mRNA (Fig. 3A). The relative amounts of 24P3 mRNA were determined after normalizing the values for band densities of 24P3 mRNA to the band densities of 18S rRNA on the same blots. The average ratio of normalized 24P3 mRNA in the turpentine-injected mice over normalized 24P3 mRNA in the control mice was 416 ± 117 (n = 12). The membranes were also hybridized with the mAGP cDNA probe. The ratio of normalized mAGP mRNA in the livers of turpentine-injected versus saline-injected animals was calculated to be 12 ± 5 (n = 6). This result matches well the 10- to 20-fold increase in mAGP mRNA after turpentine injection which was reported by Prowse and Baumann (18).

To determine the effect of turpentine injection on the level of SIP24/24P3 in the bloodstream, sera from saline- and turpentine-injected mice were collected 12 and 24 h after injection, and SIP24 was detected by Western blot analysis. As in the results for the Northern blots of 24P3 mRNA, SIP24 protein was undetectable in sera from saline-injected mice. However,
Identification of a New Acute Phase Protein

**Fig. 4. Northern blot analysis of total RNA isolated from the livers of vehicle- and dexamethasone-injected mice.** Male and female mice were injected with 2-hydroxypropyl-β-cyclodextrin (C) or dexamethasone in 2-hydroxypropyl-β-cyclodextrin (DEX). Twenty-four hours after injection, total liver RNAs were extracted and resolved by agarose gel electrophoresis. The RNAs were then transferred to a nylon membrane and hybridized sequentially with 32P-labeled 24P3, mAGP, and rat 18S rRNA probes. The results are shown from one of each of the tissues.

**Fig. 5. Tissue distribution of 24P3 mRNA in saline- and turpentine-injected animals.** Twenty-four hours after injection of saline (−) or turpentine (+), total RNAs were extracted from different tissues and resolved by agarose gel electrophoresis. Three different mice (one male and two females) were used as sources of the tissues. The RNAs were transferred to nylon membranes and hybridized with 32P-labeled 24P3 and rat 18S rRNA probes. The figure shows representative results from one of each of the tissues.

SIP24 was detected in sera from turpentine-injected mice. Sera collected at both 12 and 24 h after injection of turpentine had elevated levels of SIP24 (Fig. 3B). There was no observable difference between the sexes in the extent to which SIP24 was elevated. Thus, as for 24P3 mRNA in liver, turpentine treatment elevated the SIP24 protein level in the bloodstream.

Dexamethasone induces a modest increase in 24P3 mRNA in Liver—Because dexamethasone induces SIP24/24P3 in cultured Balb/c 3T3 cells and regulates the APR in vivo, we also examined the 24P3 mRNA and SIP24 protein levels in dexamethasone-injected mouse livers and sera by Northern and Western blot analyses, respectively. Twenty-four hours after injection of the vehicle, 24P3 mRNA was very low or undetectable in the injected control mouse livers. By contrast, 24 h after the injection of dexamethasone, there was a modest but obvious elevation in the liver 24P3 mRNA level (Fig. 4). The increase in 24P3 mRNA in the livers of dexamethasone-injected mice compared with control mice was 8 ± 3-fold (n = 12). Like turpentine, dexamethasone had a similar effect in male and female mice. Northern blot analysis of mAGP mRNA showed the expected marginal increase (1.25 ± 0.20-fold over control; n = 6) of mAGP mRNA after dexamethasone treatment. Unlike its rat counterpart, mouse AGP is induced only about 1.5-fold after glucocorticoid treatment (18). The SIP24 protein could not be detected in the sera from either vehicle- or dexamethasone-injected mice by Western blot analysis (data not shown).

**Discussion**

We have shown that 24P3 mRNA encodes a protein that is identical in all known aspects with SIP24. Thus, we propose...
that SIP24 and the protein encoded by the 24P3 mRNA are the same protein. We base our conclusion on the following structural evidence. First, the derived amino acid sequence of 24P3 has 200 amino acid residues with a putative signal peptide of 15 N-terminal hydrophobic residues, and its calculated molecular weight is 22,800 (10), whereas SIP24 is secreted, has approximately 180 amino acid residues, and its estimated molecular weight is 21,000 excluding the polysaccharide moiety (9). Second, six different peptides derived from SIP24 showed identity with the 24P3 sequence. This identity covers the length of the protein. Third, the deduced 24P3 protein sequence has a potential N-glycosylation site (10), and SIP24 is N-glycosylated (9). Fourth, antiserum raised against SIP24 purified from 3T3 cells recognizes the same protein as do antisera raised against the 24P3 protein which had been expressed in and purified from E. coli. Fifth, probes for 24P3 mRNA and SIP24 protein detected an mRNA and a protein that were regulated in the same way in vivo. Based on this information, which showed that SIP24 and the 24P3-encoded protein are structurally and immunologically related and are regulated identically in vivo and in cultured cells, we conclude that the 24P3 mRNA encodes the SIP24 protein. Thus, we refer to this protein as SIP24/24P3.

Our results of turpentine and dexamethasone injection experiments have clearly shown that SIP24/24P3 is induced in the liver in a manner indicative of a positive APP. Because SIP24/24P3 is expressed at about the same levels in both males and females it is unlikely that sex hormones play a major role in regulating its expression. By comparison, the related lipocalin and APP rat α2-microglobulin is synthesized only in male liver (19).

The results of our tissue distribution studies show that SIP24/24P3 is primarily expressed in liver during the APR. However, it is also expressed at lower levels in the brain and uterus during the APR. As reviewed by Aldred et al. (4), the liver is not the only site of APP expression, even though it is the most important one in terms of magnitude of production. For example, the brain is an organ for which the extracellular compartment is separated by the blood-brain barrier from the main vascular/extravascular body compartment. So, it may be desirable for the brain to synthesize its own APPs when needed. The levels of messenger RNAs encoding several APPs are altered in the brains of various species during the APR.

These proteins include rat transferrin, transthyretin, ceruloplasmin, and retinol-binding protein (4).

SIP24/24P3 was also found expressed in the pregnant uterus in the unstressed animal. Besides liver, several APPs are also expressed in the tissues comprising the interface between the maternal and fetal body compartment in the pregnant animal; such tissues include uterus, placenta, and yolk sac (4). Extensive tissue remodelling takes place during pregnancy with the constantly changing interaction between the fetus and the tissues of the maternal reproductive tract. The APPs may be needed to control the extent of tissue damage during pregnancy.

Although SIP24/24P3 mRNA was induced in mouse kidney primary cell culture after SV40 infection (10), we did not detect any SIP24/24P3 mRNA expressed in kidney in either saline- or turpentine-treated mice. There are several possible explanations for these observations: 1) SIP24/24P3 mRNA may be expressed in the kidney at a level below the sensitivity of our assays; 2) conditions in vivo are different from those to which cultured cells are subjected; or 3) different factors may be required to induce SIP24/24P3 in nonparenchymal tissues such as liver.
Identification of a New Acute Phase Protein

as the kidney. It has been reported that hepatic nonparenchymal cells produced IL-6 in response to intraperitoneal endotoxin (lipopolysaccharide) but not in response to intramuscular turpentine injection (20).

The regulation of APP expression is extremely complex with many regulatory humoral factors being involved (2, 3, 5, 20, 21). The regulation of SIP24/24P3 seems to be no exception. In Balb/c 3T3 cells, we have previously shown that SIP24/24P3 can be induced by serum, bFGF, epidermal growth factor, and dexamethasone, also signaling bFGF, epidermal growth factor, and dexamethasone, also signaling bFGF, epidermal growth factor, and dexamethasone, also signaling bFGF, epidermal growth factor, and dexamethasone.

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When the magnitudes of SIP24/24P3 induction after turpentine and dexamethasone injection are compared, it is clear that glucocorticoids cannot account for the entire increase in SIP24/24P3 during the APR. As for many other APPs, the interplay among various inducing factors may be necessary to achieve maximum induction of SIP24/24P3 in vivo and in cultured cells (2, 3, 5). This possibility and the underlying mechanism of SIP24/24P3 regulation are currently under investigation in our laboratory.

Our current knowledge of the structure and regulation of SIP24/24P3 provides several clues relating to its possible function. Based upon the fact that SIP24/24P3 protein is produced both upon viral infection and lipopolysaccharide induction in cultured cells, Meheus et al. (11) have suggested that SIP24/24P3 could play a role in the defense mechanism against infection. Our identification of SIP24/24P3 as an APP suggests that it may be involved in homeostasis and have an anti-inflammatory role. A number of factors that induce SIP24/24P3, including bFGF, epidermal growth factor, and dexamethasone, also have been shown to have effects on cultured cells which would be anti-inflammatory in vivo (2, 23). We have also shown that

### Table I

| Treatment | SIP24 |
|-----------|-------|
| Control   | 1.00 ± 0.47 |
| IL-6, 10 units/ml | 0.10 ± 0.01 |
| IL-6, 100 units/ml | 1.16 ± 0.51 |
| TNF-α, 0.1 ng/ml | 4.73 ± 1.37 |
| TNF-α, 1 ng/ml | 8.60 ± 0.48 |
| TNF-α, 10 ng/ml | 5.70 ± 2.09 |
| IL-6, 100 units/ml | 11.69 ± 0.44 |

The identification of SIP24/24P3 as a member of the lipocalin protein family suggests that SIP24/24P3 might be a binding protein for small hydrophobic molecule(s). We have reported that PGF2α also induces SIP24 production in 3T3 cells (7). This prostaglandin is a mitogen for 3T3 cells (25). Many prostaglandins, including PGF2α, are released as a result of increased metabolism of arachidonic acid during the APR (2).

Prostaglandins are also produced at different rates throughout pregnancy (26). So, it is possible that the level of SIP24/24P3 is regulated during the APR and in pregnancy in response to released PGF2α. We speculate that SIP24/24P3 may have an anti-inflammatory role and that this role might involve the ability of SIP24/24P3 to bind a prostaglandin-like molecule.

Acknowledgments—We thank Dr. Lydie Meheus for anti-24P3 sera, Dr. Suzanne Harba-Renevey for the 24P3 cDNA, Dr. Heinz Baumann for mAGP cDNA, and Drs. Richard Torczynski and Harris Busch for the cloned rat 18 S rRNA.

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