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Identification of mouse SIP24/24P3 as a new acute phase protein and study of its expression

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Identification of mouse SIP24/24P3 as a new acute phase protein

and study of its expression

by

Quansheng Liu

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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GENERAL INTRODUCTION

SIP24 is a glycoprotein secreted from quiescent mouse Balb/c 3T3 cells (1-3). It can be induced by serum, basic fibroblast growth factor (bFGF), phorbol myristate acetate, prostaglandin F-2α and dexamethasone (1-3). It is a superinducible protein because it can be further induced if the cells are pretreated with the protein synthesis inhibitor, cycloheximide (1-3). SIP24 has been purified and partially sequenced in our laboratory (3).

24P3 cDNA was originally cloned from a subtractive library made from SV-40 infected primary mouse kidney cells (4). It was found that 24P3 mRNA was induced 12-17 fold after SV-40 infection, and the induction required the synthesis of wild-type SV-40 large T antigen. Polyoma virus induced 24P3 to a lesser extent under the same conditions. Recently, I have identified SIP24 as the product of mouse 24P3 mRNA (5). Therefore, in this thesis the protein is referred to as SIP24/24P3.

SIP24/24P3 has also been identified as a new member of the lipocalin family (6,7). Lipocalins are a group of binding proteins for small hydrophobic molecules (6-11). Examples of lipocalins include serum retinol-binding protein, α-1 acid glycoprotein and prostaglandin D synthase. Lipocalins are believed to be transporters for small hydrophobic ligands. There is increasing evidence to suggest that many lipocalins bind to specific cell surface receptors (8). It has been suggested that many lipocalins may be involved in mediating cell homeostasis (6,8). Some lipocalins, including α-1 acid glycoprotein and retinol-binding protein, are also acute phase proteins (6,12, 13).
The acute phase response (APR) is a complex host response to various inflammatory or stressful stimuli such as infection, wounding, or elevated levels of stressful and tissue-damaging agents (12-14). The APR is the earliest and most immediate response among a series of reactions initiated by the host in an effort to prevent ongoing tissue damage, isolate and destroy the infective organism and return the organism to normal function.

Acute phase proteins (APPs) or reactants are a group of proteins whose plasma concentrations change rapidly during the APR (12-14). Well known APPs include C-reactive protein, \( \alpha \)-1 acid glycoprotein, serum amyloid A protein, and retinol-binding protein. Liver is the major site of APP synthesis (14,15), but many APPs are also synthesized extrahepatically (15,16). The APPs have been demonstrated both \textit{in vitro} and \textit{in vivo} to be regulated by various factors including cytokines, glucocorticoids and growth factors (12, 14, 17-26).

Even though the functions of most APPs are still unknown, the APPs are generally believed to play an anti-inflammatory role \textit{in vivo}. For example, \( \alpha_2 \)-macroglobulin, a positive APP, is a proteinase inhibitor with wide spectrum of specificity (27, 28). During APR, \( \alpha_2 \)-macroglobulin is as a general inhibitor for the proteinases that are released from damaged cells and thus helps to prevent tissue damage. Another example is the negative APP transferrin, which transports iron in the bloodstream. Downregulation of transferrin during the APR may contribute to reducing the availability of iron for bacterial growth (12).

NGAL (neutrophil gelatinase associated lipocalin) is the human homolog of SIP24/24P3 (29-32). NGAL was first purified in 1992 and the partial sequence showed homology with
SIP24/24P3 and the rat α2 microglobulin related protein (29). It was named NGAL because the protein was found to be covalently linked with neutrophil gelatinase (29, 30), a neutral metalloproteinase which is able to degrade extracellular matrix proteins and may be involved in a number of processes including inflammation and tumor invasion (33-37). However, the majorities of both NGAL and gelatinase are separated from each other and located in different secretory granules in neutrophils (32). Recent evidence suggests that NGAL may be directly involved in inflammation. NGAL was shown to be able to bind to fMLP (N-formylmethionyl-leucyl-phenylalanine) (38, 39), a chemotactic factor for neutrophils (40-42) and macrophages (43).

The rat homolog of SIP24/24P3, α2-microglobulin related protein, was fortuitously cloned by Chan et al. (44). The result of sequence comparison showed that α2-microglobulin related protein is 86% and 76% identical with SIP24/24P3 with respect to their mRNA and protein sequences respectively (31). Northern blot revealed that the mRNA of α2-microglobulin related protein is 1 kb in length and an increased expression of this mRNA can be found in liver when injured. It was determined that the mRNA of α2-microglobulin related protein increases 10-fold in the liver of male rats 18 hours after hepatectomy.

Inflammation is a localized protective response following infection, trauma, or other tissue-damaging stimuli (39-43, 45-48). Inflammation involves a series of pro-inflammatory agents that initiate and amplify the inflammatory process and an array of anti-inflammatory factors that protect the host from unnecessary inflammation as well as terminate the inflammatory process.
The host inflammatory response is both local and systemic (12, 17). The APR is part of the systemic response, whereas neutrophil infiltration into the site of inflammation is a characteristic of the local inflammatory response.

Neutrophils or polymorphonuclear leukocytes (PMNs) are specialized phagocytic cells (49). Neutrophils are generated in the bone marrow and distributed between marginated and circulating pools. Neutrophils are involved in host defenses against all classes of infectious agents as well as in the pathology of various inflammatory conditions (47, 49). Neutrophils are the first cells to arrive at a local tissue site once inflammation occurs (50). The mobilization of neutrophils to the site of inflammation (chemotaxis) is initiated by a number of chemotactic factors or chemoattractants (40-42, 51, 52). At the site of inflammation, neutrophils fulfill their function by phagocytosis and by releasing cytotoxic cellular contents which thus amplifies the inflammation process (37, 45-49).

Neutrophils possess oxidative and nonoxidative mechanisms that enable them to eliminate both invading organisms and damaged host cells (47, 48). The oxidative mechanism involves generation of reactive oxygen metabolites including hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($HO^-$). Nonoxidative mechanisms involve the production of various proteolytic and saccharolytic enzymes including collagenase and lysozyme. Microbes are eliminated by the process of phagocytosis and the subsequent oxidative and nonoxidative degradation. Neutrophil release of cytotoxic cellular contents creates a microbicidal environment which is also harmful to the host tissues.

The chemotactic factors for neutrophils include N-formyl peptides (e.g. fMLP), interleukin
8 (IL-8), platelet activating factor (PAF), leukotriene B4 (LTB4) and complement fragment 5a (C5a) (51). Among them, N-formyl peptides and their receptor have received most study (53-59). N-formyl-peptides are produced at the inflamed site by pre-existing neutrophils and macrophages from sources of bacterial and host mitochondrial proteins (40-42). Neutrophils respond to N-formyl peptides in a concentration dependent manner (51). At low concentrations ($10^{-9}$-$10^{-8}$M), neutrophils take on a polarized shape and migrate to the high end of the peptide gradient. At high concentrations ($>10^{-7}$M), neutrophils no longer move along the gradient but discharge their cellular contents.

Activation of neutrophils by N-formyl peptides is mediated by a cell surface receptor (55, 56). The N-formyl peptide receptor has been cloned in human (55). The receptor is associated with a GTP-binding protein which is sensitive to pertussis toxin (56). Upon activation, the receptor is subsequently down-regulated, and repeated exposure to agonist results in desensitization of the signal transduction pathway (55). In neutrophils, expression of the N-formyl peptide receptor is increased in septic and posttraumatic patients (57). Priming factors including tumor necrosis factor (TNF) and colony-stimulating factor (CSF) stimulate neutrophils to a primed state which is more responsive towards subsequent stimulation by N-formyl peptides (51).

Relative to its initiation, the control and termination of the inflammation process are less well understood. It is known that fMLP is also a chemotactic factor for macrophages (43). It has been suggested that macrophages may be involved in the elimination of aging or apoptotic neutrophils at the site of inflammation and therefore control the inflammation process (60, 61). Coincidently, SIP24/24P3 is also a major protein secreted from mouse PU5.1.8 macrophage cells
stimulated by lipopolysaccharide (62).

In the present study, I proved that SIP24 and 24P3 are the same protein. Then I tested the hypothesis that SIP24/24P3 is a new APP. This was accomplished by examination of SIP24/24P3 mRNA expression in various organs during turpentine induced APR. At the same time, the change of SIP24/24P3 protein level in bloodstream during APR was also examined. The results indicate that SIP24/24P3 is indeed dramatically induced during the APR, and that liver is the major site of SIP24/24P3 synthesis. The hepatic pattern of SIP24/24P3 regulation by the major APR mediators (TNF-α and IL-6) was determined in BNL (Balb/c normal liver) cells.

Because SIP24/24P3 was also found to be expressed at a significant level in the uterus during the APR as well as in midgestation, I performed a time course study of SIP24/24P3 expression during pregnancy. The expression of SIP24/24P3 mRNA was examined in the uterus and the liver, and the distribution of SIP24/24P3 protein was examined in the bloodstream and the amniotic fluid. SIP24/24P3 expression increased to a high level in the uterus around birth. The possible function of SIP24/24P3 and its involvement in the host inflammatory response are discussed.

**Thesis Organization**

This thesis includes a general introduction, two journal articles and a general summary section. One of the journal articles has been published in The Journal of Biological Chemistry, the other one is to be submitted for publication to The Journal of Molecular Reproduction. The
Papers have been presented in the form required by the specific journals. All the references cited in the general introduction and general summary are included in the reference section following the general summary section.
IDENTIFICATION OF A NEW ACUTE PHASE PROTEIN


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ABSTRACT

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 factor (bFGF), 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 SV-40-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. Injection of mice with dexamethasone caused a modest increase of SIP24/24P3 mRNA in the liver. Tissue distribution 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 TNF-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.

INTRODUCTION

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; 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 mainly synthesized 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 type-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, leukaemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor primarily induce type-2 APPs such as rat α2-macroglobulin. Third, glucocorticoids are believed to play a permissive role during the APR because 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, purification, 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 SV-40 infected mouse kidney primary cell cultures (10). The 24P3 protein is also a major
secretory protein of cultured mouse PU5.1.8 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 $\alpha_2$-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 $\alpha_1$-acid glycoprotein (AGP), $\alpha_2$-microglobulin, plasma retinol binding protein, and $\beta$-lactoglobulin. Some lipocalin proteins, including AGP, $\alpha_2$-macroglobulin 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-$\alpha$ which is a major mediator of the APR. Our results also show that SIP24/24P3 is a type-1 APP.

MATERIALS AND METHODS

Materials-Dexamethasone and cycloheximide were purchased from Sigma Chemical Company (St. Louis, MO). 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 the water-soluble form of dexamethasone encapsulated in 2-hydroxy-propyl-
β-cyclodextrin (Sigma). 2-hydroxy-propyl-β-cyclodextrin dissolved in water at the same concentration as for in the dexamethasone-containing solutions was used for the control (vehicle) injections. The turpentine used for inducing the APR in mice was purchased from E. E. Zimmermann (Pittsburgh, PA). Polyclonal anti-SIP24 serum was raised in a rabbit in our laboratory (9). Two preparations of rabbit polyclonal anti-24P3 sera (376 & 377) were gifts from Dr. Lydie Meheus (Innogenetics Institute, Ghent, Belgium). A 5′ segment of 24P3 cDNA was a gift from Dr. Suzanne Hraba-Renevey (Embryologie Moléculaire et Morphogénèse Institute, Geneva, Switzerland). The mouse α1-acid glycoprotein (mAGP) isoform 3X cDNA from mouse strain M. caroli was a gift from Dr. Heinz Baumann (Roswell Park Cancer Institute, Buffalo, NY). Cloned rat 18S rRNA in plasmid pDF8 was a gift from Drs. Richard Torczynski and Harris Busch (Wadley Institutes of Molecular Biology, Dallas, TX, 14). Recombinant mouse IL-6 (1.0X10³ U/µg) and TNF-α (4.2X10⁵ U/µg) were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Trans³⁵S-label was from ICN (Costa Mesa, CA). Nitrocellulose membranes were purchased from Micron Separations Inc. (Westboro, MA), and Zeta-Probe nylon membranes were from BIO-RAD Laboratories (Hercules, CA). Dulbecco-Vogt's modified Eagle's (DME) medium, calf serum, penicillin, and streptomycin were from GIBCO/BRL (Gaithersburg, MD).

Cell culture-Stock cultures of mouse Balb/c 3T3 and BNL cells were grown in Dulbecco-Vogt's modified Eagle's (DME) medium with both 10% calf serum and 10 U/ml each of penicillin and
streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 10% CO₂ in air.

*Cell culture and preparation of medium containing SLIP24-Balb/c 3T3 cells or BNL cells were plated on plastic dishes in DME containing 10% calf serum and 10 U/ml penicillin-streptomycin. After the cells had reached confluency, the medium was changed to 2% calf serum. Two to three days later the cells were treated with cycloheximide, dexamethasone, IL-6, or TNF-α in serum free DME medium as described for each experiment. The media were then collected and centrifuged to remove cell debris, the supernatants were lyophilized, and the pellets were redissolved in distilled H₂O and stored at -20°C until use.*

*Western Blot Analysis*—Samples of concentrated conditioned culture media and diluted serum samples were resolved by SDS-PAGE (9,15), and the proteins were transferred to nitrocellulose membranes (16). The transferred proteins were detected by immunostaining using either the Stratagene Picoblue alkaline phosphatase kit to produce a blue stain or horse radish peroxidase conjugated secondary antibody (Organon Teknika Corp., West Chester, PA) and ECL Western blotting detection reagents (Amersham corp., Arlinton Heights, IL) to produce a luminescent stain.

*Induction of the Acute Phase Response in Mice*—Turpentine or 0.9% sterile saline (5 ml/kg body wt) were injected intramuscularly into five-week-old to six-month-old male and female CF-1 mice. Dexamethasone or the vehicle alone (5 mg/kg body wt) 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 hour. A spatula was used to detach the clots from the walls of the tube every 15 min. The samples were then spun 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 M guanidine isothiocyanate (GIT) buffer 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 32P-labeled 24P3 5' region cDNA probe (440 bp), mAGP cDNA probe (700 bp), and rat 18S rRNA cDNA probe (750 bp EcoRI/PstI fragment). The membranes were stripped between hybridization with the different probes. The RNA band was visualized by autoradiography. The results were quantitated by using phosphorimager (Molecular Dynamics, Sunnyvale, CA). 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 10^6 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 quiescence. Interleukin-6 or TNF-α were added at the final concentrations described for each experiment and the cells were incubated for 24 hours. The medium was then removed and the cells were rinsed twice with Tris
buffered salts. The cells were then metabolically labeled for 4 hours by incubation with 100 μCi/ml of Trans35S-label in DME (with 10% of the normal methionine concentration), 0.2% calf serum and the same additions as present during the previous 24-hour incubation. Details of this procedure have been described (7-9,15). The samples of medium were resolved by SDS-PAGE, 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 TCA-precipitable cpm in the post-nuclear 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 clostripain-digested SIP24 were chosen to sequence and to compare with GenBank and EMBL databases. 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 database. 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 to 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 SV-40. 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 peptide B peak after reverse-phase HPLC 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 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 E.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).
Figure 1. Comparison between the SIP24 and 24P3 deduced amino acid sequences. The lower horizontal line represents the 24P3 sequence. All numbers except the first and the last indicate the amino acid position on the deduced 24P3 protein sequence at which the sequences of the peptides shown for SIP24 begin. Position 1 is the first amino acid in the 24P3 sequence that includes a putative signal sequence. Identities of sequence between the SIP24 and 24P3 are indicated by a line on the 24P3 map without interruptions of the amino acid letter code. The single location in which the sequence of one of the SIP24 peptides did not match the 24P3 sequence is indicated with a letter interrupting the line representing 24P3. The mismatch was found in peptide E in which a glycine was found in the SIP24 peptide sequence in the position of a lysine in the 24P3 sequence. The sequences of peptides A, C, and D have been reported previously (9). Peptide E, F, G are newly identified and sequenced peptides from a clostripain digest of SIP24. The symbol ? in the sequence of peptide G indicates that the amino acid in this position was not detected as a recognizable peak.
Figure 2. Recognition of SIP24 by antisera raised against 24P3 by Western blot analysis. Quiescent Balb/c 3T3 cells were incubated with or without 1 μg/ml cycloheximide and 400 ng/ml dexamethasone (DEX/CHX) in serum-free DME medium for 18 hours. The media were concentrated and resolved by SDS-PAGE, and the Western blots were stained with pre-immune serum (PI), anti-SIP24 (α-SIP24), or two preparations of anti-24P3 sera (376, 377) either alone or as a 1:1 mixture. The Western blot was visualized by using the Stratagene Picoblu 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).
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 SIP24/24P3 is 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 an uniform band corresponding to an mRNA of about 1.0 kb, 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 uninjected (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±107 (N=12). The membranes were also hybridized with the mAGP cDNA probe. The ratio of normalized mAGP mRNA in the livers of turpentine-injected vs. saline-injected animals was calculated to be 12±4 (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 hours after
Figure 3A. 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 $^{32}$P-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 28 S rRNA markers and the estimated molecular weight of the 24P3 mRNA.
Saline   Turpentine

Sex      M      F
         M      M      M      F      F      F

28S

18S

1.0 kb

SIP24/24p3

mAGP

18S
Figure 3B. 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 hours after injection of saline or turpentine. Samples (30 μl) of 2.5% sera were resolved by SDS-PAGE and blotted against pre-immune serum (PI) and anti-SIP24 serum (α-SIP24) as described in Materials and Methods. 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.
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. But, 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 analysis respectively. Twenty-four hours after injection of the vehicle, 24P3 mRNA was very low or undetectable in the injected control mouse livers. By comparison, 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 similar effect in male and female mice. Northern blot analysis of mAGP mRNA showed the expected marginal increase (1.25±0.18 fold over control; N=6) of mAGP mRNA after dexamethasone treatment. Unlike its rat counterpart, mouse AGP is only induced 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).
Figure 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-hydroxy-propyl-β-cyclodextrin (C) or dexamethasone in 2-hydroxy-propyl-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 $^{32}$P-labeled 24P3, mAGP, and rat 18S rRNA probes. The same experiment was performed twice, and a total of 12 mice (6 male and 6 female) were used for each condition.
Vehicle  |  DEX
---|---
Sex  |  M  F  M  M  F  F  F

- 28S
- 18S
- 1.0 kb

SIP24/24P3  →  

mAGP  →  

18S  →  

SIP24/24P3 is mainly expressed in liver-The tissue distribution of 24P3 mRNA was determined by Northern blot analysis. Twenty-four hours after saline or turpentine injection, total RNAs from liver, heart, kidney, lung, brain, skeletal muscle, and uterus were extracted and analyzed. As shown in Fig. 5, 24P3 mRNA was almost undetectable in all tissues from saline-injected mice. Of the tissues from turpentine-treated mice, the liver showed an obvious elevation in 24P3 mRNA. The 24P3 mRNA was not detected in samples from the heart, kidney, lung, and muscle of turpentine-injected mice. However, 24P3 mRNA was detected in the brain and uterus, although its level of expression in these tissues was much lower than in liver. When 24P3 mRNA levels from turpentine-injected mice were compared in these tissues after being normalized to the levels of 18S RNA, the relative ratios were 1.00±0.80, 0.01±0.00 and 0.19±0.00 (N=3) in liver, brain, and uterus, respectively. Thus, it seems that SIP24/24P3 is mainly expressed in liver during the APR.

SIP24/24P3 expression during pregnancy-The reproductive system has been reported to express a number of APPs during pregnancy, including rat ceruloplasmin, transferrin, retinol-binding protein, α2-macroglobulin, and others (4). The expression of SIP24/24P3 during mouse pregnancy was examined by Northern blot analysis. SIP24/24P3 message was found in the liver and uterus, but not in the placenta or fetus of day 11 pregnant mice (Fig.6). The proportion of SIP24/24P3 mRNA was about the same in the uteri and livers of five midpregnant (day 11) mice.

Interleukin-6 and tumor necrosis factor-α induce SIP24/24P3-We used BNL cells, a normal liver cell line, to test the cellular responses of SIP24 production to treatment with either of two major
Figure 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 $^{32}$P labeled 24P3 and rat 18S rRNA probes. The figure shows representative results from one of each of the tissues.
Liver  Heart  Kidney  Lung  Brain  Muscle  Uterus

Turpentine  +  +  +  +  +  +  +  +

SIP24/24P3

28S

18S

1.0 kb
Figure 6. 24P3 mRNA expression during pregnancy. Total RNAs from the maternal liver, uterus, placentae, and fetuses of day 11 mid-gestation female mice were extracted and hybridized with $^{32}$P labeled 24P3 and rat 18S rRNA probes.
APR regulating factors. Quiescent BNL cells were treated with IL-6, TNF-α, and combinations of both for 24 hours, and then metabolically labeled for 4 hours. The media containing 35S-methionine-labeled proteins were analyzed for relative rates of SIP24 synthesis as described in Materials and Methods. The results are shown in Fig. 7A and Table 1. Interleukin-6 had little or no effect on SIP24 production by BNL cells, whereas TNF-α induced SIP24. The combination of 100 U/ml IL-6 and 1 ng/ml TNF-α treatment resulted in about 1.4-fold increase of SIP24 as obtained with TNF-α treatment alone. Thus, IL-6 and TNF-α acted synergistically to increase SIP24 production by BNL cells. Western blot analysis was performed in parallel to confirm the identification of the SIP24/24P3 protein produced by BNL cells. As shown in Fig. 7B, anti-SIP24 serum detected SIP24/24P3 in medium from TNF-α-treated BNL cells, but not from IL-6-treated cells.
Figure 7A. Regulation by IL-6 and TNF-α of SIP24 synthesis and secretion. Quiescent BNL cells were incubated for 24 h with IL-6, TNF-α, and the combination of both factors. The culture media containing radiolabeled secreted proteins were resolved by SDS-PAGE and analyzed by fluorography as described in Materials and Methods. The concentrations used of IL-6 (10-1000 U/ml) and of TNF-α (0.1 to 10 ng/ml) are shown for each lane. Numbers on the left show the positions of the proteins used as molecular weight markers with their molecular weights expressed in thousands.
Figure 7B. Western blot analysis of secreted SIP24. Quiescent BNL cells were incubated for 24 h with IL-6 and TNF-α in serum-free DME medium. The culture media were collected, centrifuged, lyophilized, and redissolved in distilled H2O to 1/10 of the original volume. The concentrated media were resolved by SDS-PAGE, and the Western blots were stained with pre-immune serum (PI) or anti-SIP24 (α-SIP24). The same SIP24-enriched medium as used in Fig.2 was included as positive control (DEX/CHX). Numbers on the left indicate the positions of the molecular weight markers in thousands.
Control

IL-6 100

TNF 0.1

IL-6 100

DEX+CHX

P

SIp24/24p3

12.5

18

30

45

67
Table 1. Quantitative analysis of the effects of IL-6 and TNF-α on SIP24 production by BNL cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BNL Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.00 ± 0.47</td>
</tr>
<tr>
<td>IL-6 10 U/ml</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>IL-6 100 U/ml</td>
<td>1.16 ± 0.51</td>
</tr>
<tr>
<td>IL-6 1000 U/ml</td>
<td>1.13 ± 0.28</td>
</tr>
<tr>
<td>TNF-α 0.1 ng/ml</td>
<td>4.73 ± 1.37</td>
</tr>
<tr>
<td>TNF-α 1 ng/ml</td>
<td>8.60 ± 0.48</td>
</tr>
<tr>
<td>TNF-α 10 ng/ml</td>
<td>5.70 ± 2.09</td>
</tr>
<tr>
<td>IL-6 100 U/ml</td>
<td>11.69 ± 0.04</td>
</tr>
<tr>
<td>TNF-α 1 ng/ml</td>
<td></td>
</tr>
</tbody>
</table>

Cells were incubated with IL-6 and/or TNF-α for 24 hours and then labeled for 4 hours. The relative amounts of SIP24 protein in culture media from cells treated with various concentrations of IL-6 and TNF-α were determined by scanning each band on the fluorograms. The obtained values were normalized to the TCA precipitable cpm of matched postnuclear supernatants of the labeled cells. Each value is the average of duplicates from a single experiment. The standard deviations are shown. This experiment was repeated twice with similar results. Details are described in Materials and Methods.
DISCUSSION

We have shown that 24P3 mRNA encodes a protein that is identical in all known aspects to 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 Mr. is 22,800 (10), whereas SIP24 is secreted, has approximately 180 amino acid residues, and its estimated Mr. 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 \textit{E. coli}. Fifth, probes for 24P3 mRNA and SIP24 protein detected an mRNA and a protein that were regulated in the same way \textit{in vivo}. Based on this information, which showed that SIP24 and the 24P3-encoded protein are structurally and immunologically related and are regulated identically \textit{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 only synthesized 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, 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 SV-40 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 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 (EGF), prostaglandin F2α, and phorbol myristate acetate (7-9). Here we have shown that the two major APR regulating factors, IL-6 and TNF-α, differently regulate SIP24/24P3 expression in BNL cells. BNL cells are a Balb/c normal liver cell line that has been used to study the regulation of the APP, rabbit serum amyloid A protein gene (22). The ability of SIP24/24P3 to be induced by TNF-α indicates that it is a type-1 APP (2).

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 provide 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. (13) 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, EGF, and dexamethasone, also been shown to have effects on cultured cells which would be anti-inflammatory in vivo (2,23). We have also shown that the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (PMA) induces SIP24/24P3. PMA activates PKC, which induces specific acute phase responses (2,24).

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 PG-like molecule.

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REFERENCES


A MOUSE ACUTE PHASE PROTEIN IS EXPRESSED IN THE UTERUS AROUND THE TIME OF BIRTH

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ABSTRACT

Mouse SIP24/24P3 is a 24 k Da lipocalin that has been recently identified as a new acute phase protein (APP). During the acute phase response (APR), SIP24/24P3 mRNA is dramatically increased in liver and the protein is secreted into bloodstream. The identification of SIP24/24P3 as an APP suggests that SIP24/24P3 may be involved in the systemic inflammatory response. Although liver is the major site of SIP24/24P3 synthesis during the APR, SIP24/24P3 is also expressed in the uterus. The uterus undergoes extensive tissue remodeling during pregnancy and suffers stress and tissue damage around parturition. A time course study was carried out to investigate the variation in expression of SIP24/24P3 during pregnancy and around birth. Northern blot and Western blot analyses showed that SIP24/24P3 protein and mRNA are massively expressed in the uterus around parturition. On the other hand, contrary to the expression pattern during the APR, liver expression of SIP24/24P3 is marginal or
undetectable. Despite its massive expression in the uterus, SIP24/24P3 was not detected in the blood or amniotic fluid. The results suggest that SIP24/24P3 is expressed locally in the uterus around birth and that it could be involved in the local inflammatory response associated with parturition.

**INTRODUCTION**

Inflammation, initiated in the host following infection, trauma, or tissue-damaging stimuli involves both local and systemic changes ((Raynes, 1994; Gauldie et al., 1992; Leirisalo-Repo, 1994; Smith, 1994). Local responses include the release of cellular contents such as histamine, prostaglandins, and lysosomal enzymes with neutrophil infiltration to the site of inflammation. They are accompanied by vasodilation and pain with tissue swelling and redness. The systemic responses include the acute phase response (APR), fever, leukocytosis, and changes in plasma metal ion and glucocorticoid concentrations.

One of the earliest events in inflammation is the infiltration of neutrophils into the inflamed site (Schleimer et al., 1989). Neutrophils, also called polymorphonuclear leukocytes (PMNs), are the most abundant circulating leukocytes (Smith, 1994; Schleimer et al., 1989). They are an important part of the initial host defense against all classes of infectious agents and are also involved in chronic inflammatory diseases (Leirisalo-Repo, 1994; Smith, 1994; Kubes, 1993). When inflammation occurs, neutrophils are stimulated by chemotactic factors or chemoattractants that are generated at the site of inflammation (Leirisalo-Repo, 1994; Smith, 1994; Kubes, 1993; Baboir, 1992; Klotz et al., 1994). N-formylated peptides generated from
bacterial or mitochondrial proteins and attract neutrophils to the inflamed site in a concentration-dependent manner (Schiffmann et al., 1975; Showell et al., 1976; Carp, 1982; Babior, 1992). The neutrophils then eliminate the invading organisms and damaged cells by phagocytosis and by releasing hydrolysing enzymes, lipid peroxidation products and oxygen-derived free radicals (Leirisalo-Repo, 1994; Smith, 1994; Kubes, 1993; Varani et al., 1994a; Varani et al., 1994b).

SIP24 was discovered as an inducible protein secreted by quiescent Balb/c 3T3 fibroblast cells (Nilsen-Hamilton et al., 1982). Recently, we have shown that SIP24 is identical to mouse 24P3 protein whose cDNA was cloned from SV-40 infected primary kidney cells (Hraba-Renevey et al., 1989; Liu et al., 1995). SIP24/24P3 is also expressed in mouse PU5.1.8 macrophage cells (Meheus et al., 1993) and BNL (Balb/c normal liver) cells (Liu et al., 1995). In vivo, SIP24/24P3 mRNA is expressed in the liver as well as in the uterus and the brain during the APR (Liu et al., 1995). The liver seems to be the major site of SIP24/24P3 synthesis, and SIP24/24P3 protein can also be detected in the bloodstream during the APR. The extrahepatic expression of SIP24/24P3 is also of interest. While the brain only expresses SIP24/24P3 marginally during the APR, the uterus expresses SIP24/24P3 even under normal conditions (Liu and Nilsen-Hamilton, 1995). SIP24/24P3 shows 71% mRNA sequence identity with the human NGAL which is probably its human homolog (Bartsch et al., 1995).

NGAL (neutrophil gelatinase associated lipocalin) is a 25 kDa lipocalin protein which was purified and cloned from human neutrophils (Triebel et al., 1992; Kjeldsen et al., 1993; Bartsch et al., 1995). A minor portion of NGAL is covalently linked with neutrophil gelatinase (Triebel et al., 1992; Kjeldsen et al., 1993), a neutral metalloproteinase which degrades extracellular
matrix proteins and is believed to be involved in inflammation as well as tumor invasion and metastasis by (Varani et al., 1994a; Bartsch et al., 1995; Murphy et al., 1982; Wilhelm et al., 1989; Hibbs et al., 1985; Liotta et al., 1986). More recent evidence suggests that NGAL may directly contribute to inflammation. Sengeløv et al. showed that NGAL is identical with the binding protein of the chemotactic factor, fMLP (N-formylmethionyl-leucyl-phenylalanine) (Allen et al., 1989; Sengeløv et al., 1994). Despite the biochemical evidences, an in vivo study of NGALs involvement in inflammation is still lacking.

As reviewed by Aldred et al., (1992) the reproductive system is a major site of extrahepatic synthesis of APPs during pregnancy. During pregnancy, the reproductive system undergoes extensive tissue remodelling. Local expression of the APPs might be required to control unnecessary tissue damage associated with the remodelling process. At the end of pregnancy, the sharp increase of prostaglandin F2α (PGF2α) in the uterus and amniotic fluid causes uterine smooth muscle contraction which subsequently triggers parturition (Poyser, 1981; Kelly, 1994; Strickland et al., 1982). Parturition is accompanied by inflammation (Kelly, 1994). The administration of anti-inflammatory agents which inhibit prostaglandin synthesis delays parturition (Chester et al., 1972). Also, the level of the pro-inflammatory mediator TNFα is sharply increased in uterus prior to parturition (De et al., 1992).

The identification of SIP24/24P3 as an APP implies that it may be directly or indirectly involved in inflammation in vivo. We and others have reported the expression of SIP24/24P3 in the uterus at specific stages of pregnancy (Liu and Nilsen-Hamilton., 1995; Kasik et al., 1995). Here we present a more extensive investigation of SIP24/24P3 expression in the uterus and the
liver during pregnancy and demonstrate that the highest levels of SIP24/24P3 mRNA and protein are achieved one day postparturition. Contrary to its expression pattern during the APR, SIP24/24P3 mRNA and protein were not expressed significantly in the liver. Also, despite the high levels of its mRNA expression and protein synthesis in the uterus, SIP24/24P3 was not detected in the bloodstream and amniotic fluid. Our results indicate that SIP24/24P3 can be synthesized locally in the uterus and may participate in the local inflammatory response which occurs during the birth-associated trauma.

MATERIALS AND METHODS

Materials

The 5' segment of SIP24/24P3 cDNA cloned in pBluescript plasmid was a gift from Dr. Suzanne Hraba-Renevey (Embryologie Moléculair et Morphogénèse Institute, Geneva, Switzerland). The cloned rat 18 S rRNA in plasmid pDF8 was provided by Drs. Richard Torczynski and Harris Busch (Wadley Institutes of Molecular Biology, Dallas, TX; Fuke et al., 1981). Zeta-Probe nylon membranes used for Northern blot were purchased from Bio-Rad Laboratories. Anti-SIP24 serum was previously prepared from rabbit in our laboratory (Davis et al., 1991). Nitrocellulose membranes were purchased from Micron Separation Inc.

RNA analysis

Pregnant and non-pregnant female CF-1 mice were sacrificed by cervical dislocation. Uteri and liver were frozen in liquid nitrogen and kept at -70°C until use. Organs were homogenized in 4 M guanidine isothiocyanate buffer and centrifuged through CsCl to obtain total
RNA (Davis et al., 1986). For Northern analysis, 15 µg of total RNA was loaded in each lane and the components were separated by electrophoresis through a 0.9% agarose gel containing formaldehyde and then transferred to Zeta-Probe nylon membrane. The transferred membranes were hybridized with a 32P-labeled SIP24/24P3 5' segment cDNA probe (440 base pairs) and then with a 32P-labeled rat 18 S rRNA cDNA probe (750 base pair EcoRI/PstI fragment). The membranes were stripped between hybridizations with the two probes. The RNA band was visualized by autoradiography, and the result was quantitated by using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**Protein analysis**

When mice were sacrificed, blood samples were taken by heart puncture, and sera were prepared subsequently (Liu and Nilsen-Hamilton, 1995). Amniotic fluids were taken at the same time and then centrifuged for 5 minutes in a microcentrifuge at 14,000 x g at room temperature to remove cell debris. Tissue homogenates were prepared by homogenating 0.1 g of uterus or liver organ in 1 ml of 1 X IPB buffer (0.15 M NaCl, 0.1 % SDS, 1 % NP-40, 1 % Sodium deoxycholate, 10 mM NaPi, PH 7.2) for 30 seconds, then the tissue debris was removed by centrifugation at 14,000 x g at room temperature for 5 minutes. All the protein samples were stored at -20°C until use. Western blot analysis was carried out by resolving 5% serum or neat amniotic fluid samples (12.5 µl/lane) by SDS-PAGE with electrophoresis through 15% acrylamide gels. Equal volumes of tissue extracts were loaded. Protein concentrations of each tissue extract was determined by Bradford analysis and the results normalized to the concentration of protein in each sample. The proteins were transferred to nitrocellulose
membranes (Burnette, 1981) and then SIP24/24P3 detected by immunostaining using a horseradish peroxidase-conjugated secondary antibody (Organon Teknika Corp., West Chester, PA) and ECL Western blotting detection reagents (Amersham, Arlington, IL) to produce a luminescent stain.

Statistical Analysis of Data

Comparison of two sample means was analysed by Student's t test (Steel et al., 1980).

RESULTS

The expression of SIP24/24P3 mRNA is highly regulated during pregnancy

During the period of pregnancy, CF-1 female mice were sacrificed each day and uterine total RNAs were extracted and analyzed by Northern blot. SIP24/24P3 mRNA was expressed in non-pregnant mouse uteri (Fig. 1A). At early and middle stages of pregnancy, SIP24/24P3 was expressed in the uterus at lower levels than in the non-pregnant mouse. Starting from late pregnancy (day 17 and 18), SIP24/24P3 expression increased steadily (Fig. 1B). At the end of pregnancy (day 19), the level of SIP24/24P3 mRNA in the uterus was about 16-fold higher than in the non-pregnant mouse (p<0.001). SIP24/24P3 mRNA was present at an even higher level in the postpartum uterus. On the first day after birth, the SIP24/24P3 mRNA level in the uterus reached a peak of about 37-fold compared to that of the non-pregnant mouse uterus (p<0.01). This massive increase brought the level of SIP24/24P3 mRNA to about 2.3-fold more than in turpentine treated liver (Liu and Nilsen-Hamilton, 1995) when both were measured on the basis of wet tissue weight. After the first day postpartum, uterine expression of SIP24/24P3 was still
Fig. 1A. Northern blot analysis of uterus total RNA from female mice at various stages of pregnancy and around birth. Total RNAs were extracted from the uteri and resolved by agarose gel electrophoresis. The RNAs were transferred to nylon membranes and hybridized sequentially with $^{32}$P-labeled 24P3 and rat 18S rRNA cDNA probes. Northern blots show the result of hybridization with the 24P3 probe (upper panel) and the 18S probe (lower panel). One sample is shown for each day of gestation. Numbers on the top indicate the number of days of gestation. The day of the plug is designated as day 0. NP, non-pregnant. P1, P2 and P3 represent the 1st, 2nd and 3rd day after parturition. Numbers on the right show the positions of the 18S and 28 s rRNA markers and the estimated molecular weight of the 24P3 mRNA.
Fig. 1B. Quantitated results of SIP24/24P3 expression level after normalizing with the corresponding 18S rRNA level. Error bars are the sample standard error of the mean (N=2-7).
SIP24/24P3 EXPRESSION IN UTERUS DURING PREGNANCY

DAYS OF GESTATION

SIP24/24P3

NP 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 P1 P2 P3

57
high but began to decrease. SIP24/24P3 expression was determined to be respectively about 20- and 5-fold over the control nonpregnant uteri on the second and third day after birth respectively.

The above results indicate that SIP24/24P3 is expressed in the uterus at high levels around birth. It also seems that uterine expression of SIP24/24P3 is a highly regulated event. Interestingly, the expression profile of SIP24/24P3 parallels that of prostaglandin F2α production in the uterus (Poyser, 1981). This observation may be related to our previous observation that the expression of SIP24/24P3 is regulated in 3T3 cells by prostaglandin F2α.

SIP24/24P3 mRNA is not present at significant levels in the liver during pregnancy

Because the liver is the major site of SIP24/24P3 synthesis during the APR (Liu and Nilsen-Hamilton, 1995), we also examined SIP24/24P3 expression in the liver at various stages of pregnancy. The expression level of SIP24/24P3 in liver was very low (Fig. 2). In fact, SIP24/24P3 message was undetectable in the liver at most time points examined. There was a marginal expression of SIP24/24P3 in midgestational liver, which agrees with our previous observation, but the expression level was much lower than observed in the liver from turpentine treated animals (Liu and Nilsen-Hamilton, 1995).

SIP24/24P3 protein levels in the uterus and liver during pregnancy

To determine whether the high levels of mRNA observed in the late gestational uterus were translated into protein, we screened these tissues using Western blot analysis (Fig. 3).
Fig. 2. Northern blot of liver total RNA from female mice at various stages of pregnancy and around birth. The RNAs were extracted and analyzed as in Fig. 1A. NP, non-pregnant. P1 and P2 represent the 1st and 2nd day after parturition. Numbers on the right show the positions of the 18 S and 28 S rRNA markers and the estimated molecular weight of the 24P3 mRNA. Numbers on the top indicate the number of days of gestation.
Fig. 3. SIP24/24P3 protein in the uterus and liver during pregnancy and around birth. Uterine and liver homogenates were prepared and analyzed by Western blot as described in "Experimental Procedures". The samples were probed with both preimmune serum (PI) and anti-SIP24 serum. D16, D19 represent 16, 19 days of gestation, P1, P2 represent the 1st and 2nd day after parturition. - and +T liver samples were prepared from saline- and turpentine-injected mouse livers (Liu and Nilsen-Hamilton, 1995). The protein concentrations of each extract in mg/ml were: uterus (D16, 4.9; D19, 4.4; P1, 5; P2, 3.8) and liver (D9, 6.1; D19, 7.2; P1, 7.5). The control is the concentrated SIP24-enriched medium (Liu and Nilsen-Hamilton, 1995). Numbers on the left indicate the positions of the molecular markers expressed in thousands, from top to bottom: bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), and cytochrome (18,000).
These results demonstrated comparable levels of SIP24/24P3 protein in the uterus in late gestation and post-parturition as in the liver during the APP.

**SIP24/24P3 protein distribution in the bloodstream and amniotic fluid**

Blood and amniotic fluids were collected from the same animals as used for the liver and uterine samples. The resulting serum and amniotic fluids were analyzed by Western blot to detect the SIP24/24P3 protein. Serum and amniotic fluids are shown in Fig. 4 for the time points in pregnancy when SIP24/24P3 was found to be expressed in uterus or liver. At all time points examined, SIP24/24P3 was not detected in either sera or amniotic fluid samples. By comparison, SIP24/24P3 protein could be detected in serum from turpentine injected mouse (Liu and Nilsen-Hamilton, 1995).
Fig. 4. Distribution of SIP24/24P3 protein in the blood and amniotic fluid during pregnancy and around birth. Serum (S) and amniotic fluid (AF) samples were prepared and analyzed by Western blot as described in "Experimental Procedures". The same set of samples were probed with both preimmune serum (PI) and anti-SIP24 serum. NP, non-pregnant. D9, D18, D19 represent 9, 18, 19 days of gestation. P1, P2 represent the 1st and 2nd day after parturition. T is 5% serum from a mouse 24 hours after turpentine injection (Liu and Nilsen-Hamilton, 1995). A sample of SIP24-enriched cell culture medium was used as positive control (27). Numbers on the left indicate the positions of the molecular markers expressed in thousands, from top to bottom: bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), and cytochrome (18,000).
DISCUSSION

Our results show that the expression of SIP24/24P3 during pregnancy is highly regulated. Starting from late pregnancy, the level of SIP24/24P3 mRNA and protein in the uterus increased through birth. A 16-37 fold increase in SIP24/24P3 mRNA was seen around birth with the peak on the first day after birth. Compared to the expression in other organs (Liu and Nilsen-Hamilton, 1995), the uterine expression of SIP24/24P3 is quite remarkable. For example, the peak magnitude of SIP24/24P3 mRNA expression (P1) is about twice the level of its expression in the liver during the APR (Liu and Nilsen-Hamilton, 1995). While a typical APR is thought to last 36-48 hours (Schreiber et al., 1982), the high level expression of SIP24/24P3 in uterus lasts 3-4 days. With the assumption that the period of induced liver synthesis of SIP24/24P3 during APR covers the period of the APR, the extent of uterine expression of SIP24/24P3 around birth is even more dramatic.

During the APR, a systemic host inflammatory response, the APPs are synthesized in the liver, secreted into the bloodstream and distributed throughout the body (Raynes, 1994; Baumann et al., 1994). Our previous investigation suggests that SIP24/24P3 expression is regulated is a classic APP during the APR and therefore it may be involved in a systemic host inflammatory response (Liu and Nilsen-Hamilton, 1995). On the other hand, as reviewed by Aldred et al. (1992), many APPs can also be synthesized extrahepatically in the reproductive system during pregnancy when extensive tissue remodeling occurs. For example, rat α2-macroglobulin is a typical APP which is almost solely produced by the liver during the APR. During pregnancy, while the liver still produces a measurable but small amounts of
α₂-macroglobulin mRNA, the uterus and the placenta are the major organs producing α₂-macroglobulin mRNA. A large amount (70-80% of that produced by the inflamed liver) of α₂-macroglobulin mRNA was found in the uterus and the placenta at days 12-15 of gestation (Panrucker and Lorscheider, 1983). Here our results indicate that, contrary to its expression pattern during the APR, SIP24/24P3 mRNA is not measurably expressed in the liver during most of pregnancy and after birth. At the same time, despite the massive expression of its mRNA and protein in the uterus, SIP24/24P3 protein is not present in the blood or in the amniotic fluid. Our results suggest SIP24/24P3 might also be synthesized and retained locally during an inflammatory condition in which the liver is not involved. The localized expression of APP(s) such as SIP24/24P3 in the uterus might be beneficial to the host during the localized inflammation that occurs during parturition.

The function of SIP24/24P3 during parturition is unknown. Recent studies of its human homolog human NGAL shows that it is a binding protein for fMLP (Allen et al., 1989; Sengeløv et al., 1994). fMLP is produced at the site of inflammation from sources like bacterial proteins from invading organisms and mitochondrial proteins from damaged host cells (Schiffmann et al., 1975; Showell et al., 1976; Carp, 1982; Babior, 1992). It has been proposed that SIP24/24P3 may also serve as a fMLP carrier and help to attract neutrophils to the site of inflammation (Kasik et al., 1995). Our results are consistent with this hypothesis.

The APPs are generally believed to play an anti-inflammatory role in vivo (Raynes et al., 1994; Baumann et al., 1994). The possible involvement of SIP24/24P3 in attracting neutrophils
implies that SIP24/24P3 may contribute to the amplification of inflammation process and therefore is more likely to be a pro-inflammatory instead of anti-inflammatory protein. However, it is also possible that SIP24/24P3 may be involved in subduing inflammation. For example, fMLP is also a chemotactic factor for macrophages (Synderman et al., 1980). Macrophages been proposed to help control and terminate inflammation by phagocytosing aging and apoptotic cells including neutrophils at the inflamed site (Haslett et al., Savill et al., 1993). Alternatively, SIP24/24P3 might bind and sequester fMLP from neutrophils, thus suppressing the inflammatory response.

In summary, we have demonstrated that the APP, SIP24/24P3 is present in large amounts in the uterus in late gestation and post-parturition. Increased expression of this protein may be part of the local inflammatory response in this tissue at parturition. Studies are underway to establish the role of SIP24/24P3 in parturition and during inflammation.

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GENERAL SUMMARY

Previous studies in our laboratory identified SIP24 protein as a highly regulated protein in Balb/c 3T3 cells (1-3). In this study, the analysis of sequence data previously reported (3) along with other data which had been collected at the same time but not analyzed, revealed that six out of seven peptides of SIP24 were identical to the derived amino acid sequence of 24P3 mRNA (4). Western blot analysis of control and SIP24-enriched media with both anti-SIP24 and anti-24P3 antibodies showed that both antibodies recognized the same sized protein with the same induction pattern. Combined with other evidence (same Mr and glycosylation pattern and parallel regulation patterns of mRNA and protein), we concluded that SIP24 and 24P3 are the same protein/mRNA. We renamed the protein as SIP24/24P3.

Knowing its relationship with lipocalins (6, 7), and that its expression was regulated by dexamethasone in cultured cells (3), I tested the hypothesis that SIP24/24P3 is a new APP. A series of experiments were carried out to examine changes in levels of SIP24/24P3 mRNA and protein during the APR. It was found that after turpentine injection, SIP24/24P3 message is dramatically induced in the liver (over 400-fold). Dexamethasone injection induced a relatively moderate increase of SIP24/24P3 mRNA in liver (8-fold). When serum samples were examined, SIP24/24P3 protein was detected in sera after turpentine injection, whereas dexamethasone treatment did not induce a detectable amount of SIP24/24P3 protein in the serum. The induction pattern of SIP24/24P3 mRNA in the liver and that of its protein in blood clearly indicate that
SIP24/24P3 is an APP. The induction of SIP24/24P3 during the APR is rapid, presumably within 12 hours since the protein is detected in serum 12 hours after turpentine injection.

The tissue distribution study was carried out to determine the site of SIP24/24P3 synthesis in normal and turpentine-injected mice. Northern blot analysis revealed that during the APR, SIP24/24P3 message is expressed in the liver, uterus and perhaps the brain, but not in the heart, kidney, lung and muscle. The liver was found to be the major site of SIP24/24P3 synthesis during the APR. In mock-injected mice, SIP24/24P3 expression was low in all organs examined except the uterus, where SIP24/24P3 was expressed at a significant level (6% of the level in the livers of turpentine-injected animals).

Because the liver seems to be the major site of SIP24/24P3 synthesis during the APR, I examined the pattern of regulation of SIP24/24P3 expression in BNL (Balb/c normal liver) cells. Results of $^{35}$S-methionine metabolic labeling experiments showed that one of the major mediators of the APR, TNFα, induces SIP24/24P3 protein with a peak induction of 8.6 fold. IL-6 seemed to have no effect on SIP24/24P3 expression alone, but had a synergistic effect on SIP24/24P3 induction when added together with TNFα. The results indicate that SIP24/24P3 is a type-1 APP and that TNFα might mediate hepatic SIP24/24P3 expression.

After discovering SIP24/24P3 expression in the uterus, I examined its uterine expression in midgestation. SIP24/24P3 mRNA was detected in the liver and the uterus, but not in the placenta and the fetus. The results suggest that, as well as the liver, the uterus is also an important site of SIP24/24P3 synthesis during pregnancy.
A time course study was performed to determine the variation in level of expression of SIP24/24P3 in the uterus and liver during pregnancy. Northern blot analysis revealed that at early and middle stages of pregnancy, SIP24/24P3 is expressed in the uterus at a level comparable to or much lower than that of non-pregnant mice. Starting at late pregnancy, SIP24/24P3 expression increases in the uterus through birth. The highest level of expression of SIP24/24P3 was found before and after parturition with the peak on the first day after birth of 37-fold increase compared with non-pregnant mice. This magnitude of SIP24/24P3 expression is quite massive even compared with that of liver expression during the APR.

The time course study with liver samples showed that during pregnancy and after parturition, the liver did not express SIP24/24P3 mRNA at significant level compared with the uterus. In fact, at most time points examined, liver expression of SIP24/24P3 was undetectable. Meanwhile, an examination of SIP24/24P3 protein showed that despite the massive expression of its mRNA in the uterus, SIP24/24P3 protein is not present in circulating blood or amniotic fluid. Therefore, the high level expression of SIP24/24P3 around birth seems to be localized in the uterus.

Despite the different patterns of its expression under various conditions, the expression of SIP24/24P3 seems to be associated with inflammatory responses. Our lab has previously reported that SIP24/24P3 can be induced in Balb/c 3T3 cells by serum, bFGF, TPA, dexamethasone and PGF2α (1-3). These factors have also been reported to be involved in regulating the expression of various other APPs (18-27). 24P3 cDNA was originally cloned from, and its mRNA was found to be induced in SV-40 infected primary kidney cells (4). The
regulation studies using BNL and Balb/c 3T3 cells indicate that SIP24/24P3 is induced by the mediators of APR including IL-6, TNFα and glucocorticoid (dexamethasone) (appendix A). SIP24/24P3 has also been identified as a major protein secreted from mouse macrophage PU5.1.8 cells stimulated by lipopolysaccharide (62). Lipopolysaccharide is an endotoxin and induces severe inflammation in vivo. NGAL, the human homolog of SIP24/24P3, is purified and cloned from neutrophils (29-31), which directly participate in the inflammation process (45-48, 51). α₂-microglobulin related protein is the rat homolog of SIP24/24P3 and has been found to be upregulated 10-fold in liver after injury (partial hepatectomy) (44). Here, the in vivo identification of SIP24/24P3 as an APP indicates that SIP24/24P3 may be involved in a systemic inflammatory response (APR) where it is synthesized in liver and secreted into bloodstream. The study of SIP24/24P3 expression and distribution during pregnancy and after parturition suggests that SIP24/24P3 is expressed locally in the uterus and may participate in local inflammatory response accompanying parturition.

Extensive connective tissue remodelling takes place in uterus during parturition (15, 63, 64). It is known that selective use of the inflammatory response is an important part of the effective mechanism of delivery (63, 64). The uterus has high concentration of smooth muscle cells which are embedded in a matrix of connective tissue consisting mainly of collagen. The cervix is predominantly fibrous connective tissue with relatively few smooth muscle cells. Parturition involves effective uterine contraction as well as ripening of the cervix which softens and dilates to allow the passage of the fetus. The softening of the cervix is primarily due to
striking changes in the connective tissue. Cervical connective tissue is made up of collagen fibrils and elastin separated by the ground substance. Collagen is resistant to most extracellular proteases except to collagenase and leucocyte elastase. Measurement of collagenase activity in human cervix shows that the enzyme activity is highest during the active phase of labor. Such an increase of collagenase activity is thought to be generated from neutrophils infiltrated into the tissue around parturition.

The function of SIP24/24P3 is unknown. We have previously speculated that SIP24/24P3 might bind a PG-like small hydrophobic ligand (5). With the identification of human NGAL as a fMLP binding protein (38, 39), it seems more likely that SIP24/24P3 might bind fMLP. As proposed by others (65), SIP24/24P3 may function by attracting neutrophils to the inflamed site during inflammation. In this respect, SIP24/24P3 would be a pro-inflammatory instead of an anti-inflammatory protein. However, the presumed ability of SIP24/24P3 to bind fMLP may contribute to inflammation in opposite way(s). For example, SIP24/24P3 may also help to bring macrophages to the site of inflammation and thus contribute to the control and termination of inflammation process (58, 59). Alternatively it may sequester fMLP and limit the movement of neutrophils to the uterus, thus acting as a break on the system.

The exact cell type(s) of SIP24/24P3 synthesis in vivo is still unknown. Neutrophils are known to be involved in certain chemically-induced liver injuries (66-71). For example, neutrophils are massively present in rat liver after bacterial endotoxin injection. TNFα seems to mediate this endotoxin-induced hepatotoxicity through a neutrophil-dependent mechanism (69). Pretreatment of rats with an antiserum to TNFα afforded protection against liver injury 6 h after
lipopolysaccharide exposure, but such a pretreatment did not affect hepatic accumulation of neutrophils. Meanwhile, depletion of neutrophils protects against liver injury from endotoxin (70). It is completely possible that another cell type(s) is responsible for the major synthesis of SIP24/24P3 in vivo. It has been proposed that SIP24/24P3 is produced by neutrophils in vivo and the expression of SIP24/24P3 at the inflamed site is due to contaminating neutrophils infiltrated into local tissues following inflammation (65). Our results are consistent with this hypothesis because SIP24/24P3 is expressed at inflamed site in vivo where neutrophils are presumably accumulated.

The direction of future studies shall include investigations in vitro and in vivo. In order to examine whether SIP24/24P3 binds fMLP as does human NGAL, it is necessary to obtain the protein and characterize its binding specificity. The full length cDNA of SIP24/24P3 has been cloned by RT-PCR (Appendix B), so it is now possible to express the protein and carry out binding studies in vitro. On the other hand, it is necessary and possible to further investigate the role and involvement of SIP24/24P3 in various inflammation processes. For example, to address the question whether and to what extent SIP24/24P3 is produced by neutrophils under different inflammatory conditions, in situ hybridization can be performed to examine the cell type(s) which expresses SIP24/24P3 in vivo. Also, it is known that fMLP-activated neutrophils damage cultured hepatocytes as evaluated by the release of alanine aminotransferase into the medium (71). The effect of SIP24/24P3 on neutrophil activation by fMLP can be measured by pre-incubating SIP24/24P3 with fMLP and neutrophils. The results will help us to understand what role(s) SIP24/24P3 plays during inflammation.
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APPENDIX A
REGULATION OF SIP24/24P3 IN BALB/C 3T3 AND BNL CELLS
BY IL-6, TNF-α AND DEXAMETHASONE

The APPs are regulated by interleukin-1- and interleukin-6-type cytokines, glucocorticoids and growth factors (see "Introduction" in Section I). The effect of growth factors (bFGF, EGF) on SIP24/24P3 in Balb/c 3T3 cells are known (1-3). Here I studied the effects of IL-6 (interleukin-6-type cytokine), TNF-a (interleukin-1-type cytokine) and dexamethasone on SIP24/24P3 production in Balb/c 3T3 and BNL cells.

As described in the "Materials and Methods" of Section I, Balb/c 3T3 and BNL cells were grown to quiescence. IL-6, TNFα and dexamethasone were added at the final concentrations described for each experiment, and the cells were incubated for 24 hours. The medium was then removed, and the cells were rinsed twice with Tris-buffered salts. The cells were then metabolically labeled for 4 hours by incubation with 100 uCi/ml Tran^35S-label in DME (with 10% of the normal methionine concentration), 0.2% calf serum, and the additions as present during the previous 24-h incubation. The samples of medium were resolved by SDS-PAGE, 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 have been shown to be proportional to the relative amount of radioactive label associated with each protein band. These results were
normalized to the TCA-precipitable cpm in the post-nuclear 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. The results are shown in Fig. 1. (Balb/c 3T3 cells) and Fig. 2. (BNL cells), and the quantitative analysis of the results is summarized in Table 1. From the results, it can be concluded that: 1) In Balb/c 3T3 cells, SIP24/24P3 is induced by IL-6, TNF-α and dexamethasone. While IL-6 and TNFα have no additive or synergistic effect on SIP24/24P3 production, dexamethasone seems to have a synergistic effect on SIP24/24P3 production with IL-6 and TNFα. 2) In BNL cells, IL-6 alone has little or no effect on SIP24/24P3 production. TNFα induces SIP24/24P3 at all three concentrations tested. Dexamethasone strongly induces SIP24/24P3 when treated alone and causes an even greater induction when treated with IL-6 and TNFα. The results confirmed the speculation that the interplay of different regulating factors may be necessary to achieve the maximum induction of SIP24/24P3.
Fig. 1. Regulation of SIP24/24P3 production by IL-6, TNF-α and dexamethasone in Balb/c 3T3 cells. Quiescent 3T3 cells were incubated for 24 h with IL-6 and/or TNFα and/or dexamethasone and then metabolically labeled for 4 h. The culture media containing radiolabeled secreted proteins were resolved by SDS-PAGE and analyzed by fluorography. The X-ray films were exposed for 6 days at -70°C. The concentrations used of IL-6 (10-1000 U/ml) and of TNFα (0.1-10 ng/ml) are shown for each lane, dexamethasone (DEX) was added at 400 ng/ml. Numbers on the left show the positions of the proteins used as molecular weight markers with their molecular weights expressed in thousands.
Fig. 2. Regulation of SIP24/24P3 production by IL-6, TNFα and dexamethasone in BNL cells. The experiment was carried out and analyzed in the same way as for Fig. 1. except BNL cellw was used and the films were exposed for 32 days.
Control
IL-6 10
IL-6 100
IL-6 1000
TNF 0.1
TNF 1.0
TNF 10.0
IL-6 100 + TNF 1.0
DEX
DEX + IL-6 100
DEX + TNF 1.0
DEX + IL-6 100 + TNF 1.0
Table 1. Quantitative analysis of the effects of IL-6, TNFα and dexamethasone on SIP24/24P3 production in Balb/c 3T3 and BNL cells. The relative amounts of SIP24/24P3 protein were determined by scanning each band on the fluorograms. The obtained values were normalized to the TCA precipitable cpm of matched postnuclear supernatants of the labeled cells. Each value is the average of duplicates from a single experiment. The standard deviations are shown. This experiment was repeated three times with similar results.
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>3T3</th>
<th>BNL</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.24</td>
<td>1.00 ± 0.47</td>
</tr>
<tr>
<td>IL-6 10 U/ml</td>
<td>1.00 ± 0.02</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>IL-6 100 U/ml</td>
<td>1.90 ± 0.14</td>
<td>1.16 ± 0.51</td>
</tr>
<tr>
<td>IL-6 1000 U/ml</td>
<td>4.10 ± 0.31</td>
<td>1.13 ± 0.28</td>
</tr>
<tr>
<td>IL-6 100 U/ml</td>
<td>1.03 ± 0.35</td>
<td>4.73 ± 1.37</td>
</tr>
<tr>
<td>TNF-α 0.1 ng/ml</td>
<td>2.49 ± 0.17</td>
<td>8.60 ± 0.48</td>
</tr>
<tr>
<td>TNF-α 1.0 ng/ml</td>
<td>4.05 ± 0.32</td>
<td>5.70 ± 2.09</td>
</tr>
<tr>
<td>TNF-α 10.0 ng/ml</td>
<td>2.30 ± 0.22</td>
<td>11.69 ± 0.44</td>
</tr>
<tr>
<td>DEX 400 ng/ml</td>
<td>2.25 ± 0.17</td>
<td>14.64 ± 0.36</td>
</tr>
<tr>
<td>DEX 400 ng/ml</td>
<td>9.69 ± 0.12</td>
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</tr>
<tr>
<td>DEX 400 ng/ml</td>
<td>9.28 ± 0.33</td>
<td>19.45 ± 0.33</td>
</tr>
<tr>
<td>DEX 400 ng/ml</td>
<td>10.79 ± 0.20</td>
<td>23.68 ± 0.14</td>
</tr>
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</table>
RT-PCR was carried out to clone the full length SIP24/24P3 cDNA. Based on the published cDNA sequence (4), the forward PCR primer was designed to be identical with the 5' end of the sense strand and the reverse primer was designed to be complementary to the 3' end of the sense strand. To facilitate the subsequent cloning, Xho I and Hind III restriction enzyme sites were added to the ends of the two primers respectively. The RNA template was total RNA extracted from Balb/c 3T3 cells superinduced with dexamethasone and cycloheximide (5). The cDNA template was obtained by reverse transcribing 5 ug of the total RNA template, and PCR was carried out to amplify the cDNA template. The amplified DNA was extracted, digested with Xho I and Hind III. Then the cDNA was extracted again and ligated into the plasmid pBluescript. The plasmid construct was electroporated into XL-1 blue cells and examined. Six out of 10 colonies examined were found to contain the expected insert of about 0.87 kb. Three clones were purified and sequenced using T3, T7-2 primers and the newly synthesized QL685 primer which corresponds to position 594-613 in published 24P3 cDNA. All three clones were found to be identical to 24P3 cDNA with the exception of a single point mutation in each at three different positions within the coding region. Two clones were digested with Xmn I respectively and the mutation-free regions from these two clones were ligated together to generate a mutation free full length cDNA. An alignment of the religated full length cDNA sequence with the reported 24P3 sequence is shown in Fig. 1.
The cloning of full length 24P3 cDNA makes it possible to express the protein and subsequently characterize its property and function.
Fig. 1. Alignment of RT-PCR cloned full length cDNA with 24P3 sequence. The sequence on the top is the RT-PCR cloned full length cDNA. The sequence at the bottom is the 24P3 cDNA.