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MOLECULAR MECHANISMS OF TRANSCRIPTIONAL REPRESSION BY THE ORPHAN RECEPTOR SHP

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To my daughter Alice.

ABSTRACT

SHP (Small Heterodimer Partner) is an atypical orphan member of the mammalian nuclear receptor family that only consists of a putative ligand-binding domain and thus cannot bind DNA. The aim of this thesis was to investigate the molecular mechanisms of the transcriptionally inhibitory effect of SHP. This was achieved by analysis of structural motifs within SHP, by studying nuclear receptor interactions, by isolation of upstream target co-factors and by characterization of SHP mutations.

In the first study, we provide evidence that SHP binds directly to the estrogen receptors α and β via LXXLL-related motifs. Similar motifs, referred to as nuclear receptor (NR) boxes, are usually critical for the binding of co-activators to the ligand-regulated activation domain AF-2 within nuclear receptors. We demonstrate that SHP variants, carrying either interaction-defective NR-box mutations or a deletion of the repressor domain, have lost the capacity to inhibit agonist-dependent estrogen receptor activation. Our study suggests that SHP acts as a transcriptional co-regulator by inhibiting the activity of nuclear receptors via occupation of the co-activator binding surface and via active repression. However, active repression mechanisms have remained elusive and may involve factors distinct from known nuclear receptor co-repressors.

In the second study, we describe the isolation of mouse EID1 (E1A-like inhibitor of differentiation 1) as the first co-inhibitor for SHP. We characterize the interactions between SHP and EID1 and identify two repression-defective SHP mutations that have lost the ability to bind EID1. We suggest histone acetyltransferases and histones as targets for EID1 action, and we propose that SHP inhibition of transcription involves EID1 antagonism of p300/CBP-dependent co-activator functions.

Recent evidence suggests the existence of a larger family of EID1-related proteins. In the third study, we describe a third family member designated EID3 that is highly expressed in testis and shows homology to a region of EID1 implicated in binding to p300/CBP. We demonstrate that EID3 acts as a potent inhibitor of nuclear receptor transcriptional activity by a mechanism that is independent of direct interactions with nuclear receptors, including SHP. However, EID3 directly binds to the C-terminus of CBP, which has been implicated to act as the interaction surface for nuclear receptor co-activators. Consistent with this idea, EID3 prevents recruitment of CBP to a natural nuclear receptor-regulated promoter. Our study suggests that EID-family members act as inhibitors of p300/CBP-dependent transcription in a tissue-specific manner.

In the fourth study, we characterize GPS2 (G-protein pathway suppressor 2), a subunit of the N-CoR/HDAC3 co-repressor complex, as an interaction partner for SHP. Specific interactions between GPS2 and SHP have been verified *in vitro* and *in vivo* and appear physiologically relevant as GPS2 mRNA is expressed in SHP target tissues. It is shown that GPS2 increases SHP-mediated repression of nuclear receptor target genes by enhancing intrinsic repression of the SHP ligand-binding domain. Taken together, these results suggest that SHP recruits a conserved co-repressor complex to nuclear receptor target genes via direct interactions with the GPS2 subunit.

SHP fulfils specific roles as inducible co-repressors in the feedback regulation of LRH-1 (liver receptor homologue 1) target genes involved in bile acid synthesis. In the fifth study, we have identified and characterized the homeodomain protein Prox-1 (Prospero-related homeobox 1) as an additional co-repressor for LRH-1. We show that

Prox-1 binds to LRH-1 via two LXXLL motifs and represses the LRH-1 dependent expression of SHP. Our hypothesis is that Prox-1 may modulate LRH-1 target gene expression during development and under normal physiological conditions, whereas SHP may primarily serve as a metabolic sensor under conditions of increased metabolic activity such as a high-cholesterol diet.

LIST OF PUBLICATIONS

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II. Båvner, A., Johansson, L., Toresson, G., Gustafsson, J-Å., and Treuter, E. (2002) A transcriptional inhibitor targeted by the atypical orphan NR SHP. EMBO Rep. 3 (5): 478-484

III. Båvner, A., Matthews, J., Gustafsson, J-Å., and Treuter, E. (2005) EID3; a novel EID family member and an inhibitor of CBP dependent coactivation. (Manuscript under revision for Nucleic Acids Research)

IV. Båvner, A., Sanyal, S., Gustafsson, J-Å., and Treuter, E. (2005) SHP recruits the N-CoR/HDAC3 co-repressor complex through direct interaction with GPS2. (Manuscript)

V. Steffensen, K.R., Holter, E., Båvner, A., Nilsson, M., Pelto-Huikko, M., Tomarev, S., and Treuter, E. (2004) Functional conservation of interactions between a homeodomain co-factor and a mammalian FTZ-F1 homologue. EMBO Rep. 5 (6): 613-619

CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acid(s)
AD	Activation Domain
AF	Activation Function
AHR	Aryl Hydrocarbon Receptor
AMF-1	Activation domain Modulating Factor (GPS2)
AR	Androgen Receptor
ARNT	Aryl hydrocarbon Receptor Nuclear Translocator
bp	Base pair(s)
CA	Cholic Acid
CAR	Constitutive Androstane Receptor
CBP	CREB-Binding Protein (similar to p300)
CDCA	Chenodeoxycholic Acid
CPF	CYP7A1 Promoter binding Factor (FTF/LRH-1)
CREB	cAMP Response Element-Binding protein
CYP7A1	Cytochrome P450 7A1, Cholesterol 7α-hydroxylase
CYP8B1	Cytochrome P450 8B1, Sterol 12α-hydroxylase
CYP1A1	Cytochrome P450 1A1
CYP2B	Cytochrome P450 2B
CYP3A	Cytochrome P450 3A
DBD	DNA-binding domain
DAX-1	Dosage-sensitive sex reversal Adrenal hypoplasia congenital critical region on the X chromosome, gene 1
E1A	Adenoviral E1A
EID1	E1A-like Inhibitor of Differentiation 1
EID3	E1A-like Inhibitor of Differentiation 3
ER	Estrogen Receptor
ERR	Estrogen receptor-Related Receptor
FTF	Alpha-Fetoprotein Transcription Factor (CPF/LRH-1)
FXR	Farnesoid X Receptor
GPS2	G-Proteinpathway Suppressor 2 (AMF-1)
GR	Glucocorticoid Receptor
HAT	Histone Acetyltransferases
HDAC	Histone Deacetylase
HNF-4	Hepatocyte Nuclear Factor-4
LBD	Ligand-Binding Domain
LBP	Ligand-Binding Pocket
LRH-1	Liver Receptor Homologue 1 (FTF/CPF)
LXR	Liver X Receptor
mSin3	mammalian Switch-independent 3 protein
N-CoR	Nuclear hormone receptor-Co-Repressor
NF-κB	Nuclear Factor-κB
NR	Nuclear Receptor
NR-box	Nuclear Receptor-box (LXXLL motif)
p300	E1A binding protein, p300 (similar to CBP)

PCR	Polymerase Chain Reaction
PPAR	Peroxisome Proliferator-Activated Receptor
RT-PCR	Reverse Transcriptase-PCR
Prox-1	Prospero-related homeobox 1
PXR	Pregnane X Receptor
RF	Repression Function
RAR	Retinoid Acid Receptor
RXR	Retinoid X Receptor
SHP	Small Heterodimer Partner
SF-1	Steroidogenic Factor 1
SMRT	Silencing Mediator of Retinoid and Thyroid hormone receptors
SRC-1	Steroid Receptor Co-activator 1, (NCOA1)
SRC-2	Steroid Receptor Co-activator 2, (TIF-2/GRIP1)
SRC-3	Steroid Receptor Co-activator 3, (ACTR, p/CIP, RAC-3, AIB-1, and TRAM-1)
SREBPs	Sterol Regulatory Element Binding Proteins
TBL1	Transducin Beta-Like protein 1 (Ebi)
TBL1-R	TBL-1-Related protein
TR	Thyroid Hormone Receptor

1 GENERAL INTRODUCTION

Molecular mechanisms of transcriptional repression by the orphan receptor SHP

This thesis investigates molecular and cellular mechanisms governing the metabolic control of gene expression. The work focuses on the orphan nuclear receptor SHP (Small Heterodimer Partner, NR0B2), which acts as a transcriptional co-repressor and is implicated to play important physiological roles in maintaining cholesterol and bile acid homeostasis.

1.1 TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

A gene is defined as "the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons)"(84). Chromatin is a dynamic and interactive structure that is assembled, remodeled and modified in response to cellular signaling. Rearrangements of chromatin structure are essential in regulating gene expression and other nuclear processes, such as DNA replication, recombination and repair, but also cell cycle progression and developmental transitions. The basic repeating unit of chromatin is the nucleosome, a protein octamer of core histones around which approximately 146 bp DNA are wrapped (91). Free N-terminal tails of the core histones protrude from the core octamer (93). The flexible N-terminal extensions contain conserved amino acid (aa) residues that are subject to various posttranslational modifications. The most prominent of these is acetylation, a process where an acetyl group of acetyl coenzyme A is linked to the ε-amino group of lysine by histone acetyltransferases (HATs) and may be removed by histone deacetylases (HDACs). A number of gene regulatory proteins have been identified as HATs or HDACs, or have been shown to act in complexes with such enzymes (91).

1.1.1 Transcription factors

1.1.1.1 Basal transcription factors

Basal transcription factors are typically defined as the minimal complement of proteins necessary to reconstitute accurate transcription from a minimal promoter (such as a TATA element or an initiator sequence). They are distinct from the regulatory transcription factors, which bind to sequences further away from the initiation site and serve to modulate levels of transcription. This regulation presumably occurs through interactions between the regulatory and basal transcription factors. The basal transcription complex assembles through an extensive series of protein-protein interactions. Although the basal factors can assemble on the promoter in a step-wise manner in vitro, there is some evidence that many of the factor interactions can occur in the absence of DNA and that some of the factors may pre-assemble into a "holoenzyme" [1].

[1] http://www.gene-regulation.com/pub/databases/transfac/cl.html

1.1.1.2 Regulatory transcription factors

Gene regulatory proteins recognize short defined segments of double-helical DNA and thereby determine which of the thousands of genes in a cell will be transcribed. Hundreds of gene regulatory proteins have been identified in a wide variety of organisms. Each of these proteins have unique features and most bind to DNA as homodimers or heterodimers and recognize DNA through one of a small number of structural motifs, including the helix-turn-helix motif, the homeodomain motif, zinc finger motif, the leucine zipper motif, and the helix-loop-helix motif. The precise amino acid sequence that is folded into the motif determines the particular DNA sequence that is recognized (1). Eukaryotic regulatory transcription factors are divided into five super classes which in turn are divided into a varying number of classes and subclasses[2].

Table 1. *Classification of regulatory transcription factors.*

Super class	**1:Basic Domains**	**2: Zinc-coordinating DNA-binding domains**	**3: Helix-turn-helix**	**4: Beta-Scaffold Factors with Minor Groove Contacts**	**0: Other Transcription Factors**
Class	*Leucine zipper factors (bZIP), Helix-loop-helix factors, NF-1, RF-X, bHSH*	*Cys4 zinc finger of NR type, diverse Cys4 zinc fingers, Cys2His2 zinc finger domain, Cys6 cysteine-zinc cluster*	*Homeo domain, Paired box, Fork head/ winged helix, Heat shock factors, Tryptophan clusters, TEA domain*	*RHR (Rel homology region), STAT, p53, MADS box, beta-Barrel alpha-helix transcription factors, TATA-binding proteins, HMG, Heteromeric CCAAT factors, Grainyhead, Cold-shock domain factors, Runt*	*Copper fist proteins, HMGI(Y), Pocket domain, E1A-like factors, AP2/EREBP-related factors*

[2] http://www.gene-regulation.com/pub/databases/transfac/cl.html

1.1.2 The nuclear receptor superfamily

Nuclear receptors (NRs) are regulatory transcription factors, which belong to super-class 2 "zinc-coordinating DNA-binding domains" and the "Cys4 zinc finger of NR type" class[3]. The binding of small lipophilic ligands (steroid hormones, thyroid hormone, retinoids, fatty acids, cholesterol metabolites) to their conserved C-terminal ligand-binding domain (LBD) allows them to switch between active and inactive states (11). Proteins sharing the same structure but without identified ligands are called orphan receptors. With the discovery of orphan receptors came the new concept of "reverse endocrinology". Historically, purified hormones were used as targets to find their partner receptors. With the advent of orphan receptors the method was reversed, with the orphan receptors now used to screen for binding and activating hormones (72). Several ligands for orphan receptors are still not identified and recent reports on the structure of the Nurr1 family orphan receptor binding domain show that these particular receptors are unable to bind ligands. This suggests that they are regulated exclusively via ligand-independent mechanisms (7).

NRs bind via a conserved DNA-binding domain (DBD) to hormone response elements (HREs) in promoter and enhancer regions belonging to their respective target genes. The HREs supply specificity to receptor monomer, homodimer, and RXR-heterodimer binding (2). Moreover, NRs bind stably and with quite high affinity to DNA even when their cognate HREs are assembled into chromatin (151). The transcriptional functions of NRs are guided by the activation function 1 (AF-1) located in the N-terminus, the activation function 2 (AF-2) located in the C-terminal LBD (42), and the repressor function (RF) also located in the C-terminus. Members of the NR superfamily are important regulators of cell differentiation, organ physiology and development (96).

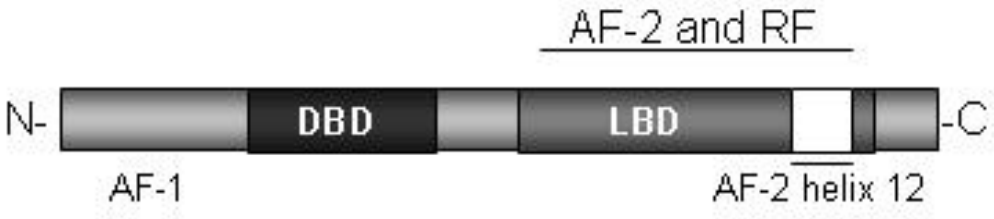

Figure 1. *Nuclear receptor structural organization.*

[3] http://www.gene-regulation.com/pub/databases/transfac/cl.html

NRs are of vital importance in many aspects of vertebrate development and adult physiology. They are divided into subfamilies (0-6) based on similarities of amino acid (aa) sequences and some other properties (25). The number of human NRs is limited to 48 family members that can be grouped into classes according to how they bind ligands (7) as demonstrated in table 2.

Table 2. *Nuclear receptor classification.*

Endocrine or paracrine	Nutritional sensors	Structural ligand	Orphan
Steroid hormone receptors			**Empty LBP**
ER α, β (NR3A1, 2) PR (NR3C3) AR (NR3C4) GR (NR3C1) MR (NR3C2)	PPAR α, β, γ (NR1C1,2,3) LXR α, β (NR1H3,2) FXR (NR1H4) PXR (NR1I2) CAR (NR1I3)	HNF4 α, γ (NR2A1,2) ROR α (NR1F1)	LRH-1 (NR5A2) ERR α, β, γ (NR3B1,2,3) ROR β, γ (NR1F2,3)
Non-steroid hormone receptors			**No LBP**
RAR α, β, γ (NR1B1,2,3) TR α, β (NR1A1,2) VDR (NR1I1)			NGFI-B (NR4A1) Nurr1 (NR4A2) Nor1 (NR4A3)
			Unknown LBP
			SHP (NR0B2) DAX-1 (NR0B1) COUP-TF α, β, γ (NR2F1,2,3) SF-1 (NR5A1) GCNF (NR6A1) TLX (NR2E1) PNR (NR3E3) TR2 (NR2C1) TR4 (NR2C2) RevErb α, β (NR1D1,2)

Table adopted from (7). Ligand-binding pocket (LBP). ER (estrogen receptor), PR (progesterone receptor), AR (androgen receptor), GR (glucocorticoid receptor). MR (mineralocorticoid receptor), RAR (retinoic acid receptor), TR (thyroid hormone receptor), VDR (vitamin D receptor), PPAR (peroxisome proliferator activated receptor), LXR (liver x receptor), FXR (farnesoid x receptor), PXR (pregnane x receptor), CAR (constitutive androstane receptor), HNF4 (hepatocyte nuclear factor 4), ROR (RAR-related orphan receptor), LRH-1 (liver receptor homologue 1), ERR (estrogen related receptor), NGFI-B (nerve growth factor IB-like), Nurr 1(Nur-related factor 1), Nor 1(neuron-derived orphan receptor 1), SHP (small heterodimer partner), DAX-1 (Dosage-sensitive sex reversal Adrenal hypoplasia congenital critical region on the X chromosome, gene 1), COUP-TF (chicken ovalbumin upstream promoter-transcription factor), SF-1 (Steroidogenic factor 1), GCNF (germ cell nuclear factor), TLX (tailless homolog), PNR (photoreceptor-specific nuclear receptor), TR2 (orphan nuclear receptor TR2), TR4 (orphan nuclear receptor TAK1), RevErba (V-erbA related protein EAR-1), Rev-erbβ (Rev-erb-beta).

1.1.2.1 Transcriptional regulation of nuclear receptor-dependent gene expression

The transcriptional functions of NRs are mediated by co-factors that are widely defined as co-repressors and co-activators on the basis of their need for gene transcriptional repression or activation (42,96,101).

Histone deacetylases (HDACs) arrest transcription by modification of the chromatin template and many HDACs interact with SMRT (silencing mediator of retinoid and thyroid hormone receptors) and N-CoR (nuclear hormone receptor co-repressor), which

were the first co-repressors identified for NRs (20,53). They are paralogous of one another and function in similar ways. Both N-CoR and SMRT make contact with the repression domain (RF) that exists within several NR LBDs. Furthermore, additional subunits of the co-repressor complex function as scaffolds, assist in substrate recognition, or regulate co-repressor function. TBL1 (transducin beta-like protein 1) and TBL1-R (TBL1 related protein) form a complex with SMRT and N-CoR and stabilize the quaternary structure of the complex by making contacts with HDAC3 (123). Additionally, TBL1 and TBL1-R both bind to histones H2B and H4 and may help in chromatin recognition (168). Recently, both TBL1 and TBL1-R have been shown to work as specific adaptors for the recruitment of the ubiquitin/19S proteasome complex. Moreover, TBL1-R was shown to mediate a required exchange of N-CoR and SMRT for co-activator upon NR ligand binding and the authors conclude that TBL1 and TBL1-R are a specific class of co-regulators, playing important roles in both co-repression and co-activation (121).

The GPS2 (G protein pathway suppressor 2) protein interacts with both N-CoR and TBL1 and stabilizes the co-repressor complex (168). GPS2 was originally isolated and shown to suppress lethal G-protein subunit-activating mutations in the yeast pheromone response pathway (143). By using the yeast two-hybrid screening method with the transactivation domain of bovine papillomavirus type 1 E2, the activation domain modulating factor (AMF-1/(GPS2)) was isolated and was shown to stimulate E2 transcriptional activation (12). The transactivation by AMF-1/(GPS2) has been found to depend on direct interaction with p300 (119). Furthermore, GPS2 has been shown to interact with and be degraded by human papillomavirus E6 proteins (27). AMF-1/(GPS2) has also been shown to interact with p53 and stimulate p53-dependent transcriptional activity (120). In addition, GPS2 was shown to suppress small G-protein (RAS)- and MAP kinase mediated signal in mammalian cells (143). The TNFα (Tumor necrosis factor α) receptor induces a stress-activated protein kinase cascade and some components of this cascade are MEKK1 (mitogen-activated protein kinase/extra cellular signal-regulated kinase kinase kinase 1) and SEK (stress-activated protein kinase/extracellular signal regulated kinase kinase) which regulate the activity of JNK1 (c-Jun N-terminal kinase 1). The HTLV-I (human T-cell lymphotropic virus, type 1) oncoprotein Tax can activate JNK1. Moreover, Tax can bind to GPS2 which can suppress Tax activation of JNK1. GPS2 could also suppress TNFα activation of JNK1 but not TNFα activation of p38 kinase (56). N-CoR and SMRT can also interact with mSin3 (mammalian switch-independent 3 protein) which is a co-repressor for several non-receptor transcription factors. Conclusively, there are multiple SMRT and N-CoR complexes in the cell that contact different protein subunits. Moreover, many NRs make contact with the transcriptional machinery. These contacts can disturb or stimulate transcriptional initiation (123). Furthermore, NRs can interact with the mediator complex in the presence of hormone agonists which contributes to the formation of a preinitiation complex on the target promoter (124). On the contrary, SMRT and N-CoR can, in addition to recruiting HDACs to the chromatin template, also make inhibitory contacts with some components of the transcriptional machinery (107,161). Binding of unliganded NRs to N-CoR/SMRT is mediated via a conserved motif, called the CoRNR-box or as LXXI/HIXXXI/L (49,108,122,162).

The action of co-repressors/HDACs is opposed by co-activators functioning at distinct steps of the activation pathways. Studies have shown a link between the acetylation of specific lysine residues in the N-terminal tails of core histones and the

activation of transcription (152,153).The co-activators p300 and CBP (p300/CBP) and PCAF (p300/CBP-associated factor) were found to have intrinsic nucleosomal HAT activity (5,114,165). The p300/CBP proteins are global regulators of transcription (141), and contain other protein domains, including a bromodomain and three cysteine-histidine rich domains (C/H-1, C/H-2 and C/H-3) which are thought to mediate protein-protein interaction (97). NRs mostly have indirect interactions with p300/CBP, mediated by the SRC (steroid receptor co-activator) family of bridging factors (68,86,89,137). Recruitment to ligand-bound NRs is generally mediated by conserved LXXLL motifs that exist in most of the co-activators (47,149). The mediator complex interacts with RNA polymerase II and is probably a component of the RNA polymerase II holoenzyme. The human complex, also called TRAP (39) or DRIP (125), interacts with several different activators that include not only the NRs TR and VDR but also other viral and cellular activators such as p53, VP16, Sp1, SREBP, nuclear factor κB (NF- κB) and E1A. This action seems to function via direct interaction with both RNA-pol II and DNA-bound activators, resulting in activator-enhanced recruitment of RNA-pol II and other general transcription factors to the promoter (54).

EID1 (E1A-like inhibitor of differentiation 1) binds to the A-B pocket of Rb similarly to the adenoviral E1A. Moreover, it is an unconventional co-factor that does not associate with histone deacetylases (HDACs) but instead functions as an "anti-co-activator" that interacts with p300/CBP (95,103). EID1 is degraded by the proteasome when cells exit the cell cycle, and ubiquitination occurs through the EID1 C-terminus via binding of pRb which in turn interacts with the ubiquitin ligase MDM2 (103). Furthermore, EID1 has been shown to be able to interact with histones and histone tails and has an acidic domain in its N-terminus (6) (paper II) and preliminary data shows that EID1 can associate with chromatin *in vivo* (A. Båvner, unpublished results). The INHAT complex inhibits the histone acetyltransferase activity of p300/CBP and PCAF. Furthermore, INHAT functions as a transcriptional regulator by masking histones via a glutamic and aspartic acid (E/D) rich INHAT domain (133).

Additional chromatin modifications are histone methylation, histone phosphorylation, histone ubiquitination, and DNA methylation. Histone methylation can be both stimulatory and inhibitory to transcription (123). Core histones, especially H3 and H4, are targets for methylation and several histone methyltransferases (HMTs) have been identified, including: 1. the H3 lysine 9 (H3-K9)-specific HMTs Suv39H1 and G9a, which are involved in transcriptional repression, or silencing (128,147); 2. the H3 lysine 4 (H3-K4)-specific HMT Set 9, which is involved in transcriptional activation (111,156); and 3. members of the protein arginine methyltransferase (PRMT) family, such as PRMT1 and CARM1, also involved in transactivation (18,75,145,157). NRs can also recruit ATP-dependent chromatin remodeling complexes, which change the chromatin structure in a non-covalent manner. The Swi/Snf family is an ATP-dependent remodeling complex which serves as a co-activator by opening the nucleosome structure making it possible for specific and general transcription factors to access DNA (46,100). The human SWI/SNF consists of eight or nine subunits, with either hBrg1 or hBrm as the ySnf2-related ATPase subunit (70). However, there is evidence suggesting that this complex can function in both transcriptional activation and repression (17,106).

2 SMALL HETERODIMER PARTNER

2.1 AIMS OF THE PRESENT STUDY

The aim of this thesis was to investigate the molecular mechanisms of transcriptional inhibition by SHP (Small Heterodimer Partner, NR0B2). This aim was achieved by analysis of structural motifs within SHP, by studying NR interactions, by isolation of upstream target co-factors and by characterization of SHP mutations.

2.2 STRUCTURE AND EXPRESSION

SHP is an unusual orphan member of the NR family without a DBD. Alignment of SHP with the RXRα LBD, whose three dimensional structure has been determined revealed that SHP consists of a putative LBD with a nine amino acid residue (aa) long N-terminal extension. Furthermore, our work revealed that SHP has two functional LXXLL motifs with which SHP binds to the AF-2 of ligand-activated NRs (paper I). In contrast to almost all other NRs, the conserved glutamic acid residue (E) is exchanged with aspartic acid (D) within the helix 12 in the SHP AF-2. SHP has intrinsic repression activity and until now no ligand has been found.

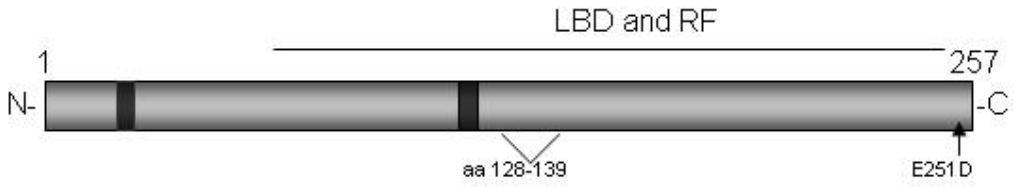

Figure 2. *Schematic representation of human SHP with the two NR-boxes indicated as black boxes, repression function (RF), and ligand-binding domain (LBD).*

Figure 3 shows an alignment based on the human (h)SHP amino acid sequence comparing the SHP protein in different species which were found using the BLAST data-base[4]. Mouse (Mus musculus) (m)SHP and rat (Rattus Norvegicus) (r)SHP show a 77.8% and 77.4% aa homology to human SHP protein, with the fish Danio rerio, Oreochromis niloticus, and Tetraodon nigroviridis showing respectively a 52.8, 45.9 and 43.6% sequence identity to the hSHP protein. Chimpanzee (Pan troglodytes) is predicted to be 777 aa long with an extended C-terminus of unknown function. In the figure only the N-terminus (aa 1-257) corresponding to SHP is shown, demonstrating a 98.8% identical aa sequence to hSHP. Red jungle fowl (Gallus gallus) is 838 aa long and showed similarities in its C-terminal extension with the chimpanzee SHP C-terminus, and the N-terminus (aa 1-249) showed a 54.6% aa sequence identity. The functional significance of the extended C-terminus is unknown. Evidently, no human orthologue contains a C-terminal extension. Apparently, the two functional NR-boxes seem to be quite conserved across species.
The closest relative to SHP is the orphan receptor DAX-1 (NR0B1) with 35 % aa sequence identity.

[4] http://www.ncbi.nlm.nih.gov/BLAST/

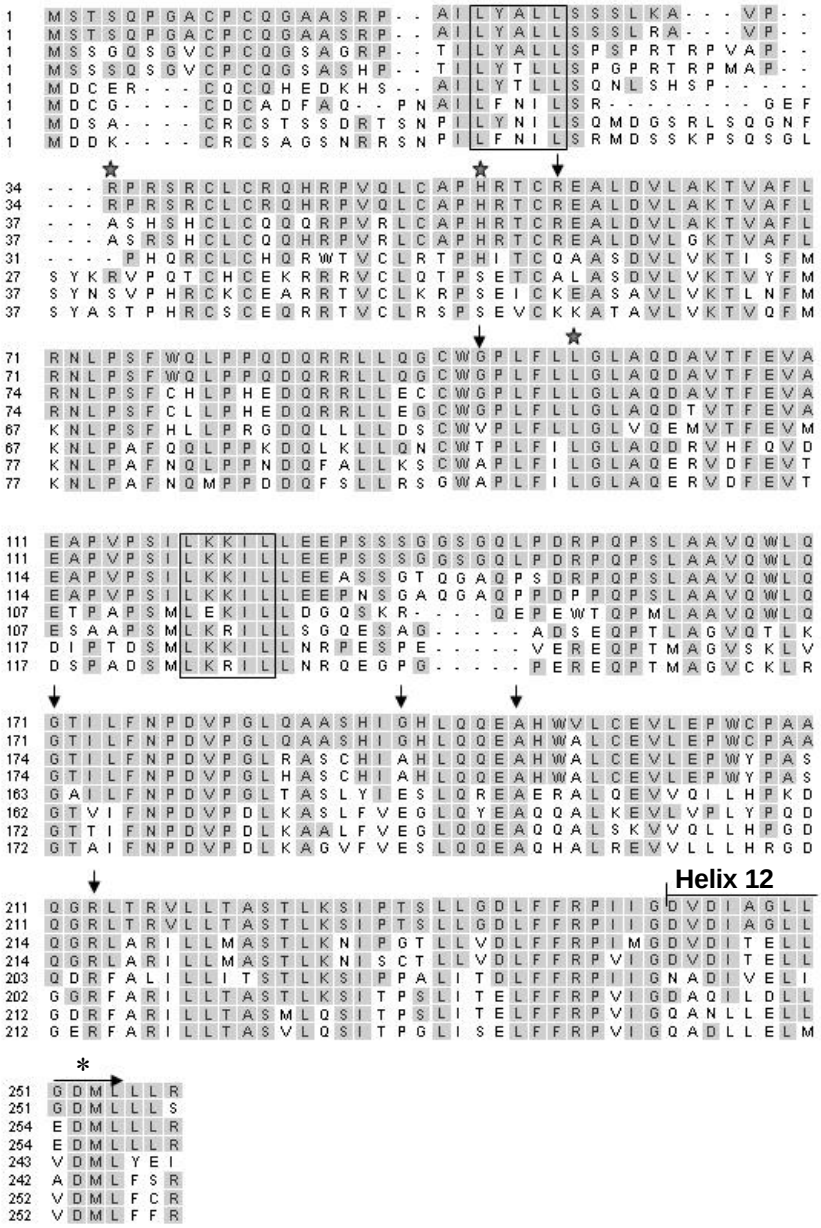

Figure 3. *SHP species alignments, line 1-Homo sapiens, 2-Pan troglodytes, 3-Mus musculus, 4-Rattus norvegicus, 5-Gallus gallus, 6-Oreochromis niloticus, 7-Danio rerio, 8-Tetraodon nigroviridis. NR-box 1 and 2 are boxed, Helix 12 with the E to D "mutation" that is indicated with an asterisk. Natural human SHP deletions are indicated with a star and mutations with an arrow (see chapter 2.6).*

Several studies have investigated the tissue expression of SHP mRNA, which is summarized below and in table 3.

Human SHP mRNA was detected by Northern blot in heart, kidney, pancreas, small intestine, spleen, liver, adrenal gland, adrenal medulla, adrenal cortex, stomach but not in brain, placenta, lung, skeletal muscle, thymus, prostate, ovary, colon, peripheral blood leukocytes, testis, thyroid, thymus and heart (79,130), (paper IV). Human

hepatocytes (FT 215) treated with the FXR agonist CDCA (chenodeoxycholic acid) increased SHP mRNA expression, which was detected by northern blot (116). Moreover, human SHP mRNA was significantly up-regulated by treatment with the bile acids GCDCA (glycoCDCA) and GDCA (glycodeoxyCA) which activates FXR in cultured human primary hepatocytes, which was detected by quantitative single plex real-time PCR (35). Human SHP mRNA was upregulated by FXR ligands in primary human hepatocytes detected by Northern blot (44). Human HL-60 promyelocytic leukemia cells treated with phorbol ester (TPA) to induce monocytic differentiation showed up-regulated SHP mRNA and protein expression (24). It was also found to be expressed in human adipose stromal cells (76) and HepG2 cells (98).

Mouse SHP mRNA is expressed highly in liver and less in heart, pancreas, kidney, smooth muscle, submaxillary gland, and epididymis (not in brain, placenta, lung, skeletal muscle, kidney, eye, testis, prostate) which was shown by northern blot (130,134). Mouse liver SHP mRNA levels which are diminished in CYP7A1$^{-/-}$ mice are restored by feeding FXR ligands (92). Mice fed with cholic acid (CA) showed increased SHP mRNA in liver detected by Northern blot (116). Mouse SHP mRNA in mouse hepatic (BNL-CL.2) cells is induced by LRH-1 which was detected by reverse transcriptase-PCR (RT-PCR) (14). Mouse SHP mRNA was detected by RT-PCR in kidney, heart, smooth muscle, pancreas, submaxillary gland, and epididymus in mesenteric, epididymal, and subcutaneous fat tissues (112). In resting mouse macrophages, oxLDL suppresses SHP expression, possibly due to modulation of mRNA stability (69).

Rat SHP mRNA was upregulated by FXR agonists and detected in rat liver and rat hepatocyes by northern blot (44). Rat SHP mRNA was readily detected by northern blot in liver, heart, H411EC3 cells and less in skeletal muscle, kidney, testis, lung, and spleen (98). Rat SHP mRNA was detected by RT-PCR in epididymis, prostate, testis, uterus, colon, small intestine, bladder, stomach, spleen, lung, liver, thymus, heart, adrenal, spinal cord, olfactory lobes, and cerebellum (not pituitary and kidney) (58). Real time PCR analysis showed that rat hepatic mRNA expression of SHP was low during the embryonic period and that mRNA levels peaked close to that of adult rats (>6 weeks-old rats) by 4 weeks of age. Moreover, protein levels for SHP corresponded to the mRNA expression (4). Moderate consumption of red wine reduces risk of death from cardiovascular disease. Interestingly, the polyphenols procyanidins which are present in red wine changed the expression of liver CYP7A1 and SHP in male rats, as analyzed with microarray hybridization (28).

Nile tilapia SHP mRNA was detected by northern blot in male and female liver but not in testis and ovary. By RT-PCR, SHP mRNA was detected in all adult tissues examined including; brain, pituitary, gill, heart, spleen, liver, intestine, ovary, kidney, muscle, and testis (155).

In summary, SHP mRNA is readily detectable in many tissues. Surprisingly, no *in situ* hybridization data has been reported with SHP so far, but there is some unpublished data detecting SHP mRNA in liver and prostate (S. Mäkelä, unpublished results). Moreover, detection of endogenous SHP protein has only rarely been reported (4,14,19,37,167), probably due to lack of highly specific antibodies and possibly naturally low basal expression of the SHP protein. However, there is one study detecting endogenous SHP in mouse pancreatic islets (66).

Table 3. *Summary of SHP mRNA expression in different tissues detected by Northern blot (-, +) and RT-PCR (-, +).*

Tissue	**Human SHP**	**Mouse SHP**	**Rat SHP**	**Fish SHP**
Liver	+, +, +	+ , +	+, +,+, +	+, +
Liver CYP7A1$^{-/-}$ +FXR agonist		+		
Heart	-	+, +	+, +	+
Pancreas	+, +	+, +		
Brain	-	-, -	-	+
Placenta	-	-		
Lung	-	-	+, +	
Skeletal muscle	-	-, -	+	
Kidney	+	-, +	+, -	+
Testis	-, -	-	+	-, +
Spleen	+, +		+	+
Epididymus		+	+	
Prostate	-, -	-	+	
Uterus			+	
Colon	-, -		+	
Small intestine	+, +, +		+	+
Bladder			+	
Stomach	+		+	
Spleen			+	
Thyroid	-			
Thymus	-, -, -		+	
Adrenal	+		+	
Adrenal medulla	+			
Adrenal cortex	+			
Spinal cord			+	
Olfactory lobes			+	
Cerebellum			+	
Pituitary			-	+
Smooth muscle		+		
Eye		-		
Peripheral blood leukocyte	-, -			
Ovary	-, -			-, +
Gonads				
Gill				+
Muscle				+
Mesenteric fat		+		
Epididymal fat		+		
Subcutaneous fat		+		
Primary human hepatocytes	+			

Table 3 *continued*.

Cell line	**Human SHP**	**Mouse SHP**	**Rat SHP**
HL60 cells + TPA	+		
FT 215 cells	+		
Caco-2 cells	+		
HepG2 cells	++		
HeLa	-		
Human adipose stromal	+		
Hepa 1		-	
Hepatic BNL-CL.2 cells +LRH-1		+	
Resting macrophages RAW 264		+	
H411EC3 cells			++
dRLH84 cells			-

2.3 REGULATION OF THE SHP PROMOTER

The mouse and human SHP promoter contains five NR5A binding sites and can be potently transactivated by SF-1 and FTF (82). The human, rat and mouse SHP promoters contain binding sites (IR1-type FXREs) for the FXR/RXR heterodimer which has been shown to bind to all three promoters (44). Furthermore, hSHP represses its own transcription through binding to both LRH-1 and the ligand induced FXR/RXR heterodimer on its own promoter (92). Similarly, in paper V we show that the homeodomain protein Prox-1 binds to LRH-1 and represses the human SHP promoter (144). The transactivation by FXR/RXRα has been shown to be increased by PGC-1α (PPARγ co-activator-1α) in presence of CDCA and SHP can repress its own transcription by competing with PGC-1α (61). The SHP promoter contains a conserved and functional HNF-4 binding site and it has been shown that HNF-4 activates the transcriptional activity of the SHP gene (139). Moreover, ERRγ has been reported to bind to one of the five SF-1REs and activate the transcription from the SHP promoter. SHP also binds to ERRγ and thereby inhibits the transactivation of its own promoter suggesting a potential autoregulatory loop controlling SHP gene expression (130). Estrogens have been shown to induce the expression from the mouse, rat and human SHP promoters and this induction is absent in ERαKO mice (77). Another study has suggested that 12-O-tetradecanoylphorbol-13-acetate (TPA) -induced ERK and JNK activation subsequently leads to activation of c-Jun protein by specific phosphorylation which in turn leads to increased expression of the SHP gene in a monocytic differentiation procedure. They also found that the SHP gene promoter contains one functional TPA response element (TRE), a predicted c-Jun response element (24). The human SHP promoter was recently shown to contain PXR RE and SHP mRNA was upregulated by the PXR agonists rifampicin and LCA (40).

However, recent studies have reported differences between the mouse, rat and human SHP promoters, indicating differential species regulation. For example, the LXRα/RXRα heterodimer has been shown to upregulate the human SHP promoter but not the rat SHP promoter in rat primary hepatocytes (45). One study shows that the basic helix-loop-helix (bHLH) transcription factors E2A proteins (E47, E12, E2/5) activate the human but not the mouse SHP promoter and the E47 heterodimer partner BETA2 inhibits the E47-mediated transactivation of the human SHP promoter. This suggests that the E2A proteins regulate the human and mouse SHP promoter differentially (66). SREBPs (sterol regulatory element-binding proteins) regulate the transcription of genes involved in cholesterol and fatty acid synthesis. SREBP-1 has been found to bind to an SRE1 located in the region from -186-195 in the human SHP promoter leading to a stimulation of human SHP gene expression. However, the SRE1 was not conserved in the mouse SHP promoter indicating differential regulation of SHP in human and mouse (65).

2.4 TARGETS AND FUNCTION

Several *in vitro* studies have reported that SHP interacts with a variety of NRs, which usually requires the LBD (58,136). Intriguingly, in paper I we show that SHP may play an important role in regulation of NR-co-activator interactions via the LXXLL motifs. This fact makes it highly possible that SHP might be able to interact with virtually all AF-2 containing NRs. The interactions predominantly lead to a reduction of the transactivation of NR target genes.

Table 4A. *Reported interactions of SHP with NRs (subfamily 1).*

NR	Y2H	M2H	Pull-down	Co-IP	Refs
TRβ NR1A2	**mSHP/TRβLBD** **mSHPΔ148/rTRβ LBD** **rSHP/ rTRβ**	*hSHP/hTRβLBD*			(134) (135) (98) (44)
RARα NR1B1	**mSHP/RARαLBD** **mSHPΔ148/mRARαLBD** **rSHP/hRARα +**	*hSHP/hRARαLBD*			(134) (135) (98) (44)
PPARα NR1C1	**rSHP/mPPARα** **rSHP/rPPARαLBD**		**rSHP/rPPARα**		(98) (58) (62)
PPARγ NR1C3			**hSHP aa 88-110/ hPPARγ2DBD**	**hSHP /hPPARγ2**	(112)
LXRα NR1H3		*hSHP/hLXRαLBD*	**mSHP/hLXRα** **mSHPaa 92-260 /hLXRα** **mSHPmt1.2/hLXRα** **mSHP/hLXRαLBD**	**SHP/LXRα**	(44) (14)
LXRβ NR1H2			**mSHP/mLXRβ**		(14)
FXR NR1H4		*hSHP/mFXR LBD*	*mSHP/mFXR*		(44) (92)
PXR/SXR NR1I2			**mSHP/mPXR** **mSHP/hPXR**		(116)
CAR NR1I3	**mSHP/mCAR** **mSHPΔ148/mCAR** **SHP/ mCAR** **SHPΔ128-139/ mCAR**		**SHP/ mCAR** **SHPΔ128-139/ mCAR** **mSHP/mCAR**		(134) (135) (117) (3)

Positive interactions are written in **bold** and negative interactions are written in *italics.*

Yeast two- hybrid (Y2H), GST/HIS pull-down (Pull-down), Co- immuno precipitation (Co-IP), mammalian two-hybrid (M2H).

NRs not found to be tested for SHP interactions are;
TRα (NR1A1), RARβ/γ (NR1B2/NR1B3), PPARβ (NR1C2), RevErbα/β (NR1D1/2), RORα/β/γ (NR1F1/2/3), VDR (NR1I1), HNF-4γ (NR2A2), RXRγ (NR2B3), TR2 (NR2C1), TR4 (NR2C2), TLX (NR2E1), PNR (NR2E3), COUP-TFα/γ (NR2F1/3), MR (NR3C2), PR (NR3C3), Nurr1 (NR4A2), Nor1 (NR4A3), GCNF (NR6A1).

Table 4B. *Reported interactions of SHP with NRs (subfamilies 2-5, 0)*

NR	Y2H	M2H	Pull-down	Co-IP	Refs
HNF4α NR2A1		**mSHPΔ148** **/rHNF4L366E** *hSHP* */rHNF4αLBD*	**mSHP/rHNF4L366E** **hSHP/hHNF4 end.** **hSHP/hHNF4 AF2** **hSHP/hHNF4 N-ter** **hSHP 1-160/hHNF4** *SHP 161-257/HNF4*		(80) (44) (10) (142)
RXRβ NR2B2	**rSHPD2** **/RXRβLBD-/+**				(58)
ERα NR3A1	**mSHP/hERαLBD+** **rSHP/hERαLBD+** *rSHPmt1.2/hERαLBD+* **rSHPbox2/hERαLBD+**	**mSHP** **/hERαLBD** **hSHP** **/hERαLBD**	**mSHPΔ148/hERαLBD** **rSHPD2/ERα** **rSHP/ERαLBD** *rSHPmt1.2/mERαLBD* *rSHP/hERαAF-2mut* **rSHPbox2/hERα** **mSHP/hERα**		(136) (58) (57) (44) (73)
ERβ NR3A2	**rSHP/hERβLBD+** *rSHPmt1.2/rERβLBD+* **rSHPbox2/rERβLBD+**		**rSHPD2/hERβ+** *rSHP/hERβAF-2mut+* **rSHPbox2/hERβ+**		(58) (57)
ERRα NR3B1	**hSHP/ ERRα** **mSHP/ ERRα**		**SHP/ ERRα**		(130)
ERRβ NR3B2	**hSHP/ ERRβ** mSHP/ ERRβ		**SHP/ ERRβ**		(130)
ERRγ NR3B3	**hSHP/ ERRγ** **mSHP/ ERRγ** **SHP/ ERRγ** **SHPΔ128-139/ ERRγ** **rSHP/rERRγ**		**SHP/ ERRγ** **SHP/ ERRγ** **SHPΔ128-139/ ERRγ**		(130) (117) (127)
GR NR3C1				**rSHP** **/hGR**	(8)
AR NR3C4	**rSHP/hAR** *rSHPmt2/hARLBD*		*mSHP/ hAR-LBD* **rSHP/hAR**		(136) (43)
NGFI-B NR4A1				**SHP** **/Nurr77**	(167)
SF-1 NR5A1		*hSHP/mSF-1* *LBD*	*mSHP/mSF-1*		(44) (92)
LRH-1 NR5A2	**rSHP/mLRH-1LBD**	**mSHP/hLRH-** **1LBD** *mSHP/hLRH-* *1DC* **hSHP/hLRH-** **1LBD** *hSHP/hLRH-* *1ΔC* **hSHPR213C** **/LRH-1LBD** **mSHP/mLRH-** **1LBD** **hSHP/hLRH-** **1LBD** **hSHP/mLRH-** **1LBD**	**mSHPaa1-160** **/hLRH-1LBD** *mSHP/hLRH-1ΔC* **mSHP/mLRH-1** **mSHP/hLRH-1** **mSHP/hLRH-1 LBD** **hSHP/hLRH-1** **hSHP/hCPF**		(80) (81) (92) (44) (14) (10) (144)
DAX-1 NR0B1	*rSHP/hDAX-1*	*rSHP/hDAX-1*			(E.T.)*
SHP NR0B2	*rSHP/rSHP*	*rSHP/rSHP*			(E.T.)*

Positive interactions are in written in **bold** and negative interactions are written in *italics.* *E. Treuter, unpublished data.

Mouse SHP was originally isolated with mouse CAR and interacted in a ligand-dependent manner with TR-LBD and RAR-LBD, and in the presence or absence of ligand with RXR-LBD. Moreover, rat SHP was isolated with mPPARα in a yeast two-hybrid assay using a rat liver cDNA library and interacted ligand dependently with rTRβ, hRARα and mRXRα and ligand independently with PPARα and rHNF-4 (98). Similarly, rat SHP was isolated using the rat PPARα LBD in a yeast two-hybrid screening using a rat liver cDNA library (58). Furthermore, SHP has been shown to bind to and inhibit transcription by PPARα/RXRα heterodimers from the acyl-CoA oxidase (AOx) – peroxisome proliferator-response elements (PPRE) (62).

SHP interacts with the AF-2 of the estrogen receptors α and β (ERα/β) in a ligand (agonist) dependent manner and represses the transcriptional activity mediated by the ERs on an estrogen response element luciferase reporter (ERE-luc) (57,136). SHP has also been shown to be an inhibitor of 4-OHT agonist activity in RL95-2 human endometrial carcinoma cells which express endogenous ERα (73).

The androgen receptor (AR) is targeted by SHP leading to repression of AR dependent transactivation of the MMTV-luciferase reporter in CV1 and CHO cells (43). However, in another study SHP was shown to be unable to inhibit AR dependent transactivity from the MMTV promoter in HepG2 cells (136). SHP interacts with the three estrogen related receptors α, -β and -γ (ERR) and inhibits the transcriptional activity mediated by them (130). Moreover, rat ERRγ was isolated using rat SHP as bait in a yeast two hybrid screening (127).

Furthermore, LRH-1 regulates the aromatase expression downstream of PGE_2 (prostaglandin E_2) and SHP mRNA was expressed in human adipose stromal cells, and was shown to inhibit the aromatase transcription mediated by LRH-1 in preadipocytes. Therefore SHP is thought to play a role in the expression of aromatase expression and estrogen production in breast adipose tissue (76). Recently, SHP was shown to interact with Nurr77 and repress the Nurr77 mediated transactivation of the CYP17 promoter (167).

2.4.1 SHP in liver

The degradation of cholesterol to bile acids in the liver is initiated either by cholesterol 7α-hydroxylase (CYP7A1) or by mitochondrial sterol 27-hydroxylase (CYP27A1). CYP7A1 is the first step and rate limiting enzyme of the classic (neutral) pathway and CYP27A1 is the first and rate limiting step in the alternative (acidic) pathway. The classic pathway consists of a cascade of fourteen steps catalyzed by enzymes located in the cytoplasm, microsomes, mitochondria and peroxisomes. The acidic pathway is not well defined but some metabolites (formed by CYP27A1, CYP7B1) are mainly produced in peripheral tissues and must be transported to the liver to be converted to bile acids (23).

SHP is highly expressed in liver and plays an important role in cholesterol metabolism by interacting with transcription factors and thereby modulating transcription of enzymes involved in the pathway converting cholesterol into bile acids. Bile acids have been shown to activate the orphan receptor farnesoid X receptor (FXR) (118). Moreover, the feedback repression of CYP7A1 has been shown to occur by the binding of bile acids to the FXR, leading to transcription of SHP. SHP then inactivates LRH-1 (liver receptor homologue 1) by binding to it which results in a promoter-specific repression of CYP7A1 and SHP (44,92). This finding was the first crucial step in establishing the physiological function of SHP *in vivo* and to this date it is the only physiological function described for SHP. The human cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from high density lipoproteins to triglyceride-rich lipoproteins. Similarly to the inhibition of CYP7A1, the sterol-dependent induction of CETP via LXR and LRH-1 is repressed by SHP (94). CYP8B1 (Sterol 12α-hydroxylase) catalyzes the synthesis of CA (cholic acid) and controls the ratio of CA over CDCA in the bile. SHP has been shown to repress rat CYP8B1 via binding to LRH-1 in HepG2 cells (30). However, an in vivo study in rats showed that SHP seemed to play a minor role in the inhibition of the rat CYP8B1 gene (166). Another study in mice showed that SHP inhibits the expression of CYP8B1 through interaction with LRH-1 (29). The apical sodium-dependent bile acid transporter (ASBT) partly mediates the intestinal reclamation of bile salts. An LRH-1 cis-acting element has been identified in the mouse but not the rat ASBT promoter and SHP diminished the activity of the mouse promoter and partly repressed its activation by LRH-1 (19). Furthermore, there is a functional FTF binding site in the rabbit ASBT promoter and only FXR-activating ligands can repress rabbit ASBT expression via the regulatory FXR-SHP-FTF cascade (85). The human ASBT is positively regulated by retinoic acid and bile acids induce a negative feedback regulation via the FXR-LRH-1-SHP cascade when the RAR/RXR heterodimer activates ASBT (109). Furthermore, LRH-1 has recently been shown to regulate the apolipoprotein AI (APOAI) gene which is involved in cholesterol homeostasis and SHP was shown to suppress APOAI gene expression by inhibiting LRH-1 transcriptional activity (31).

Moreover, bile acid activated FXR induces SHP expression which represses SREBP-1c expression by inhibiting the activity of LXR and possibly other SREBP-1c stimulating transcription factors (160). Interaction has also been shown with LXRα and –β, and inhibition of trans-activity has been shown on an artificial reporter driven by an LXR-response element and the human ATP-binding cassette transporter 1 (ABCA1) (14).

The interaction of SHP with HNF-4 (hepatocyte nuclear factor 4) has been shown to occur within the AF-2 domain, leading to repression of HNF-4 mediated transactivation (80). HNF-4α up-regulates the expression of CYP8B1 and bile acids have been shown to repress human CYP8B1 transcription through interaction of SHP with HNF-4α (169). CYP27A1 catalyses the sterol side-chain oxidation and cleavage to synthesize two primary bile acids, CA and CDCA in humans (22). SHP represses the HNF-4 stimulated CYP27A1 transcription (21). The hepatocyte nuclear factor 1 (HNF-1) promoter is activated by HNF-4α, and SHP has been shown to inhibit the HNF-4α mediated transactivation of the HNF-1α promoter in co-transfection assays (59). The MTP (microsomal triglyceride transfer protein) gene is involved in lipoprotein synthesis and secretion in the liver. Its expression is regulated by HNF-4 (and HNF-1) and MTP has been shown to be down-regulated by bile acids. This is believed to occur via inhibition of HNF-4 by interaction with SHP which is up-regulated by bile acids via FXR (48). Angiotensinogen (ANG) is the precursor of vasoactive octapeptide angiotensin II and is mainly synthesized in the liver and secreted into the circulation (26). The human ANG gene was repressed by bile acids and SHP represses the human ANG gene promoter by inhibiting HNF-4 binding to the promoter (142). Moreover, in paper II, we show that SHP represses the HNF4- responsive apolipoprotein CIII promoter (6).

Ntcp (sodium/taurocholate cotransporting polypeptide) is a cell membrane transport protein involved in bile formation, responsible for bile acid uptake (150). The liver is also the main storage organ for vitamin A, a ligand for RXR/RAR which is an activator of rat Ntcp gene expression. SHP is induced by bile-acid activated FXR and SHP has been shown to inhibit retinoid activation of the Ntcp RXR/RAR element, leading to down regulation of bile acid uptake (32). The maintenance of bile acids and decreased expression of the Ntcp is associated with cholestasis. One study has demonstrated that bile acid mediated induction of SHP precedes down regulation of Ntcp (170). The glucocorticoid receptor (GR) is a target receptor for SHP which interacts with the dexamethasone bound GR and represses the PEPCK (phospho (enol) pyruvate carboxykinase) promoter (8). When activated by agonistic ligands, PXR (pregnane x receptor) inhibits the CYP7A1 and induces CYP3A genes. Recently, SHP was shown to interact with the agonist bound PXR and to be a potent inhibitor of PXR transactivation of CYP3A in human hepatocytes and in mice (116). Bile acids have been shown to inhibit the expression of gluconeogenic genes, including glucose-6, phosphate (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), and fructose 1,6-bis phosphate (FBP1) in mouse liver and HepG2 cells. SHP was shown to repress the gluconeogenic gene promoters by disrupting the association of CBP-Foxo1 or CBP-HNF-4 (163). However, SHP was shown to induce the transcriptional activity by PPARα/RXRα heterodimer from the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD)-PPRE }(62). Furthermore, SHP interacts ligand independently with the DBD/hinge region of PPARγ and potentiates the transcriptional activity (112).

To date, the most common SHP binding transcription factors (TFs) reported are the NRs mentioned above. However, a few other TFs have been reported to interact with SHP and may represent alternative downstream targets to SHP and link SHP to NR-independent transcriptional pathways (summarized in table 5).

Table 5. *Reported SHP- interacting transcription factors.*

TF class	Y2H	Pull-down	Co-IP	Refs
Forkhead				
Foxo1		**hSHP/Foxo1 CT** **hSHP 1-91/Foxo1**	**hSHP/Foxo1**	(163)
HNF3α		**SHP/rHNF3α 168-269**	**SHP/rHNF3α**	(67)
HNF3β		**SHP/rHNF3β**	**SHP/rHNF3β**	(67)
HNF3γ		**SHP/mHNF3γ**	**SHP/mHNF3γ**	(67)
RHR				
NFκB		**SHP/p65 195-283** *SHP/p50*	**SHP/p65**	(69)
bHLH				
BETA2/NeuroD	**mSHP/BETA2** **mSHP 1-159/BETA2**	**hSHP/BETA2**	**hSHP/BETA2**	(66)
Id-2	**mSHP/hId-2**	**hSHP/hId-2**		(66)
E47	*mSHP/E47*	*hSHP/E47*	*hSHP/E47*	(66)
Per1		*hSHP/mPer1*		(66)
AHR		*mSHP/hAHR*		(74)
ARNT		**mSHP/hARNT**		(74)

Positive interactions are in written in **bold** and negative interactions are written in *italic.*

Yeast two-hybrid (Y2H), GST/HIS pull-down (Pull-down), Co- immuno precipitation (Co-IP), mammalian two-hybrid (M2H).

One example is the SHP-mediated inhibition of TCDD-stimulated reporter activity from the AHR-responsive CYP1A1 and UGT1A6 gene promoters, via direct interaction with aryl hydrocarbon receptor nuclear translocator (ARNT) (74). Another example is that SHP functions as a transcription co-activator for the NF-κB (nuclear factor-κb) (69). A third example is the recently reported interaction with the forkhead transcription factor HNF3 (hepatocyte nuclear factor 3/Foxa) which leads to inhibition of DNA binding. SHP inhibits the three isoforms; HNF3α, β and γ, resulting in regulation of G6Pase, CYP7A1 and PEPCK gene expression (67). SHP has also recently been shown to interact with the basic helix-loop-helix transcription factor BETA2/NeuroD but not its heterodimer partner E47. The interaction leads to direct repression of BETA2/NeuroD transcriptional activity by inhibition of interaction between BETA2 and p300. SHP repressed the BETA2-dependent activity of glucokinase and cyclin-dependent kinase inhibitor p21 promoters (66).

2.4.2 SHP in pancreas

The highest SHP mRNA expression is reported in liver and pancreas (see Structure and expression, and table 3) yet the physiological functions of SHP in pancreas have not been established. The transcription factors HNF-4α, HNF-1α, PDX-1, HNF-1β and BETA2 have been demonstrated to be key factors involved in pancreas development and β-cell function (138). Furthermore, mutations in HNF-1α in mice resulted in a decrease in mRNA expression of Pdx-1, HNF-4α, NEURO-D1/Beta-2 and SHP (139). Moreover, SHP and BETA2 has been shown to colocalize in islets of mouse pancreas (66). SHP therefore may serve as an important checkpoint to balance the activity of the islet transcriptional network. The physiological significance of this negative regulation needs to be further characterized in mutant SHP mouse models (140).

HNF-4α has recently been shown to contribute to the regulation of the major fraction of the transcriptosomes in liver and pancreatic islets by binding to almost 50% of the transcribed genes. Moreover, HNF-1α target genes were identified in human pancreatic islets and the SHP promoter was one of 106 occupied promoter regions (113). The HNF-4α genes are correlated with maturity-onset diabetes of the young 1 (36). Isoforms of HNF-4α result from alternative splicing and alternate usage of promoters P1 and P2 and are expressed in human pancreatic β-cells. Both isoforms were repressed by SHP on the HNF-1α promoter which is required for β-cell function (34). In obesity related diabetes UCP2 (mitochondrial uncoupling protein) is up-regulated in β-cells, which results in impaired glucose-stimulated insulin secretion (GSIS). It has been shown that over-expression of SHP in β-cells increases GSIS more likely by increasing the ATP/ADP ratio than via transactivation of PPARγ (146).
Carboxyl ester lipase (CEL) has recently been shown to be a target for LRH-1 in exocrine pancreas. CEL hydrolyzes dietary cholesteryl esters in the presence of trihydroxylated bile salts (71) in the small intestine (9). Furthermore, LRH-1 regulate CEL transcription and SHP has been shown to decrease the LRH-1 mediated induction of CEL promoter activity (38).

Table 6. *SHP target genes.*

Target gene	TF	Target gene function	Ref
		Cholesterol metabolism	
CYP7A1 ↓	LRH-1	Bile acid synthesis	(44,92)
CYP7A1↓	HNF3α/β/γ		(67)
rat CYP8B1 ↓	LRH-1	Bile acid synthesis	(30)
rat CYP8B1 -	LRH-1		(166)
mouse CYP8B1 ↓	LRH-1		(29)
Human CYP8B1 ↓	HNF-4α		(169)
CETP ↓	LRH-1	Cholesterol ester transfer	(94)
CETP ↓	LXR		(94)
Mouse ASBT ↓	LRH-1	Bile acid transport	(19)
Rabbit ASBT ↓	LRH-1		(85)
Human ASBT ↓	LRH-1		(109)
Rat NTCP ↓	RAR/RXR	Bile acid transport	(32)
APOA1 ↓	LRH-1	Lipogenesis	(31)
Apoc III ↓	HNF-4	Lipogenesis	(6)
MTP ↓	HNF-4	Lipogenesis	(48)
AOx-PPRE ↓	PPARα/RXRα	Lipogenesis	(62)
HD-PPRE ↑	PPARα/RXRα	Lipogenesis	(62)
CEL ↓	LRH-1	Hydrolyzes dietary cholesteryl esters	(38)
SREBP-1c ↓	LXR	Lipogenesis	(160)
ABCA1 ↓	LXR	Cholesterol efflux	(14)
HNF-1α ↓	HNF-4α	Regulation of several liver specific genes	(34,59)
		Glucose metabolism	
PEPCK ↓	HNF-4	Rate limiting gluconeogenic enzyme	(163)
PEPCK ↓	GR		(8)
PEPCK ↓	HNF3α/β/γ		(67)
FBP1↓	HNF-4	Gluconeogenesis	(163)
G6Pase ↓	HNF3α/β/γ	Regulate blood glucose levels	(67)
G6Pase ↓	Foxo1		(163)
GK ↓ (glucokinase)	BETA2/NeuroD		(66)
		Steroid hormone biosynthesis	
CYP19 ↓	LRH-1	Catalyzes aromatic estrogens from androgens	(76)
CYP17 ↓	Nurr77	CC21-steroid hormone biosynthesis	(167)
		Xenobiotic metabolism	
CYP1A1 ↓	ARNT	Drug and hormone metabolism	(74)
CYP2B (PBRU)↓	CAR/RXR	Drug and hormone metabolism	(3)
CYP3A ↓	PXR	Drug and hormone metabolism	(116)
UGT1A6 ↓	ARNT	Drug and hormone metabolism	(74)
		Other	
CDK inhi p21 ↓	BETA2/NeuroD	Cell cycle arrest	(66)
Human ANG ↓	HNF-4	Regulation of blood pressure	(142)

Down regulated ↓, up regulated ↑, no regulation –

2.5 MECHANISMS OF FUNCTION

The ability of SHP to repress transcription of NR target genes was recognized early on to be crucial for its physiological function as an inducible co-repressor. SHP has been shown to repress the transcriptional activity of ERs through direct antagonism of co-activator function via competition (58). Furthermore, in paper I, it is shown that SHP binds directly to ERs via LXXLL-related motifs which can be critical for the binding of co-activators to the AF-2 domain (57). However, SHP has been shown to inhibit ER dimerization in vitro and inhibit E2-ERα binding(73). Several examples of co-activator antagonism via competition are mentioned below and surprisingly also one example of co-repressor antagonism.

SHP inhibits HNF-4 and RXR mediated trans-activity by interacting with their AF-2 domains and by competing with SRC-3 for binding. For HNF-4, the C-terminus of SHP was necessary for repression and efficient interaction, in contrast to RXR which was not affected by the SHP C-terminal deletion (80). Similarly, SHP inhibits AR-mediated gene activation by competing with the AR co-activators TIF-2 and FHL2. However, both the AR AF-2 and N-terminal domain (NTD) interacted with SHP in a ligand dependent manner. The SHP interaction with the AF-2 was dependent on NR-box2 but not the interaction with the NTD (43). The SHP inhibitory effect on ERRγ transactivation has been reported to depend on competition with the co-activator GRIP-1/SRC-2 and the natural human SHP mutations L98 and H53 showed no interaction with ERRγ. Furthermore, interaction was dependent on intact AF-2 surfaces of the ERRs (130). SHP antagonizes PGC-1 coactivation of GR and the interaction with GR is dependent on a functional NR-box 2 and helix 12 within SHP (8). The inhibitory effect by SHP on LRH-1 trans-activity is dependent on competition with SRC-3. Furthermore, binding requires an intact helix 12 within LRH-1 and the mouse SHP mutant lacking the C-terminal repression domain (aa 160-260) represses with significantly decreased efficiency but still binds well to LRH-1. Furthermore, the human SHP mutation R213C, showed a decreased repression activity but still bound as efficiently as the wild type (81). LXRα interaction is dependent on helix 12 for interacting with SHP and SHP is dependent on NR-boxes 1 and -2 and the C-terminus (aa160-260) to interact with LXRα. Furthermore, SHP repressed TIF-2 mediated activation (14). The N-terminal region of SHP binds to and represses the trans-activity of BETA2/NeuroD by competing with p300 for the BETA2/NeuroD interaction (66). Finally, the trans-activity of PPARγ is up-regulated by SHP in the presence and absence of ligand via direct binding of SHP to the DBD/hinge region of PPARγ. The C-terminus of SHP is important for the activation and SHP inhibited repressor activity of N-CoR (112). SHP interacts with the NF-κB component p65 and up-regulates the trans-activity by binding to the C-terminal sub-regions of SRC-1 (69).

However, other mechanisms of function have been reported, for example, inhibition of DNA binding and recruitment of a co-activator. SHP inhibits PXR/RXR heterodimer DNA binding to the ER6 in the CYP3A4 promoter. The SRC-1 antagonizes the inhibitory effect of SHP on PXR transactivation in mammalian cells (116). SHP binds to ARNT and inhibits AHR/ARNT binding to XRE in vitro (74). Similarly, the forkhead DNA binding domain of HNF3 is targeted by SHP resulting in inhibition of DNA-binding of HNF3. Furthermore, the aa 1-97 and 128-139 of SHP were not required for repression of NRs (67).The N-terminus (aa 1-160) of SHP was also shown

to interact with the N-terminus of HNF-4 in addition to the AF-2 domain, and inhibited HNF-4 DNA binding to the ANG gene promoter (142).

In addition to the mechanism of function described above, SHP has been shown to have an intrinsic repression activity that has been mapped to the C-terminal LBD (see discussion). In paper I, we identified the first candidate co-inhibitor for SHP called EID1 using the yeast two-hybrid screening method. EID1 interferes with the function of NR co-activators such as p300/CBP but surprisingly does not link SHP to conventional co-repressors. Recently, the first connections of SHP to known components of co-repressor complexes have been postulated. For example, one study has suggested that SHP represses the phenobarbital (PB) induced CYP2B (PBRU) gene expression by binding to the constitutive androstane receptor (CAR). This repression is thought to be the result of either inhibition of co-activator recruitment by GRIP1 or active recruitment of co-repressors. These studies also suggested that possible co-repressors to be recruited are mSin3A and HDAC1 (3). Another recent study indicates that SHP repression of the cholesterol 7α hydroxylase gene (CYP7A1) by bile acids is mediated by recruitment of mSin3A-Swi/Snf to the promoter. The recruitment is thought to occur via direct interactions with Brm and mSin3A through SHPs C-terminal repression domain (63). SHP has also been shown to associate with unmodified and lysine 9-methylated histone-3 and to interact with HDAC1 and the G9a methyltransferase in euchromatin. The authors suggest that SHP represses transcription by a multistep mechanism including first; histone deacetylation, second; H3-K9 methylation and third; stable association of SHP with chromatin (10). Notably, none of these interactions were found using the yeast two-hybrid screening method. However, in paper IV the identification and characterization of GPS2 as a novel co-repressor for SHP and as the missing link between SHP to the N-CoR/HDAC3 complex is reported. Furthermore, the SHP-GPS2 interaction was found using the yeast two-hybrid screening method, indicating a functional interaction. Moreover, SHP has also been shown to interact with RNA polymerase II but not with TFIID or TFIIE in GST pull-down assays (14).

In table 7, published SHP-interacting co-regulators are listed. However, not all of them are mentioned in the text above. Notably, except EID1 and GPS2, none of the co-regulators reported to interact with SHP was verified by additional methods or identified by unbiased protein-protein interaction screenings. Furthermore, none of the LXXLL containing co-regulators interacted with SHP in yeast two-hybrid assays (E. Treuter, unpublished data).

Table 7. *Reported interactions of SHP with transcriptional co-regulators.*

Coreg.	Y2H	M2H	Pull-down	Co-IP	Refs
EID1	**rSHP/mEID1 SHP/EID1 SHPD128-139/EID1**	**rSHP /mEID1 M**	**rSHP/mEID1 SHP/EID1 SHPD128-139/EID1**	**rSHP/mEID1**	(6) (117)
mSin3A			**mSHP/mSin3A mSHP/r mSin3Aend. SHP C-term/mSin3A mSHP/mmSin3Aend.**	**mSHP /hmSin3Aend.**	(3) (63) (A.B.)◦
HDAC1			**mSHP/HDAC1 mSHP/rHDAC1end. hSHP/hHDAC1 SHP/HDAC1end. mSHP/HDAC1 mSHP/mHDAC1end.**	**hSHP/HDAC1**	(3) (10) (63) (A.B.)◦
HDAC3			**mSHP/HDAC3 mSHP/mHDAC3end.**		(A.B.)◦
BAF155			**SHP/hBAF155 end**		(63)
BAF57			**SHP/hBAF57pur**		(63)
Brm			**SHP C-term/Brm SHP/hBrm pur**	**mSHP/hBrm end.**	(63)
G9a, Lys 9 Meth H3			**hSHP/hG9a end. hSHP/hH3**	**hSHP/G9a**	(10)
GPS2	**rSHP/hGPS2**	**rSHP /hGPS2 100-156**	**h/m/rSHP/hGPS2 mSHP/mGPS2end**	**hSHP/hGPS2**	(A.B.)◦
SMRT			**mSHP/SMRT mSHP/rSMRTend.**		(3)
N-CoR			*mSHP/N-CoR* **mSHP/rN-CoR end. mSHP/NCoR N-**		(3) (A.B.)◦
Basal TF					
RNApolII			**mSHP/RNApolII**		(14)
TFIID			*mSHP/TFIID*		(14)
TFIIE			*mSHP/TFIIE*		(14)
Co-activator					
CBP	*rSHP/mCBP-N*		*hSHP/hCBP end.*	**SHP/CBP**	(10) (167) (E.T.)*
SRC1	*rSHP/hSRC1*		**SHP/SRC1aa 759-1141**		(69) (E.T.)*
TIF-2	*rSHP/mTIF-2*				(E.T.)*

Positive interactions are in written in **bold** and negative interactions are written in *italic. Yeast two-hybrid (Y2H), GST/HIS pull down (Pull-down), Co-immuno precipitation (Co-IP), mammalian two-hybrid (M2H).* ◦A. Båvner, Paper IV, * E.Treuter, unpublished data.

2.6 IN VIVO STUDIES IN HUMANS AND MICE

SHP was originally isolated due to its binding to CAR in 1996 with the yeast two-hybrid method (134). Closely thereafter two independent studies reported the isolation of SHP with PPARα using the yeast two-hybrid method (58,98). In year 2000, the first step in understanding the physiological function of SHP *in vivo* was the finding that feedback repression of CYP7A1 occurred by the binding of bile acids to the FXR, leading to transcription of SHP. SHP then inactivated LRH-1 (liver receptor homologue 1) by binding to it which resulted in a promoter-specific repression of CYP7A1 and SHP (44,92). In 2002, SHP deficient mice were reported to show gross accumulation and increased synthesis of bile acids caused by derepression of rate-limiting CYP7A1 and CYP8B1 hydroxylase enzymes in the biosynthetic pathway. Dietary bile acids induced liver damage and restored feedback regulation but a synthetic FXR agonist was not hepatotoxic and had no regulatory effects. Cholestyramine enhanced the expression of CYP7A1 and CYP8B1 and reduced the bile acid pool. The authors conclude that there are three negative regulatory pathways controlling bile acid synthesis, two SHP-independent and one SHP-dependent pathway. The input of FXR and SHP was sufficient to mediate negative feedback-regulation of bile acid synthesis. However, when the organism is stressed by liver damage etc, alternate pathways of regulation are activated to change the bile acid synthesis (64). A similar study shows that mice lacking the SHP gene fed normally show no large defects in cholesterol metabolism. Moreover, when the SHP null mice are fed with the FXR agonist GX4064 the repression of CYP7A1 is lost. However, when fed with bile acids the CYP7A1 was suppressed which shows the importance of several additional SHP independent negative regulatory pathways (159). Long term studies on the effect of cholesterol and CA rich diets in the same SHP null mice surprisingly show that they were protected against cholestasis. Furthermore, CYP8B1 was re-expressed but not CYP7A1 and other FXR and SHP targets suggesting that lack of SHP might have a beneficial effect in cholestasis (158). Future phenotypical analysis of the SHP knock-out mice and the production of tissue-specific knock-outs and transgenic mice will ultimately uncover physiological pathways involving SHP.

In 2001, a study on obese people in Japan revealed that analysis of mutations (R34X, A195S, R57W, G189E, H53fsdel10 and L98fsdel9insAC) in SHP lead to decreased SHP function suggesting that genetic variation in the SHP gene contributes to increased body weight (110). On the contrary, another study in U.K. Caucasians concludes that it is unlikely that genetic SHP variations are a common predisposition to obesity, increased birth weight or diabetes. However, they identified a common G171A coding polymorphism and the rare homozygous subjects may be predisposed to mild obesity and possibly increased birth weight (102). Furthermore, a third study concludes that SHP mutations are not a common cause of obesity but children with the rare G171A allele showed a trend toward increased birth weight and BMI through insulin secretion defects. Moreover, carriers with the minor allele of the -195CTGAdel promoter polymorphism, showed the opposite trend compared to the trend connected with the G171A allele (51). In a study in 2004 among Danish men the authors conclude that the prevalence of functional SHP variants associated with obesity is considerably lower compared to the Japanese study. However, obese subjects tended to have a higher prevalence of the codon 171 polymorphism and a codon 93D variant displayed a lower

ability to inhibit HNF-4α transactivation of the HNF-1α promoter compared to the wild type (33).

Finally, chromatin immunoprecipitation assays have been performed with HNF1α, HNF4α, and HNF6 in isolated human pancreatic islets and hepatocytes. The enriched extracts were subsequently hybridized to a promoter microarray. An overview of the transcriptional regulatory networks in hepatocytes and pancreatic islets was made and interestingly, SHP was shown to be an important regulatory downstream target of HNF transcription factors. In hepatocytes SHP was regulated by HNF1α and HNF4α and not by HNF6, but in islets SHP was regulated by HNF1α and HNF6 and not by HNF4α (113).

2.7 RESULTS

2.7.1 SHP LXXLL motifs interact with ERs (Paper I)

In this article we identified LXXLL-related motifs, the so-called NR-box motifs within SHP. Furthermore, SHP bound directly to the ERs via the LXXLL-related motifs in GST pull-down and yeast two-hybrid assays. Moreover, the SHP NR-box 2 motif was sufficient for ligand-dependent interaction with ERα and ERβ. NR-boxes are usually critical for the binding of co-activators to the ligand-regulated activation domain AF-2 within NRs. In concordance with the NR-box dependency, SHP required the intact AF-2 domain of agonist-bound estrogen receptors for interaction. GST pull-down assays demonstrated that mutations within the ligand binding domain helix 12 or binding of antagonistic ligands, which are known to result in an incomplete AF-2 surface, abolished interactions with SHP. Supporting the idea that SHP directly antagonises receptor activation via AF-2 binding, we demonstrated that SHP variants, carrying either interaction-defective NR-box mutations or a deletion of the repressor domain, have lost the capacity to inhibit agonist-dependent transcriptional estrogen receptor activation. Using DNA-dependent protein-protein interaction assays and mammalian two-hybrid assays, we demonstrated that SHP interacted with the ER dimer on DNA via ternary complex formation. The results of this study provide the mechanistic explanation for the previously less understood interaction characteristics of SHP and allow envisaging how SHP, independently of DNA-binding and conventional dimerization-type interactions, might exert its inhibitory effect on NRs.

2.7.2 EID1 is the first isolated SHP-associated co-factor (Paper II)

In this study we describe the isolation of mouse EID1 (E1A-like inhibitor of differentiation 1) using the yeast two-hybrid screening method, as a candidate co-inhibitor for SHP. EID1 represents the first SHP-associated upstream target protein that could be directly linked to transcription inhibitory mechanisms and is different from conventional co-repressors. We characterized the interactions between SHP and EID1 using yeast two-hybrid, mammalian two-hybrid, GST pull-down assay and subcellular colocalization studies. Furthermore, repression analysis, mammalian two-hybrid and an HNF4 responsive system in mammalian cells identified two repression-defective SHP mutations. Interestingly, both of these mutations had lost the ability to bind to EID1. These results indicate that SHP has a co-regulator-binding surface encompassing putative helices 3 and 12, distinct from those of other NRs presumably because critical residues (yet to be identified) account for the selectivity of SHP towards EID1. We suggest that naturally occurring SHP mutations possibly manifest in physiological disorders because they affect the inhibitory capacity and interactions with associated co-factors such as EID1. EID1 bound to acetyl transferases but not co-activators without HAT activity and to histones in vitro. The binding of histones and histone tails may be mediated by an acidic stretch within in the EID1 N-terminus. Moreover, EID1 significantly inhibited p300/CBP-dependent functions in transient transfection

experiments, and the inhibition may be HAT-independent for co-activators relevant for NR activation. Inhibitory mechanisms may include the disruption of p300/CBP-co-activator interactions and the direct inhibition of HAT activity and possibly histone binding. Conclusively, mutational analysis of SHP repression and EID1 binding highlighted the divergence of repression mechanisms between SHP and repressing receptors that depend on the C-terminus of N-CoR/SMRT co-repressors and identified two mutations that both abolished repression and EID1 binding.

2.7.3 EID3 is a novel EID-family member (Paper III)

BLAST databases were searched with the EID1 open reading frame to identify potential functional homologues and revealed the existence of several unpublished open reading frames. In this study we focused on a predicted human cDNA with an open reading frame encoding 333 aa. Recently, EID2 was characterized as an inhibitor of muscle differentiation and an antagonist of both p300/CBP and HDACs. In paper III we described a third EID1 (E1A-like inhibitor of differentiation 1) family member designated EID3 that is highly expressed in testis according to northern blot analysis and show homology to a region of EID1 implicated in binding to p300/CBP. Mammalian two hybrid assays and subcellular colocalization studies were performed to test if this putative homologue could interact with SHP and other NRs, but the assays showed no binding to SHP or the NRs tested. However, in transient transfection assays we demonstrated that EID3 acts as a potent inhibitor of NR transcriptional activity by a mechanism that is independent of direct interactions with NRs, including SHP. EID3 as well as EID1 directly bind to and block the C-terminal domain of CBP, which has been implicated to act as the interaction surface for NR co-activators. Consistent with this idea, both EID3 and EID1 prevented recruitment of CBP to the naturally ERα regulated pS2 promoter in chromatin immunoprecipitation assays. This study suggested that EID-family members act as inhibitors of p300/CBP-dependent transcription by binding to the C-terminus of P300/CBP in a tissue-specific manner.

2.7.4 GPS2 links SHP to a co-repressor complex (Paper IV)

Our previous reports described EID1 that acts co-inhibitory by antagonizing co-activator action. However, we were curious to see if SHP possibly uses co-factors directly linked to conventional co-repressors. Therefore yeast two-hybrid experiments were performed and we recently succeeded in identifying an additional SHP-associated protein called G-protein pathway suppressor 2 (GPS2) which has been demonstrated to be a novel subunit of the N-CoR/HDAC3 co-repressor complex. We characterized the interactions between human SHP and human GPS2 with the GST-pull down assays and co-immunoprecipitation assays. Northern blot analysis shows that GPS2 is expressed in all SHP expressing tissues. Furthermore, we demonstrated that GPS2 acts as a potent inhibitor of NR transcriptional activity already repressed by SHP in mammalian cells. Moreover, we identified individual co-repressor proteins in the SHP associated co-repressor complex using liver tissue extracts and extracts from cultured cells using the GST or HIS pull-down methods. Collectively, these data indicate that there are probably two separate mechanisms linked to SHP co-repression; co-activator inhibition via EID1 and N-CoR/HDAC recruitment via GPS2.

2.7.5 Prox-1 is a putative regulator of SHP expression (Paper V)

Members of the Fushi tarazu factor 1 (FTZ-F1, NR5A3) subfamily belong to the category of ancient orphan receptors with homologues from Drosophila to human. Mammalian homologues include SF-1 (NR5A1) and LRH-1(NR5A2). Structure and function analysis indicates that the LRH-1 ligand binding surface may not be optimized for common co-factors (129). Whereas MBF1 (13) and TIF-2 (129) may act as co-activators, as discussed earlier SHP functions as an inducible co-repressor for LRH-1 activity (44,92). In this study, we described the identification of Prox-1 (Prospero-Related Homeobox 1) as an additional co-repressor for LRH-1. We showed that LRH-1 and Prox-1 interact in yeast, mammalian cells and *in vitro*, and that these interactions require LRH-1 aa residues 317-560, i.e. the entire LBD, and Prox-1 aa 48-132 containing two NR-box motifs. Our data suggested that Prox-1 is expressed in all LRH-1 target tissues and is likely to be a physiologically relevant co-factor of LRH-1, particularly in the adult liver of mammals which was independently confirmed by other research groups (88). Using the *shp* promoter, which is an established LRH-1 target, transfection experiments suggested a co-repressor function of Prox-1 in LRH-1 transcriptional activity. Possibly, Prox-1 action is involved in the regulation of SHP expression levels in liver and pancreas. We concluded that Prox-1 has intrinsic transcriptional repression potential that is largely dependent on an N-terminal repression domain and that may involve recruitment of HDAC3. Characteristics of this interaction as well as overlapping expression patterns of LRH-1 with Prox-1 in liver, pancreas, and intestine in mice and humans (15,41,115,126,148) and developmental functions suggest that the relationship between LRH-1 and Prox-1 might be particularly important in regulating gene expression patterns during mammalian liver and pancreas development and in physiology of the adult enterohepatic system.

2.8 COMMENTS ON METHODOLOGY

Here I briefly describe the major methods I have used during my PhD period. The advantages and disadvantages of each method have become apparent to me during the years and I do emphasize that these are highly personal and subjective observations/comments.

2.8.1 Protein interaction studies

Elucidating gene function involves determining the function of each gene's encoded protein product. In the cell, proteins participate in extensive networks of protein-protein interactions. These interactions take the form of dynamic "protein machines", which assemble and disassemble in concert with an ever-changing influx of intra, inter and extracellular cues. A preliminary step in understanding protein structure and function is to determine which proteins interact with each other, thereby identifying the relevant biological pathways.

2.8.1.1 Yeast two-hybrid assay

In a yeast two-hybrid experiment a protein of interest is fused to a GAL4 DNA-binding domain and transfected in a yeast host cell that has a reporter gene controlling this DNA binding domain. If this fusion protein does not activate transcription on its own, it can be used as "bait" to screen a cDNA library of clones that are fused to the GAL4 activation domain. The cDNA clones within the library that encode proteins that can form protein-protein interactions with the bait are identified by their ability to cause activation of the reporter gene (154).

*Advantage*s; unbiased, detects weak binding, convenient.
Disadvantages; false positives, might miss proteins that need other proteins in equivalent amount and post-transcriptional modifications (mammalian proteins), dependent on the quality of the cDNA library.

2.8.1.2 Mammalian two-hybrid assay

The mammalian two hybrid system is very similar to the yeast two-hybrid system. The differences are that the reporter must be transfected and that the activator protein is VP16 in mammalian cells.
Advantages; mammalian protein in mammalian system (post-translational modifications).
Disadvantages, repressing proteins may suppress the trans-activity of the reporter resulting in false negative result, not all proteins are expressed in the nucleus which results in false negatives (observed by sub-cellular localization studies).

2.8.1.3 Co-immunoprecipitation assay

Co-immunoprecipitation is a purification procedure to determine if two different molecules (usually proteins) interact. An antibody specific to the protein of interest is added to a cell lysis. Then the antibody-protein complex is pelleted usually using protein-G sepharose which binds most antibodies. If there are any protein/molecules that bind to the first protein, they will also be pelleted. Identification of proteins in the

pellet can be determined by western blot (if antibody exists) or by sequencing a purified protein band[5].
Advantages; sees interactions both in nucleus and cytoplasm, two over-expressed proteins probably interact directly since putative endogenous linker protein probably is in too small amount to mediate the interaction.
Disadvantages; using endogenous proteins does not reveal direct interaction due to putative "linker proteins".

2.8.1.4 GST pull-down assay

The pull-down assay is an in vitro method used to determine physical interaction between two or more proteins. In a pull-down assay, a tagged (here GST or HIS) bait protein is captured on an immobilized affinity ligand specific for the tag, thereby generating a 'secondary affinity support' for purifying other proteins that interact with the bait protein. The secondary affinity support of immobilized bait can be incubated with a variety of other protein sources that contain putative prey proteins. Confirmation of previously suspected interactions typically utilizes a prey protein that has been expressed in an artificial protein expression system. Protein expression system lysates (i.e., E. *coli* or baculovirus-infected insect cells), *in vitro* transcription/translation reactions, and previously purified proteins are appropriate prey protein sources for confirmatory studies. Discovery of unknown interactions contrasts with confirmatory studies because the research interest lies in discovering new proteins in the endogenous environment that interact with a given bait protein. Any cellular lysate in which the bait is normally expressed, or complex biological fluid (i.e. blood, intestinal secretions, etc.) where the bait would be functional, are appropriate prey protein sources for discovery studies[6].
Advantages; in vitro direct interaction, easy
Disadvantages; false positives due to low stringent washing, or sticky proteins.

2.8.2 Sub-cellular localization of proteins

2.8.2.1 Immunocytochemistry and fluorescent tagged proteins visualized by confocal microscopy

I have only worked with over-expressed proteins transfected into mammalian cells. Proteins were detected either by fluorescent tagging or immuno staining.
Advantages; study localization and pattern of a protein, study colocalization of two associated proteins.
Disadvantages; over-expressed proteins may not behave like endogenously expressed proteins. Two interacting proteins may show the same individually distribution which can give a "false negative" result if you expect them to together form another pattern. Two non-interacting proteins can give a false positive result if you think they associate only because they are distributed equally. Primary and secondary antibodies must be carefully studied for background and specificity.

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[5] http://www.biochem.northwestern.edu/holmgren/Glossary/Definitions/Def-C/coimmunoprecipitation.html

[6] http://www.piercenet.com/Products/Browse.cfm?fldID=F3FD3612-415F-42A5-8922-736F9FDD36FB

2.8.3 Reporter studies in mammalian cells

2.8.3.1 Transient transfections

Mammalian cells can be used to study transcriptional effects mediated by one or more proteins on a reporter gene. The plasmids containing the protein/s of interest is/are transiently transfected together with an appropriate reporter into the cells. Thereafter the cells are usually harvested and reporter gene expression is measured, which tells if the reporter gene is activated or repressed by the proteins of interest.
Advantages; relatively simple method, not very time consuming.
Disadvantages; not a natural promoter context (no chromatin), to get even amounts you must be very careful and accurate when preparing transfection mixes and cell seeding.

2.9 DISCUSSION

A conserved "signature motif" within co-regulators is called the NR-box and has been shown to mediate co-activator-NR interaction. The NR-box is a short α-helical LXXLL motif and aa residues flanking the core motif are important for the recognition of NRs. The number and sequences of the LXXLL motifs also vary considerably among co-activators and may be important for the differences in binding to selected NRs or a class of NRs (reviewed in (131)).

In paper I we demonstrated that SHP binds to the AF-2 domain of the ERs through two NR-box motifs in similarity to the co-activator TIF-2. Moreover, SHP does not interact with any of the tested LXXLL co-factors (E.T., unpublished data) in agreement with the lack of a functional AF-2 in SHP. In contrast to the situation with other repressing NRs like TR and RAR, SHP does not bind to the CoRNR-box in the C-terminus of N-CoR/SMRT (135). In conclusion, the SHP LBD as evidenced by protein interaction analysis appears structurally and functionally different from other LBDs.

Furthermore, the NR-box 2 motif within SHP was functionally independent supporting the theory that competition between SHP and TIF-2 occurs on the AF-2 surface of the ERs and that SHP interacts with ERs in the same fashion as TIF-2 did. After our findings, a number of papers have followed presenting similar results with other NRs (see first section in mechanisms of function). Moreover, the repression-defective SHP aa 1-159 containing the NR-box motifs could not inhibit ER transactivation. Therefore, SHP most likely functions in a two-step mechanism: first by binding to the AF-2 domain and second by recruiting co-repressors to the C-terminus/LBD. Conclusively, SHP is not just a repressor of transcription but an LXXLL-containing co-repressor that recognizes the active LBD conformation. Alternative molecular mechanisms were thought to exist connecting the intrinsic repressor function of the SHP LBD to conventional co-repressor complexes. However, molecular mechanisms of SHP repression have remained enigmatic until recently, particularly with respect to the intrinsic repressor function of the SHP LBD.

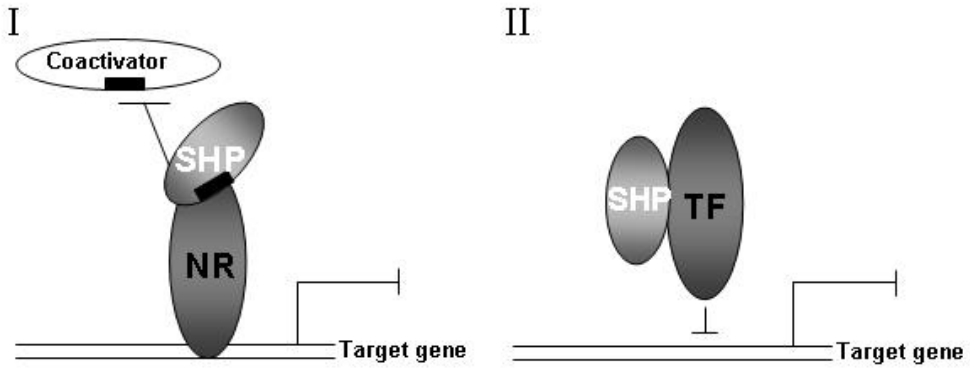

Figure 4. I. *SHP blocks binding of co-factors to a NR.* II. *SHP prevents DNA-binding of the TF.*

Furthermore, in paper V we demonstrate that Prox-1, like SHP, contains two NR-boxes and binds via those to LRH-1 and represses the transcriptional activity. However, yeast two-hybrid and colocalization studies (E. Holter, unpublished data) suggest that the interactions of SHP and Prox-1, respectively, with the LRH-1 LBD are not identical, although they both depend on a functional AF-2 of LRH-1. These facts raise the possibility of LRH-1 co-repressor complexes containing Prox-1 and SHP. Intriguingly, putative LRH-1 ligands could regulate co-regulator recruitment. Moreover, the Prox-1 repression domain is mapped and shown to interact with HDAC3. Interestingly, Prox-1 could repress the LRH-1 activated SHP promoter, and is a putative negative regulator of SHP expression. One hypothesis is that Prox-1 may modulate LRH-1 target gene expression during liver development and under normal physiological conditions, whereas SHP may primarily serve as a metabolic sensor under conditions of increased metabolic activity such as high-cholesterol diet.

Recent work has indicated the existence of cellular proteins that share intriguing features with E1A. The first of these proteins, designated EID1 (E1A-like inhibitor of differentiation 1) was cloned on the basis of interactions with the Rb (Retinoblastoma) tumor suppressor protein and was subsequently characterized as inhibitor of p300/CBP-dependent functions on differentiation (95,103). In paper II, we report the identification of EID1, the first SHP-associated upstream target protein that could be directly linked to transcription inhibitory mechanisms and that is different from conventional co-repressors. EID1 inhibits genes regulated by a variety of NRs and inhibitory mechanisms may include the disruption of p300/CBP-co-activator interactions, probably due to direct inhibition of HAT activity (95,103). EID1 bound to the human GCN5, the orthologue of the HAT co-activator PCAF (164), while the co-activators RAP250 (16) and TIF-2 (83) did not bind, indicating EID1 selectivity towards HATs. EID1 antagonized NR transactivation by inhibiting the p300/CBP-dependent transcription activation functions of NR associated co-activators such as TIF-2 and RAP250. The EID1 inhibition could possibly be due to histone binding via its N-terminal acidic stretch (133). Interestingly, immunocytochemical data indicated that EID1 associated with chromatin *in vivo* (A. Båvner, unpublished data). Furthermore, mutational analysis of the intrinsic SHP repression and EID1 binding highlights the divergence of repression mechanisms between SHP and repressing receptors that depend on N-CoR/SMRT co-repressors (as discussed in (42,135)) and identified two mutations that abolished both repression and EID1 binding. These results indicate that SHP has a co-regulator binding surface encompassing putative helices 3 and 12. Moreover, it is distinct from those of other NRs (reviewed in (42)) presumably because critical residues (yet to be identified) account for the selectivity of SHP towards EID1. The homology of SHP M1 to the naturally occurring DAX-1 mutation R267P as well as the requirement of helix 12 (M4) for repression (78) highlights the close functional relationship of these two atypical NRs. Finally, naturally occurring SHP mutations (110) possibly manifest in physiological disorders because they affect the inhibitory capacity and interactions with associated co-factors such as EID1.

In paper III, EID1 was used to identify potential functional homologues, revealing the existence of several unpublished open reading frames. We identified EID3, a third previously uncharacterized EID family member. The exclusive expression of EID3 in testis contrasts to the more ubiquitous expression pattern of the other two members (6,55,95,103,104), and indicates that EID3 might have testis specific functions.

However, unlike EID1, EID3 had an extended C-terminal domain of unknown function. EID3 showed homology to a region of EID1 that is implicated in binding to p300/CBP (95). Furthermore, EID3 had a profound inhibitory effect on the transcriptional activity of CBP, indicating functional similarities to EID1 (95,103). This observation is most likely due to the binding of EID3 to the C-terminus of CBP (aa 1678-2441) indicating a functional conservation of these interactions within the EID family. The more potent inhibition of NR transcriptional activity mediated by EID3 compared to EID1 could be due to the fact that a larger fraction of EID3 is primarily located in the nucleus. Furthermore, the inhibition seems to be independent of direct interaction of EID3 with the tested NRs, as judged from mammalian two-hybrid interaction assays and sub-cellular co-localization studies (data not shown). These data suggest that EID3 functions as a co-inhibitor of NRs and thus could be involved in the transcriptional control of testicular tissue homeostasis. Moreover, transcriptional activation by NRs, including SF-1 (105), RXR, ER, GR (60), LXR (52) and AR (50) requires the action of P300/CBP. A sequence within the C-terminus of CBP, termed the SRC1 interaction domain (SID), has been mapped to aa 2058-2130 (137). The SID domain has also been reported to interact with other nuclear factors in addition to the p160 proteins including IRF-3, E1A, p53, Tax, Ets-2 and KSHV IRF-1 (87,90,132). Proteins that bind to the SID domain have recently been shown to share a sequence motif similar to an amphipathic α-helix in the AD1 domain of the p160s (99). Interestingly, we have localised putative amphipathic α-helices in EID1 and -3 within the conserved EID regions. Therefore, it is possible that the EID family interacts with the SID domain of CBP via these amphipathic α-helices, thereby competing with the p160s of the same surface. This might disrupt an NR/co-activator complex at the promoter, leading to a decrease in NR target gene expression. However, there is also a possibility that the EIDs bind elsewhere in the C-terminus of CBP which could lead to structural changes, which in turn leads to disruption of proper co-activator assembly. Consistent with this idea, we have demonstrated that EID1 and -3 prevent recruitment of CBP to a natural NR-regulated promoter using chromatin immunoprecipitation (ChIP) assays. These results suggest that EID1 and -3 interfere with the recruitment of CBP to the promoter-bound ERα/co-activator complex and demonstrate a functional interaction between EID1 and -3 and CBP *in vivo*. We propose a model where EID1 and EID3 act as inhibitory "co-regulators of the co-regulators" that could be critically involved in the fine-tuning of transcription, by targeting the promiscuous co-activators P300/CBP without interfering with the present transcription factor.

In an attempt to identify additional SHP co-factors, we identified and characterized GPS2 as a novel co-repressor for SHP and as the missing link between SHP to the N-CoR/HDAC3 complex (paper IV). This suggests that all repressing NRs utilize similar co-repressor complexes via contacting alternative subunits. We mapped the putative SHP interaction domains within GPS2 which are distinct from the GPS2 N-terminal N-CoR interaction domain (168) and the central non-conserved region. GPS2 mRNA was expressed in all tissues where SHP mRNA was expressed, indicating a physiological relevance for the observed interaction. Furthermore, transient transfection assays suggested that SHP recruits GPS2 to a target promoter of a SHP target NR. This is further confirmed by using a SHP mutation that is unable to interact with the target NR. Moreover, repression assays show that GPS2 further increases the intrinsic repression activity of SHP. In vitro binding studies show that SHP binds to HDAC1 which also has been shown by others (3,63). Interestingly, the observed binding of SHP to the N-

terminus of N-CoR and to HDAC3 suggests that SHP can potentially recruit and interact with several subunits of a co-repressor complex consisting of GPS2, N-CoR and HDAC3. Moreover, binding studies with SHP and endogenous proteins in liver nuclear extracts show that SHP can associate with GPS2 and HDAC3 as well as with HDAC1 and mSin3 in vivo. It remains to be investigated whether or not HDAC1 or 3 in complex with SHP are enzymatically active or silent, as previous evidence implies, and if alternative deacetylation-independent repression mechanisms would account for SHP repression via GPS2/N-CoR/HDAC complexes. Irrespective of this important issue, our results suggest a model where SHP recruits the N-CoR/HDAC3 co-repressor complex via direct interactions with GPS2 to a promoter-bound SHP target NR. Considering the possibility that SHP contacts subunits of functionally distinct co-regulator complexes such as SIN3/HDAC1/SWI/SNF, EID1/P300/CBP, or HDAC1/G9a (3,6,10,63) it is likely that SHP differentially utilizes each of these complexes in a cell type- and target gene -dependent manner. This suggests that all repressing NRs use similar co-repressor complexes by contacting alternative subunits.

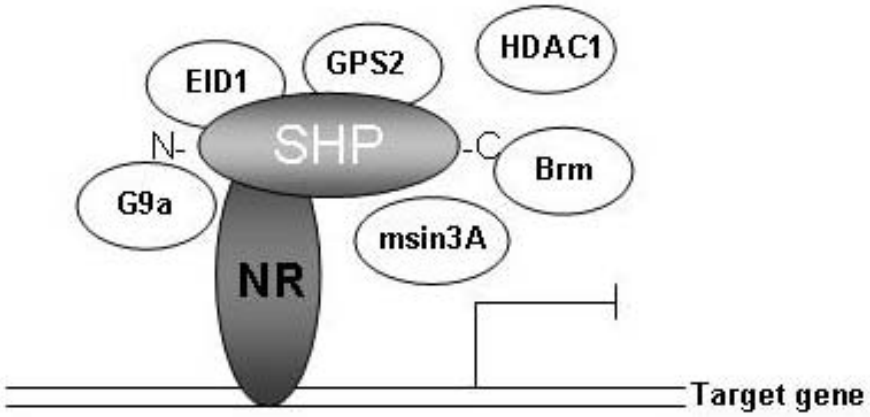

Figure 5. *SHP-interacting co-factors involved in transcriptional corepression. Only co-factors are shown that have been tested at least with two different interaction assays.*

2.10 CONCLUSIONS AND PERSPECTIVES

SHP was originally isolated in 1996 (134) and seven articles were published about different aspects of SHP such as expression, binding to NRs and repression until 1999 (58,79,82,98,134-136). In 2000, the two first articles relating to the physiological function of SHP, being involved in the negative feedback regulation of conversion of cholesterol to bile acids, were reported from studies in mice (44,92). Since that finding a large number of articles have been published, dealing with the involvement of SHP in repressing genes that are parts of metabolic pathways (see table 5). In 2002, studies on SHP deficient mice demonstrated that there are three negative regulatory pathways controlling bile acid synthesis, two SHP-independent and one SHP-dependent pathway (64,158,159). However there are still important questions to be answered, e.g. which NRs are physiologically relevant SHP targets and which physiological pathways are altered by SHP? Future phenotypical analysis of the SHP knock-out mice and the production of tissue-specific knock-outs and transgenic mice will ultimately uncover physiological pathways involving SHP also in other tissues than liver. The use of SHP NR-box mutations in transgenic mice would give information about NR-box dependent and independent physiological interactions.

Furthermore, in 2000, we reported on one of the mechanisms of the inhibitory effect of SHP and revealed that SHP binds to the ERs via LXXLL motifs and competes for binding with the co-activator TIF-2 for binding to the AF-2 within the ERs (paper I). Subsequently, several articles have shown that SHP interacts with NRs and competes with co-activators for binding (see chapter Mechanisms of function). However, more questions have to be answered, e.g. how does SHP repress NR target gene expression and which co-regulators are involved? The first article describing a SHP up-stream co-regulator (EID1) with repression potential was published in 2002 (paper II). Moreover, there are a few recent reports about putative SHP co-regulators (3,10,63). Intriguingly, we have recently identified yet another putative SHP co-repressor called GPS2 (paper IV). We will study this interaction further and test it together with human SHP mutations that show altered subcellular localization and repression function (unpublished data). Furthermore, we will characterize SHP antibodies and use them to isolate a SHP-associated multiprotein complexes.

Another important issue is species differences in the SHP function observed in mammalian two-hybrid assays and colocalization studies (A. Båvner, unpublished data). This question will be further studied using SHP protein from different species together with EID1 and GPS2 from different species.

As described earlier in this thesis SHP is an orphan receptor with no identified ligand or it may not have one. We have mapped the minimal SHP interacting EID1 peptide (A. Båvner, unpublished data) to be used to provide structure and stability of SHP, which has been shown to be very difficult to purify, in order to subsequently crystallize SHP and reveal its tertiary structure by X-ray crystallography. If the tertiary structure of SHP is uncovered, the understanding of SHP function would significantly increase and putative ligands could be designed in order to regulate SHP activity. However, if no ligands exist and SHP turns out to be a “true” orphan receptor, it will be important to identify ligand-independent pathways that regulate SHP function and expression.

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"Mix everything in same thing"….
Drawing made by 7 year old Alice, when with me in the lab. The drawing must be called lab-work ☻

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