Identification of a short form of ubiquitin-specific protease 3 that is a repressor of rat glutathione S-transferase gene expression

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The transcription rate and protein expression from both GSTA2 (glutathione S-transferase A2) and albumin genes decrease in rat liver after IL-6 (interleukin 6) plus DEX (dexamethasone) treatment of primary hepatocytes or after LPS (lipopolysaccharide)-induced acute-phase response in animals. The down-regulation is associated with the induced expression of a nuclear protein (termed IL6DEX-NP for IL-6/DEX-induced nuclear protein) that binds to a specific site on the promoter of GSTA2, leading to a decrease in transcriptional activity. IL6DEX-NP is not similar to other transcription factors, and, for identification, we functionally cloned it from a rat liver library using a yeast one-hybrid screen based on DNA-binding activity. The cloned sequence was a truncated form of USP3 (ubiquitin-specific protease 3) and the truncated USP3 protein in a yeast extract bound to DNA containing the IL6DEX-NP recognition sequence. Using 5′- and 3′-RACE (rapid amplification of cDNA ends), the complete sequence of USP3 was found in liver from LPS-treated rats. However, using Western blot analysis, only truncated forms of USP3 could be identified in nuclear extracts from LPS-treated rat livers. A GSTA2 promoter–reporter gene plasmid and USP3-expressing plasmids were transfected into rat hepatoma cells. Expression of the short form of USP3, but not the full-length protein, abolished expression from the reporter gene. Chromatin immunoprecipitation localized USP3 to the GSTA2 promoter in rat hepatocytes *in vivo*. We believe that the short form of USP3 is IL6DEX-NP and that it may play an important role in the negative regulation of proteins during the acute-phase response.

Key words: acute-phase response, albumin, glutathione S-transferase (GST), hepatic nuclear factor 1 (HNF1), sepsis, ubiquitin-specific protease 3 (USP3).

INTRODUCTION

The acute-phase response is an innate immune system reaction to infection, burns and other acute injuries. In the acute-phase response, pro-inflammatory factors such as the cytokines TNFα (tumour necrosis factor α), IL-1 (interleukin 1) and IL-6 are elevated in the circulation and stimulate hepatocytes to synthesize and secrete large amounts of specific proteins termed positive acute-phase proteins. There are also negative acute-phase proteins whose synthesis declines during this response. Rat GSTA2 (glutathione S-transferase A2), albumin and other GST isoenzymes are negative acute-phase liver proteins [1] and their expression decreases during LPS (lipopolysaccharide)-induced acute-phase response in rats or following IL-6 plus DEX (dexamethasone) treatment of rat hepatocytes in primary culture. In the case of GSTA2 and albumin, the decrease in expression is due both to a decline in HNF1 (hepatic nuclear factor 1) activity early in the acute-phase response and to the increase in the levels of a nuclear DNA-binding protein (termed IL6DEX-NP for IL-6/DEX-induced nuclear protein) that binds to the GSTA2 and albumin gene promoters late in the acute-phase response and decreases their transcriptional activity [2–4]. The IL6DEX-NP-binding site overlaps the promoter binding site for HNF1, a transcription factor that is necessary for the basal and induced expression of GSTA2, albumin and presumably other liver proteins with HNF1-binding sites in their promoters [5].

Previous studies showed that IL6DEX-NP binds to a core nucleotide sequence, TGATT, and that its molecular mass was approx. 28 kDa. Despite numerous studies, IL6DEX-NP could not be shown to be related to known transcription factors, suggesting that it may be unique. Therefore, to identify IL6DEX-NP, we used a yeast one-hybrid screening system to functionally select IL6DEX-NP from a cDNA expression library constructed from rat liver mRNA based on the ability of expressed proteins to bind to core IL6DEX-NP-binding sites [6]. Using a variation of the core IL6DEX-NP-binding sequence that reduced HNF1 binding (TGATC), clones were identified that coded for a protein with a molecular mass of approx. 38 kDa. Sequence analysis showed the cloned sequence to be identical with that which codes the C-terminal region of USP3 (ubiquitin-specific protease 3) [also called UBP3 (ubiquitin protease 3)] (EC 3.1.2.15). USP3 is a member of the UCH (ubiquitin C-terminal hydrolase) type 2 subfamily of the deubiquitinating enzyme superfamily [7,8] with a homologue in humans that has ubiquitin hydrolase activity, but which has not been well characterized functionally [9]. In the present study, we provide evidence that shUSP3 (short form of USP3) is IL6DEX-NP and is formed by either alternative splicing or post-translational modification of USP3.

EXPERIMENTAL

Rat liver library screening

To identify rat IL6DEX-NP, a yeast one-hybrid expression system (MATCHMAKER One Hybrid System; BD Biosciences Clontech) was used to screen a cDNA expression library

Abbreviations used: 3-AT, 3-aminotriazole; ChIP, chromatin immunoprecipitation; DEX, dexamethasone; EMEM, Eagle's minimal essential medium; EMSA, electrophoretic mobility-shift assay; GAL4AD, activation domain of GAL4; GST, glutathione S-transferase; HNF1, hepatic nuclear factor 1; IL, interleukin; IL6DEX-NP, IL-6/DEX-induced nuclear protein; LPS, lipopolysaccharide; ORF, open reading frame; RACE, rapid amplification of cDNA ends; USP3, ubiquitin-specific protease 3; shUSP3, short form of USP3.

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The nucleotide and predicted amino acid sequences for rat USP3 have been deposited in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number NM_001025424.

constructed from rat liver mRNA (MATCHMAKER rat liver cDNA library; BD Biosciences Clontech). Double-stranded target or bait oligonucleotides were synthesized [Y1H1, 5′-d(AATTCCTAAGTTATGATCAATAACCACTAAGTTATGATCAATAACCACTAAGTTATGATCAATAACCAT)-3′ for pHISi-1 with EcoRI and XbaI cuts; Y1H2, 5′-d(AATTCCTAAGTTATGATCAATAACCACTAAGTTATGATCAATAACCACTAAGTTATGATCAATAACCAC)-3′ for pLacZi with EcoRI and XhoI cuts] to contain three consecutive IL6DEX-NP consensus binding sites (underlined) that were mutated to minimize HNF1 binding as determined previously by EMSA (electrophoretic mobility-shift assay) [3]. The specificity of binding of bait oligonucleotides was confirmed by unlabelled competition EMSA (see below and Figure 1). The bait oligonucleotides were cloned into pHISi-1 (for histidine prototroph selection) and pLacZi vectors [for *lacZ* (β-galactosidase) gene product screens] and these reporter plasmids were used to transform yeast (strain YM4271). Liver library plasmids (pACT2 with a *Leu2* selective marker) containing in-frame fusions of a rat liver cDNA with the GAL4AD (activation of GAL4) domain were used to co-transform the yeast strains carrying target pHISi-1 and pLacZi plasmids, and positive clones were selected based on histidine (*HIS3* reporter gene) and leucine prototroph selection with 15 mM 3-AT (3-aminotriazole) to elevate the growth threshold and by β-galactosidase product screening (*lacZ* reporter gene) [6].

Functional characteristics of bait oligonucleotides

The suitability of the yeast one-hybrid bait oligonucleotides for binding IL6DEX-NP was established by unlabelled competition EMSA as described previously [3]. Briefly, radioactively labelled wild-type GSTA2 promoter sequence was incubated with nuclear extracts from IL-6 plus DEX-treated rat hepatocytes grown in primary culture and increasing amounts of unlabelled bait oligonucleotide concentrations. To determine the HNF1- and IL6DEX-NP-binding characteristics of the mutated GSTA2 promoter reporter sequences, EMSA was performed with radioactively labelled mutant oligonucleotides and nuclear extracts from rat hepatocytes grown in primary culture with DEX in the presence or absence of IL-6.

5′- and 3′-RACE (rapid amplification of cDNA ends)

The cloned nucleotide sequence of the protein selected for IL6DEX-NP-binding activity obtained from library screening matched the C-terminal portion (326 of 520 amino acids) of the ORF (open reading frame) of a predicted rat mRNA sequence for USP3 (GenBank® accession number XM_343415) based on a BLAST search of the NCBI database. This short form of USP3 is designated shUSP3. To determine whether the shUSP3 sequence was a portion of a longer mRNA sequence in liver cells, 5′- and 3′-RACE was performed with SMART RACE cDNA reagents from BD Biosciences Clontech. Total RNA was isolated from rat liver and from rat hepatoma H4-II-E cells (obtained from and cultured according to recommendations from A.T.C.C.) with Nucleospin II RNA columns. 5′-RACE- and 3′-RACE-ready cDNA was produced and amplified from the total RNA and 5′-RACE PCR was performed with Universal Primers and the gene-specific primer GSP3 (5′-GCCACCCTGAAGTTCCAAGTGCAGATG-3′) which hybridizes with residues 921–947 of the yeast clone sequence for shUSP3. 3′-RACE was performed with the gene-specific primer EGSP5 (5′-GATGCACAGATACCCTTACTCAAC-3′) which hybridizes with residues 297–320 (see Figure 3). Additional PCR primers for the complete ORF were designed and used in PCR to amplify sequences from reverse-transcribed mRNA isolated from rat liver and H4-II-E cells. PCR products were analysed on 0.9 % agarose gels; isolated DNA bands were purified with QIAquick Kit reagents (Qiagen Sciences), and sequenced directly using gene-specific primers. PCR products that overlapped the sequence of shUSP3 were identified and ligated into pCR2.1-TOPO vector, cloned in bacteria and purified. Insert lengths were verified by restriction digests, and inserts were sequenced with appropriate vector-specific and gene-specific primers.

DNA-binding activity of shUSP3

Extract from a yeast clone that expressed shUSP3 protein was tested to determine whether the expressed protein behaved similarly to IL6DEX-NP *in vitro*. Extract from a yeast strain with no pACT2 expression library plasmid and one from a pACT2 vector containing no library insert (empty vector) were used as negative controls. To prepare yeast extracts for EMSA, single colonies were shaken (250 rev./min) in 25 ml of selective medium for 48 h at 30 °C. Cells were collected by centrifugation at 1000 ***g*** for 5 min at 4 °C, washed with 15 ml of ice-cold water, and recentrifuged. The cells were resuspended in 15 ml of 67 mM potassium phosphate buffer (pH 7.4) containing 10 units/ml Lyticase (Sigma) for 90 min at room temperature (23 °C) to digest the cell walls. Cells were collected by centrifugation at 1000 ***g*** for 5 min at 4 °C and were resuspended in 10 mM Hepes buffer (pH 7.9) containing 150 mM NaCl, 1 mM EDTA, 1 × Complete™ EDTA-free protease inhibitor cocktail (Roche) and 6 % (v/v) Nonidet P40, and frozen in liquid nitrogen for 10 min. Cells were thawed at 4 °C and homogenized with a rotor/stator homogenizer. The homogenate was centrifuged at 800 ***g*** for 30 s at 4 °C to remove debris, and the supernatant was centrifuged at 3200 ***g*** for 8 min at 4 °C to pellet the nuclei. The pellet was resuspended in 300 μl of 20 mM Hepes buffer (pH 7.9) containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM EDTA, 25 % (v/v) glycerol and 1 × Complete™ EDTA-free protease inhibitor and applied to a QIAshredder column (Qiagen Sciences) that was centrifuged at 10000 ***g*** for 2 min. The eluate was divided into 50 μl aliquots and stored at − 80 °C. EMSAs of yeast extracts and competition assays were performed as described previously [3].

Production and affinity purification of USP3 antiserum

Two peptides (SQFRSKRSKNQENG and DETELYMCHKCKKKQK) were selected from the shUSP3 sequence based on their rarity, predicted antigenicity, hydrophobicity and degree of difficulty for synthesis. The peptides were conjugated to a carrier (Multiple Antigenic Peptide methodology; Invitrogen) and used to immunize rabbits for polyclonal antibody production. Specific antibodies were purified from whole serum by affinity chromatography. Equimolar amounts of the two peptides from the shUSP3 sequence that were used for polyclonal antibody production in rabbits were mixed together and coupled to a HiTrap NHS (*N*-hydroxysuccinimide)-Activated HP column (1 ml; AP Biotech) following the column manufacturer's instructions. Specific antibodies were purified from rabbit immune serum by a modification of a published method [10]. Briefly, serum was heated at 56 °C for 30 min to inactivate complement, clarified by centrifugation at 10000 ***g*** for 15 min at 4 °C, and filtered through glass filter paper. Serum was diluted into binding buffer (0.02 M Tris/HCl, pH 7.5, and 0.15 M NaCl) and recirculated three times through the affinity column at 4 °C. The column was washed with 20 ml of binding buffer and 20 ml of high-salt buffer (0.02 M Tris/HCl, pH 7.5, and 0.5 M NaCl). Acid-sensitive antibodies were eluted with 10 ml of 0.2 M glycine (pH 2.5), and fractions

were collected into 1 M Tris/HCl (pH 8) to neutralize them. The column was washed with buffer (0.02 M Tris/HCl, pH 8.8) and base-sensitive antibodies were eluted with 0.1 M triethylamine (pH 11.5), while fractions were collected into 1 M Tris/HCl (pH 8) to neutralize them. Fractions containing antibodies were combined, concentrated and diafiltered into PBS plus 0.05 % sodium azide, pH 7.5, and 100 μl aliquots were stored frozen until used.

Expression of USP3 forms in bacteria and Western blotting

The nucleotide sequence for shUSP3 was ligated into bacterial expression vector pTrcHis (which includes an N-terminal His_6 epitope tag) and that for USP3 was ligated into pET41 eK/LIC (which includes an N-terminal GST epitope tag) and both were transfected separately into competent bacterial cells. Protein expression was induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) for 3 h, and bacteria were lysed by sonication. USP3 and shUSP3 were purified by affinity chromatography. Western blotting was performed after transferring proteins from SDS/PAGE gels on to PVDF membranes (Immobilon) with anti-USP3 (1:6000–1:8000) and horseradish-peroxidase-conjugated anti-rabbit (1:2000; Amersham Biosciences) antibodies, and detection was on film with enhanced chemiluminescence reagents (PerkinElmer).

Affinity purification of IL6DEX-NP from rat liver

Affinity purification of proteins from liver nuclear extracts of LPS-treated rats was performed at 4 °C using a modified oligonucleotide affinity trapping method [11]. Briefly, anchor oligonucleotides with the repeat sequence $(AC)_5$ were attached covalently to CNBr-activated Sepharose 4B beads (Sigma) and the resin was packed into a 1 ml FPLC column and equilibrated with binding buffer (0.01 M Tris/HCl, pH 7.9, 0.001 M EDTA and 0.01 M NaCl). Rats were treated with LPS, and, 48 h later, nuclear extract was made from the livers as described previously [4]; the time point was chosen as the point of maximum IL6DEX-NP expression following a single dose of LPS. IL6DEX-NP expression and binding to GSTA2 promoter was verified by EMSA (results not shown). Trapping oligonucleotides made with single-stranded overhanging repeat sequences $(GT)_5$ and a double-stranded IL6DEX-NP-binding site with mutations to reduce HNF1 binding [4] were incubated with the nuclear extract. Approx. 95 ml of a mixture of trapping oligonucleotide and diluted liver nuclear extract was passed slowly through the anchor affinity column and then washed with 25 ml of binding buffer. A step change to a buffer of higher salt concentration (0.01 M Tris/HCl, pH 7.9, 0.001 M EDTA and 0.5 M NaCl) was then used to elute bound protein and an additional step change to a buffer of higher salt concentration still (0.01 M Tris/HCl, pH 7.9, 0.001 M EDTA and 1.2 M NaCl) was used to elute the trapping oligonucleotide from the anchor column. Eluted protein was concentrated and separated by SDS/PAGE, and Western blotting was performed with anti-USP3.

Transient transfection to determine functional activity of USP3 forms

To determine whether the yeast clone protein selected by one-hybrid screening was able to suppress transcription of the GSTA2 gene, we subcloned the coding regions of both the shUSP3 sequence (1612 nt without the GAL4AD sequence) and the full-length USP3 sequence (2305 nt) into the expression vector pcDNA3.1/His and separately co-transfected them into rat H4-II-E hepatoma cells with firefly luciferase reporter vector pGL3-Basic (Promega) that contained the wild-type full-length GSTA2 promoter [3] sequence (1.6luc). Additionally, the expression vector pcDNA3.1/His with no insert (empty vector) was co-transfected with 1.6luc as a control. All plasmids used for transfection were cloned into bacteria and purified with EndoFree Maxi Kits (Qiagen), and DNA was quantified with a Quant-iT DNA assay kit (Molecular Probes). Rat H4-II-E hepatoma cells were cultured in EMEM (Eagle's minimal essential medium) plus 10 % foetal bovine serum and 1 % penicillin/streptomycin according to A.T.C.C. recommendations. All rat hepatoma cells used for reporter assays were co-transfected with 0.08 μg/well *Renilla* luciferase vector phRL-null (Promega) to control for variation in transfection efficiency. Cells were plated at a density of 2×10^4 cells/well on 24-well dishes and were cultured overnight to approx. 90 % confluence. For transfection, the medium was changed to EMEM alone containing plasmid DNA (0.36 μg/plasmid per well) and Lipofectamine™ 2000 transfection reagent (Invitrogen) and was incubated for 6 h. The medium was exchanged for fresh EMEM containing 20 % serum without antibiotics, and the cells were cultured for an additional 12 h and then lysed. Luciferase reporter activities were measured in a luminometer (Berthold Sirius II) with Promega Dual-Luciferase Reporter Assay System reagents.

ChIP (chromatin immunoprecipitation) to localize USP3 to the GSTA2 promoter *in vivo*

To determine whether the rat liver GSTA2 promoter is a genuine *in vivo* binding site for USP3, we performed ChIP assays by modifications to the method of Ge et al. [12]. Rats were treated with LPS as described above. Hepatocytes were prepared by *in situ* collagenase perfusion of livers as described previously [2], and they were diluted to 10^8 cells/20 ml of medium for cross-linking with formaldehyde. Complete™ EDTA-free protease inhibitor cocktail tablets were substituted for individual protease inhibitors, and cells were lysed with a rotor/stator homogenizer. For immunoprecipitations, reagents and methods of the Roche Immunoprecipitation kit (Protein A) were used. Precleared chromatin samples were incubated with 6 μl of anti-USP3 antibodies or with 6 μl of pre-immune serum from the same animal; chromatin from a saline-treated animal with no immunoprecipitation was used as a positive PCR control. PCR was performed (32 cycles) with primers [5′-d(CCAACACTAATGGGGAGATGGTCGCA)-3′ and 5′-d(AGCCTGCTCTAGGTCTCAGTGCAG)-3′] specific for the GSTA2 promoter sequence containing the IL6DEX-NP-binding site. The PCR-amplified DNA product band from anti-USP3-precipitated chromatin from an LPS-treated rat liver was excised and sequenced.

RESULTS

Functional characteristics of bait oligonucleotides

Radioactively labelled probe (30 nt) spanning the HNF1 site of wild-type GSTA2 promoter bound and shifted HNF1 and IL6DEX-NP. Increasing concentrations of unlabelled bait oligonucleotides competed for binding of IL6DEX-NP, while binding of HNF1 was largely unaffected (Figure 1). These results established the suitability and specificity of the bait oligonucleotides for yeast one-hybrid screening.

Rat liver library screening

Yeast clones were selected based on their ability to grow on histidine- and leucine-deficient medium and by expression of β-galactosidase. Of the first six clones to appear during library screening that manifested both characteristics, three had

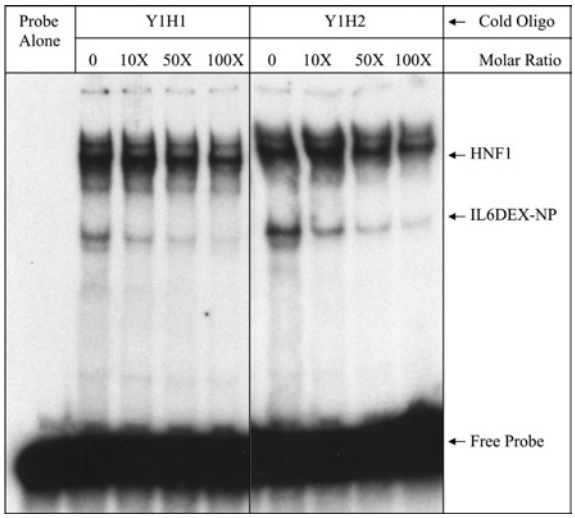

Figure 1 Bait oligonucleotides for yeast one-hybrid screening

Unlabelled oligonucleotides ('Cold Oligo') (Y1H1 and Y1H2; see the Experimental section) containing three consecutive IL6DEX-NP consensus binding sites that were used as target or bait sequences for yeast one-hybrid screening competed with radioactively labelled GSTA2 promoter for binding with IL6DEX-NP, but not for binding with HNF1. Nuclear extracts from rat hepatocytes cultured with IL-6 plus DEX were incubated with radiolabelled GSTA2 promoter sequence containing HNF1- and IL6DEX-NP-binding sites and increasing molar ratios of Y1H1 or Y1H2 and then were electrophoresed on a 4 % native acrylamide gel.

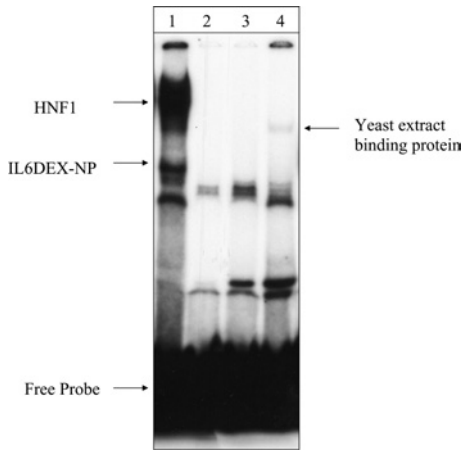

Figure 2 EMSA with extract from yeast clone that expresses shUSP3 protein showing new protein binding to radioactively labelled wild-type GSTA2 IL6DEX-NP-binding sequence

Identical results were obtained when a mutant GSTA2 probe was used (mutated to retain IL6DEX-NP binding but not HNF1 binding). The protein expressed in yeast is a fusion of GAL4AD and shUSP3 proteins and therefore the probe band is shifted to a molecular mass greater than that of the shUSP3 protein alone. Lane 1, nuclear extract from hepatocytes cultured with IL-6 and DEX (positive control); lane 2, extract from yeast with integrated target reporter plasmids (negative control); lane 3, extract from yeast with integrated target reporter plasmid and GAL4AD library plasmid with no insert (negative control); lane 4, extract from yeast expressing GAL4AD–shUSP3 fusion protein.

sequences that were identical with the C-terminal portion of the sequence for rat USP3-like protein (GenBank® accession number XM_343415) [13]. Over the course of the screening, we sequenced 50 clones, only the USP3-like protein was cloned more than once, and it was the only protein that was potentially a nuclear protein. However, a few of the selected clones with weak β-galactosidase activity coded for unknown or hypothetical proteins, but were not selected more than once.

DNA-binding activity

Extract from a yeast clone expressing shUSP3 protein in fusion with GAL4AD was used to determine whether it had DNA-binding activity in EMSA by shifting a radiolabelled wild-type GSTA2 oligonucleotide probe containing the IL6DEX-NP-binding site. A protein in extracts from the yeast clone, but not in extracts from control yeast cells, shifted radiolabelled wild-type IL6DEX-NP-binding site oligonucleotides (Figure 2); the same pattern of binding was seen with labelled oligonucleotide mutated to reduce HNF1 binding (results not shown). The radiolabelled probe bands were shifted to a higher molecular mass as compared with that of IL6DEX-NP, which is expected because, in yeast, shUSP3 is expressed as a fusion protein with GAL4AD.

5′- and 3′-RACE

The nucleotide sequence of shUSP3 from the yeast clones included 678 bp out of a total of 1560 bp of the ORF of the homologous mouse and human USP3 sequences and codes for 326 of a total of 520 amino acids; the missing sequence was contiguous and included the N-terminus of USP3. 5′- and 3′-RACE methods with gene-specific primers from the shUSP3 sequence indicated the presence of additional 5′ sequence in rat liver cells. A longer sequence of approx. 2250 bp with an ORF of 1560 bp was identified (Figure 3). The nucleotide sequence of complete, putative rat USP3 (GenBank® accession number NM_001025424) had 95 % identity with that of mouse USP3 and 87 % identity with human USP3, while the amino acid sequence had 98 % identity with that of mouse and 95 % identity with human USP3 (results not shown). An approx. 2250 bp nucleotide sequence was readily amplified with gene-specific primers by PCR from reverse-transcribed mRNA prepared from rat liver that, when sequenced, had an ORF of 1560 bp with a consensus Kozak sequence [14] at the putative ATG translation initiation codon and a consensus polyadenylation sequence and poly(A) residues in the 3′-untranslated region following a translation stop codon.

Expression of USP3 forms in bacteria

USP3 and shUSP3 were detected and affinity-purified after expression in bacteria. Purified anti-USP3 detected one primary band of each protein in Western blots (Figure 4). Both proteins necessitated short expression periods in bacteria owing to their toxicity and each had several immunoreactive bands, presumably proteolytic fragments, of lower molecular mass. Affinity-purified anti-USP3 specifically recognized both full-length USP3 and shUSP3 at the predicted molecular masses of the fusion proteins.

Affinity purification of IL6DEX-NP from rat liver

To determine which form of USP3 was present in the nuclei of rat hepatocytes, we used the IL6DEX-NP DNA-binding site to affinity-trap proteins from nuclear extracts from livers of LPS-treated rats. Two major anti-USP3 immunoreactive bands at 38 and 28 kDa were identified in the affinity-purified material by Western blotting (Figure 4). Therefore shUSP3 is present in nuclear extracts from LPS-treated rat liver. The faint band at approx. 59 kDa may represent full-length USP3, which has a predicted molecular mass of 58 906 Da.

Transient transfection to determine functional activity

We showed previously that the molecular mass of IL6DEX-NP was approx. 28 kDa, suggesting that shUSP3 and not full-length

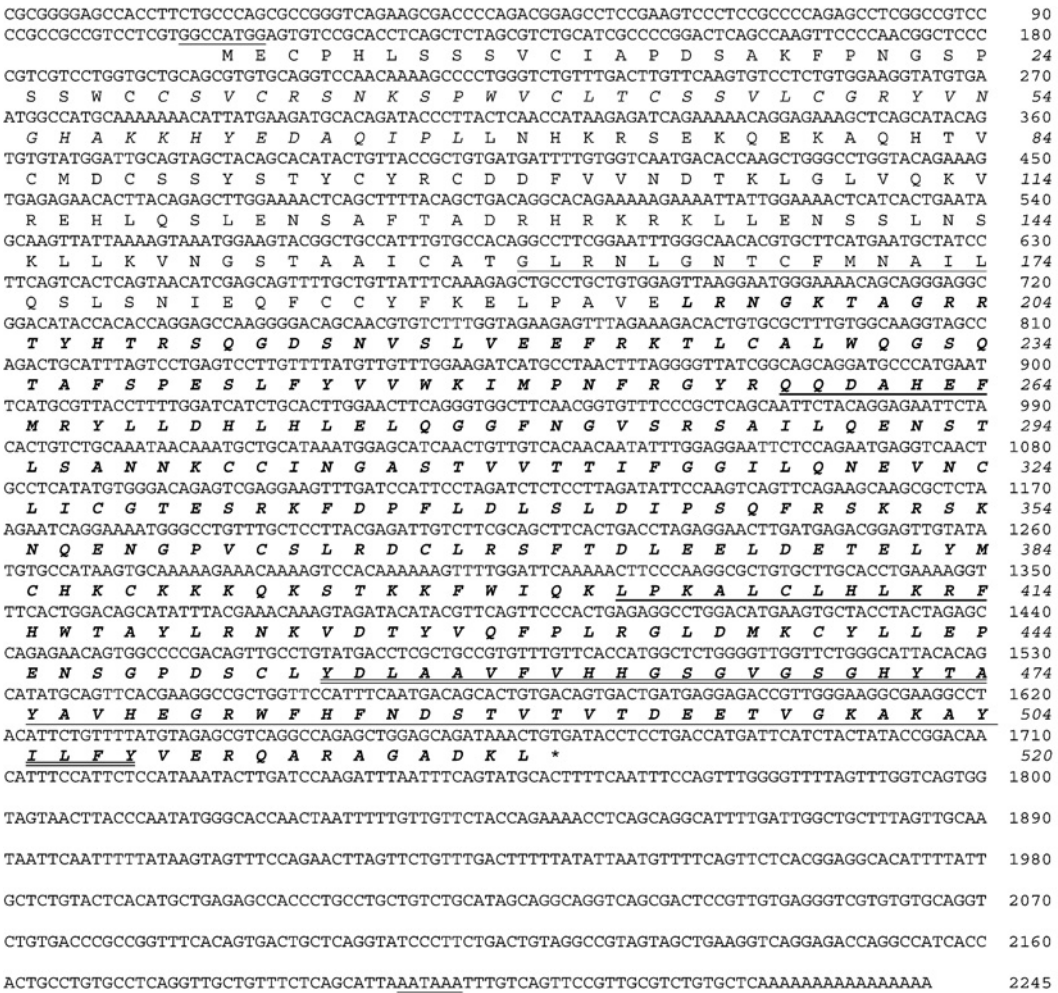

Figure 3 Complete nucleotide and amino acid sequences of rat USP3 compiled from a rat liver cDNA library clone and from 5′- and 3′-RACE experiments with rat liver mRNA

The protein sequence for shUSP3 is shown in bold italics and includes amino acids 195–520. A Kozak sequence is underlined at the translation start site, and a consensus polyadenylation sequence is underlined beginning at nucleotide 2197. Conserved cysteine (Cys-box)- and histidine (His-box)-containing regions are double-underlined; Asp- and KRF-boxes are single underlined. The zinc finger ubiquitin-binding domain (ZnF_UBF) is shown in italics (residues 29–68 of the protein sequence).

USP3 was IL6DEX-NP [3]. We performed functional reporter studies to determine which form was a negative regulator of transcription. Transient transfection of a plasmid expressing the shUSP3 protein (326 amino acids) into rat hepatoma cells suppressed luciferase reporter gene transcription driven by the full-length GSTA2 promoter to less than 10% of control, while transient transfection of the complete rat USP3 sequence (520 amino acids) into rat hepatoma cells did not suppress GSTA2 reporter gene transcription (Figure 5).

ChIP

Hepatocyte chromatin fragments that were cross-linked to USP3 immunoreactive proteins in ChIP assays contained the genuine GSTA2 promoter sequence with the IL6DEX-NP-binding site in both untreated and LPS-treated rats (Figure 6), confirming that a form of USP3 is present on the GSTA2 promoter *in vivo*.

DISCUSSION

The acute-phase response in mammals is a common reaction to a variety of injuries and it results in changes in the expression of a number of hepatic genes [15–17]. Some of the mechanisms and consequences of positive changes in hepatic gene expression, for example the increased expression and secretion of α_1-acid glycoprotein, are known to be anti-inflammatory or immunomodulating [18], while the mechanisms, functions and consequences of the negative changes in others, such as the GSTs or albumin, are not known. Since Alpha class GSTs are important for hepatoprotection, the understanding of their down-regulation during an injury such as sepsis, when they may be most needed, is important. We showed previously that GSTA2 mRNA and protein down-regulation during the acute-phase response occurs due to the loss of HNF1 DNA-binding activity and to increased expression of IL6DEX-NP, which is a negative regulator of GSTA2 gene expression [4].

The goal of the present study was to identify IL6DEX-NP to provide a better understanding of how it regulates gene expression. We used a yeast one-hybrid method [6] to screen a rat liver cDNA library for expressible proteins with the functional ability to bind a DNA target sequence that binds IL6DEX-NP. We focused on one rat liver cDNA library sequence, shUSP3, because its expressed protein met the yeast one-hybrid growth-promoting criteria under stringent 3-AT growth conditions and had high

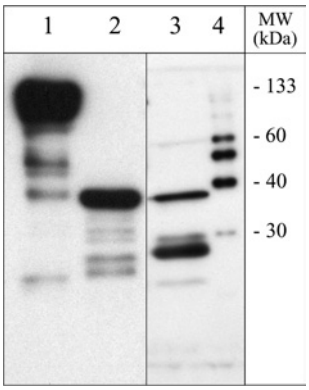

Figure 4 Expression and purification of USP3 forms from bacteria and immunoreactive affinity-trapped USP3 forms in nuclear extract from rat liver

Western blot after SDS/PAGE using affinity-purified anti-USP3 antibodies (1:6000). Secondary horseradish-peroxidase-conjugated goat anti-rabbit antibodies were used at 1:2000. Lane 1, purified (GSH affinity chromatography) protein fraction from bacteria expressing GST-tagged USP3; lane 2, purified (immobilized metal ion affinity chromatography) protein fraction from bacteria expressing His_6-tagged shUSP3 protein; lane 3, protein fraction from nuclear extract of LPS-treated rat liver eluted by first salt step (approx. 30 μg of total protein); two major immunoreactive bands (approx. 38 and 28 kDa) that were detected in the protein fraction eluted from the affinity column and were highly enriched relative to the starting material; lane 4, molecular-mass (MW) markers (sizes in kDa).

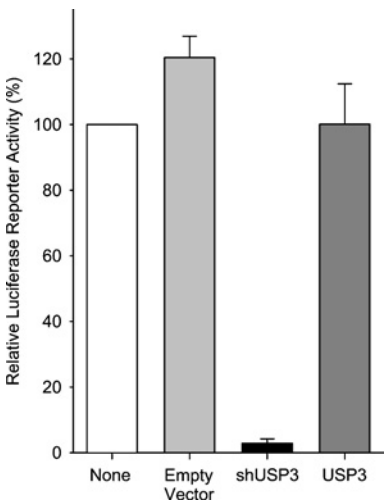

Figure 5 shUSP3 (38 kDa) suppresses luciferase reporter expression from a GSTA2 promoter–reporter construct within cells

Protein expression vectors for shUSP3, USP3 (59 kDa), and an empty vector control were co-transfected with GSTA2 promoter–reporter vector 1.6luc into rat hepatoma H4-II-E cells. Results are from three separate experiments with transfected cells from three wells of a 24-well plate for each group and were normalized for transfection variation by co-transfection with a vector that expressed *Renilla* luciferase.

β-galactosidase reporter activity. Additionally, of the first six highest growth rate yeast strains that were obtained, three expressed the shUSP3 protein sequence, while none of 50 other characterized clones was selected more than once. These results indicated that the shUSP3 protein has high selectivity for the target sequence containing the IL6DEX-NP core binding site. We believe that we have identified shUSP3 as IL6DEX-NP based on the following observations: (i) both proteins bind to the same core nucleotide sequence (Figure 2); (ii) both have similar molecular masses, 28–38 kDa (Figure 4); (iii) both are present in the nuclear extracts of liver obtained from LPS-treated rats (Figure 4); and (iv) both are effective inhibitors of the transcriptional activity of

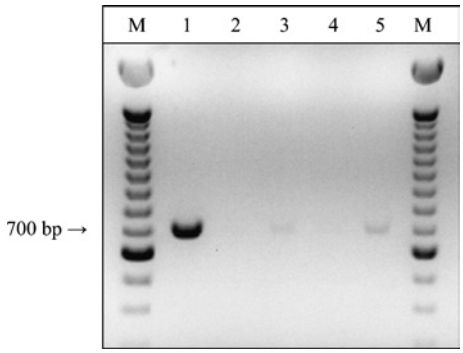

Figure 6 A form of USP3 was localized to the rat GSTA2 promoter sequence in hepatocytes from rats by ChIP

Rats were treated with saline (control) or LPS for 48 h, and hepatocytes were isolated. Formaldehyde-cross-linked chromatin was prepared from hepatocytes, sheared, pre-cleared with Protein A–agarose beads, and incubated with pre-immune serum or anti-USP3 antibodies. PCR was performed on the chromatin with a primer pair specific for a 706 bp region of the GSTA2 promoter containing the USP3-binding site. Specific GSTA2 promoter DNA was amplified only from anti-USP3-precipitated hepatocyte chromatin (lanes 3 and 5) and from input chromatin (lane 1). Lanes M, 100 bp markers; lane 1, input chromatin (positive PCR control; no immunoprecipitation); lane 2, pre-immune serum chromatin precipitation, control hepatocytes; lane 3, anti-USP3 chromatin precipitation, control hepatocytes; lane 4, pre-immune serum chromatin precipitation, LPS-treated hepatocytes; lane 5, anti-USP3 chromatin precipitation, LPS-treated hepatocytes. The amplified DNA band from anti-USP3-precipitated chromatin (lane 5) was purified from the gel and was sequenced and had 100 % identity with the genuine rat liver GSTA2 promoter sequence that includes the USP3-binding site.

the reporter construct containing the GSTA2 promoter (Figure 5). USP3 is present on the GSTA2 promoter *in vivo* in livers of both untreated and LPS-treated animals, which corresponds to our previous findings that IL6DEX-NP is present in livers of both untreated and LPS-treated rats [4]. We tried to obtain direct evidence of identity between IL6DEX-NP and USP3 by using anti-USP3 antibodies to supershift IL6DEX-NP in EMSA but were unsuccessful (results not shown).

We questioned whether shUSP3 was expressed in rat hepatocytes. Crude extracts of hepatocyte nuclei could not be shown by Western blot analysis to contain shUSP3. We therefore enriched the hepatocyte nuclear extracts by DNA affinity trapping. Two anti-USP3 immunoreactive proteins were identified in the enriched fractions from liver nuclear extract from LPS-treated rats. One immunoreactive protein was at 38 kDa, which is the same size as shUSP3 cloned from yeast, and was shown by transfection to repress expression from the GSTA2 promoter. The other protein was at 28 kDa, which is the same size as IL6DEX-NP determined previously by UV-cross-linking experiments [3]. Therefore nuclei prepared from hepatocytes from LPS-treated rats contain two short forms of USP3 (Figure 4). In previous EMSA experiments, IL6DEX-NP-binding activity was frequently resolvable into two closely spaced bands that behave essentially in parallel in terms of expression pattern, and it is likely that these bands are the 28 kDa and 38 kDa forms of USP3 [3,4].

Using 5′ and 3′-RACE on rat liver, we found mRNA that corresponded to a full-length rat USP3 sequence (520 amino acids) that was longer than shUSP3 cloned from a rat liver cDNA expression library and which was highly homologous with mouse and human USP3 sequences. Human USP3 is expressed as a 520-amino-acid protein at very low levels [9]. It is unclear whether full-length USP3 protein is expressed in rat liver because neither the mRNA nor protein has yet been isolated and characterized. However, an anti-USP3 immunoreactive band at approx. 59 kDa on the Western blot from the affinity-trapping experiment corresponds to the predicted mass of rat USP3 (58 906 Da) and

suggests that full-length USP3 is expressed at low levels and that it binds weakly to the affinity column or to column-bound short forms of USP3 or other proteins. The original NCBI sequence entry for a protein similar to rat USP3 was XM_343415 (gi:34864280), which was a computationally predicted sequence from EST (expressed sequence tag) data. The predicted protein was 435 amino acids in length (predicted molecular mass of 49.6 kDa) and consisted of several stretches of amino acids identical with those that we found in 5′- and 3′-RACE USP3 separated by several gaps. We have found no evidence for an immunoreactive 49.6 kDa form of USP3 in Western blots, even though this form would contain the peptide sequences to which anti-USP3 was directed. We conclude that the predicted sequence is in error, and in fact, the record was recently revised and superseded by XM_343415.2 (gi:62653840), which predicted an even shorter USP3-like protein of approx. 26 kDa. Again, the amino acid sequence of this predicted rat USP3 is identical with portions of the sequence that we obtained, but with several gaps. We conclude that this predicted sequence is also in error, but since it is derived from an expressed mRNA liver library source, this is additional evidence for the existence of a shorter mRNA for USP3-like protein similar to that which we cloned from a rat liver cDNA library. An additional rat sequence, XP_576424, also derived from an mRNA source, predicts a protein that aligns with our USP3 sequence with no gaps but is primarily from the N-terminus and suggests alternative mRNA splicing.

The mechanism for the generation of shUSP3 is unknown and it could be either alternative splicing or proteolytic cleavage of the full-length USP3 protein or both. Interestingly, mouse USP2 is expressed as two forms owing to alternative mRNA splicing: a 45 kDa and a 69 kDa protein, and the two proteins can form a complex [19]. In addition, there is evidence that human USP1 is post-translationally regulated by proteolytic cleavage into discretely sized protein fragments before proteasomal degradation as part of a cell-cycle-regulated pattern of expression [20].

USP3 belongs to a family of approx. 60 known or predicted deubiquitinating enzymes in rat [7] with at least as many forms in humans [21]. The specific functions of many of these proteins are unknown, but they appear to be involved in a diverse array of cellular processes including regulation of DNA repair [20], deubiquitination of oncogene protein products [22], and membrane receptor level regulation [23].

The mechanism by which shUSP3 suppresses GSTA2 and albumin transcription is unknown. An HNF1 site is required in the promoter for suppression to occur [3], and shUSP3 may interfere with the HNF1 activation of transcription in a variety of ways, including binding site competition, protein–protein interaction or other mechanisms. The DNA-binding activity of shUSP3 and its repressor activity are not dependent on the deubiquitinating activity of USP3 because shUSP3 lacks critical active site cysteine residues that are necessary for catalysis (Figure 3) [24]. Additionally, an N-terminal zinc-finger domain (znF_UBP) that is implicated in ubiquitin recognition by USP3 [25,26] is not necessary for DNA binding because it is also lacking in shUSP3 (Figure 3). Rat USP3 lacks an NLS (nuclear localization signal) [27]; however, by structural motif criteria, it is predicted to be a nuclear protein [28,29] as we have characterized it. USP3 is a member of an increasingly recognized and important group of proteins that perform several distinct cellular functions by virtue of different expression patterns, subcellular localization or adaptively emergent structural features [30,31]. We conclude that the protein with IL6DEX-NP transcriptional repressor activity during the acute-phase response in rats is either a 38 kDa or 28 kDa protein derived from a longer USP3 transcript by alternative mRNA splicing, proteolysis, or both.

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