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FUNCTION OF PIAS PROTEINS IN STEROID RECEPTOR-DEPENDENT SIGNALING

Noora Kotaja

Institute of Biomedicine/Physiology
University of Helsinki
Finland

ACADEMIC DISSERTATION

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Supervised by

Professor Olli A. Jänne
and
Docent Jorma J. Palvimo

Reviewed by

Professor Lea Sistonen
Åbo Akademi University, Turku
and
Docent Ismo Ulmanen
National Public Health Institute, Helsinki

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ABSTRACT

Steroid hormones (sex steroids, glucocorticoids and mineralocorticoids) control a wide range of biological functions connected to growth, development and homeostasis. The effects of steroid hormones are mediated by specific steroid receptors that are sequence-specific transcription factors belonging to the large family of nuclear receptors. The steroid receptors are activated upon ligand binding, which leads to dimerization of the receptors and binding to the promoter regions of their target genes. An increasing number of nuclear receptor coregulator proteins have been identified that interact with the receptors and either enhance or repress nuclear receptor-dependent transcription. ARIP3/PIASxα is an androgen receptor (AR)-interacting protein that modulates transcriptional activity of the receptor. It belongs to the PIAS (protein inhibitor of activated STAT) protein family, which also includes Miz1/PIASxβ, GBP/PIAS1, PIAS3 and PIASγ/y. Other PIAS family members have been shown to interact with and to modulate the activities of very dissimilar proteins. However, the high degree of sequence homology among PIAS proteins predicts similar functions. In this study, it was demonstrated that, in addition to ARIP3, also other PIAS proteins are able to modulate steroid receptor-dependent transcription albeit to a differential degree, depending on the receptor, the promoter and the cell type.

The activities of proteins are regulated by various covalent modifications, such as glycosylation, phosphorylation, acetylation, methylation and ubiquitination. Covalent modifications of histones and non-histone proteins involved in the regulation of transcription modulate the functions of these proteins and thus have an influence on the transcriptional output. SUMO-1 (small ubiquitin-related modifier) modification leads to attachment of SUMO-1 to specific lysine residues of target proteins in a reaction mechanistically related to ubiquitination pathway. The biological importance of SUMO-1 modification is still unclear, but there is compelling evidence for an important role of sumoylation in the regulation of protein targeting and protein-protein interactions. Activities of transcription factors are also modulated by covalent SUMO-1 modifications.

In this study, ARIP3 and other PIAS proteins were shown to interact with SUMO-1 and its E2-type conjugase Ubc9. PIAS proteins were also targets for SUMO-1 modification, and they bound other sumoylated proteins in a non-covalent fashion. Importantly, a novel function for the PIAS proteins was discovered, and ARIP3 and PIAS1 were demonstrated to act as E3 SUMO-1 ligases capable of enhancing sumoylation of several transcriptional regulators such as AR, c-Jun and the coactivator protein GRIP1. The SUMO ligase activity of PIAS proteins was dependent on the PIAS RING finger-like domain that was also critical for PIAS proteins to function as steroid receptor coregulators. The most of the effects of PIAS proteins on steroid receptor-mediated signaling were suggested to be exerted through SUMO E3 ligase and SUMO-tethering activities. Consistent with the ability of PIAS proteins to enhance sumoylation of GRIP1, PIAS proteins and GRIP1 interacted with each other and cooperated in steroid receptor-dependent transcription. In keeping with these results, mutation of the main SUMO-1 attachment sites of GRIP1 blunted its coactivator function and abolished the cooperation with PIAS proteins. In summary, this study has clarified the function of PIAS proteins and the mechanisms of steroid hormone signaling. Furthermore, these results suggest an emerging importance of SUMO-1 modifications in the regulation of transcription.

ORIGINAL PUBLICATIONS

I Kotaja N, Aittomäki S, Silvennoinen O, Palvimo JJ, and Jänne OA (2000) ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. Mol Endocrinol 14: 1986-2000

II Kotaja N, Vihinen M, Palvimo JJ, and Jänne OA (2002) Androgen receptor-interacting protein 3 and other PIAS proteins cooperate with glucocorticoid receptor-interacting protein 1 in steroid receptor-dependent signaling. J Biol Chem 277: 17781-17788

III Kotaja N, Karvonen U, Jänne OA, and Palvimo JJ (2002) PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol Cell Biol 22: 5222-5234

IV Kotaja N, Karvonen U, Jänne OA, and Palvimo JJ (2002) The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1. J Biol Chem 277: 30283-30288

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ABBREVIATIONS

AD	activation domain
AF	activation function
AIS	androgen insensitivity syndrome
AR	androgen receptor
ARE	androgen response element
ARIP3	androgen receptor-interacting protein 3
CARM1	coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
COS-1	SV40 transformed monkey kidney cell line
CREB	cAMP responsive element-binding protein
CTD	C-terminal domain of the largest subunit of RNA polymerase II
DBD	DNA-binding domain
DRIP	vitamin D receptor-interacting protein
EGFP	enhanced green fluorescent protein
ER	estrogen receptor
ERE	estrogen response element
GBP	Gu/RNA helicase II-binding protein
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor-interacting protein 1
GST	glutathione S-transferase
GTF	general transcription factor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HeLa	human cervix carcinoma cell line
HMG	high mobility group
Hsp	heat shock protein
LBD	ligand-binding domain
LUC	luciferase
Miz1	Msx-interacting zinc finger
MMTV	mouse mammary tumor virus
MR	mineralocorticoid receptor
N-CoR	nuclear receptor corepressor
NID	nuclear receptor interaction domain
NR	nuclear receptor
NTD	N-terminal domain
PIAS	protein inhibitor of activated STAT
PIC	preinitiation complex
PML	promyelocytic leukemia gene product
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
RAR	retinoid acid receptor
RNAP II	RNA polymerase II
RXR	retinoid X receptor
SAP	SAF-A/B, Acinus and PIAS motif
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
SRC	steroid receptor coactivator
STAT	signal transducers and activators of transcription
SUMO-1	small ubiquitin-like modifier 1
TAF	TBP-associated factor
TBP	TATA-binding protein
TR	thyroid hormone receptor
TRAP	thyroid hormone receptor-associated protein
UBA	ubiquitin-associated domain
Ubc9	ubiquitin-conjugating enzyme 9 (SUMO-conjugating E2 enzyme)
UIM	ubiquitin-interacting motif
VDR	vitamin D_3 receptor
ZF	zinc finger

REVIEW OF THE LITERATURE

1. PIAS PROTEIN FAMILY

The PIAS protein family is a small group of nuclear proteins, including PIAS1/GBP, PIAS3, ARIP3/PIASxα, Miz1/PIASxβ and PIASγ/y (Chung et al. 1997, Valdez et al. 1997, Wu et al. 1997, Liu et al. 1998, Moilanen et al. 1999, Sturm et al. 2000). The members of the PIAS family were found in yeast two-hybrid screens using various proteins, mostly transcription factors, as baits. In addition to these interactions, PIAS proteins have recently been reported to interact with and to modulate the activities of number of other proteins. PIAS proteins are evolutionary conserved, since homologues of mammalian PIAS proteins are found in many nonvertebrate animal species and even in plants. For example, a protein encoded by the *Drosophila melanogaster* gene *Zimp* (Mohr and Boswell 1999), a predicted *Caenorhabditis elegans* protein, the *Saccharomyces cerevisiae* septin-interacting protein Nfi1p and putative proteins from *Schizosaccharomyces pombe*, *Vicia faba* and *Arabidopsis thaliana* share sequence homology with PIAS proteins. The physiological importance of PIAS proteins is underscored by the finding that *Zimp* is an essential gene in *Drosophila* (Mohr and Boswell 1999).

1.1. Discovery of PIAS proteins

The PIAS protein family is named according to the members that were first characterized in cytokine signaling as Protein Inhibitors of Activated STATs (signal transducer and activator of transcription). PIAS3 (protein inhibitor of activated Stat3) was demonstrated to interact with Stat3 and inhibit Stat3-dependent signaling (Chung et al. 1997). PIAS1 was found in a yeast two-hybrid screen of a human B cell library with Stat1 devoid of the C-terminal transcriptional activation domain as bait (Liu et al. 1998). The other PIAS family members PIAS3, PIASxα, PIASxβ and PIASy were found by EST database searching and library screening using the PIAS1 sequence as a probe (Liu et al. 1998). PIAS proteins have also been found to interact with other proteins unrelated to the STATs. Miz1 (Msx-interacting zinc-finger) was found in a yeast two-hybrid screen using a mouse E14.5 day cDNA library and full-length Msx2 as bait (Wu et al. 1997). Msx2 is a homeodomain-containing protein that functions in the regulation of inductive tissue interactions (Wu et al. 1997). Wu et al. described Miz1 as a sequence-specific DNA-binding protein and a positive-acting transcription factor that is able to enhance the DNA binding of Msx2. Miz1 is a mouse counterpart of human PIASxβ, but the Miz1 form studied by Wu et al. lacked the nucleotides encoding the first 131 amino acids of PIASxβ. ARIP3 (androgen receptor-interacting protein 3) was discovered when a region of androgen receptor (AR) including

the DNA-binding domain and part of the hinge region was used as bait to screen a mouse embryo E10.5 library (Moilanen et al. 1999, Fig. 1). Rat ARIP3 corresponds to human PIASxα. The first 550 amino acids of PIASxα and PIASxβ are identical, and they differ only in their C termini. Thus, they most probably represent splicing variants encoded by the same gene. Gu/RNA helicase II-binding protein (GBP) bound to the helicase II in a yeast two-hybrid screen using a human B lymphocyte cDNA library (Valdez et al. 1997). GBP is almost identical to PIAS1; it lacks only the first nine amino acids, and displays a few amino acid differences when compared to PIAS1 sequence. KChAP (K^+ channel-associated protein) was discovered when a yeast two-hybrid screen of rat brain cDNA library was performed with the full-length Kvβ1.2 subunit of the K^+ channel as bait (Wible et al. 1998). Rat KChAP is almost identical to PIAS3, and it was termed PIAS3β to distinguish it from the original mouse PIAS3 clone (Wible et al. 2002). The N-terminal region of KChAP contains an in-frame insertion of 39 amino acids, which is lacking from mouse PIAS3 but not from human PIAS3.

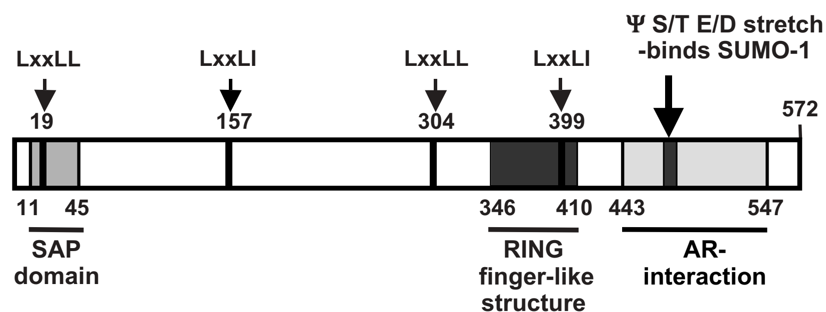

Fig. 1. Schematic structure of ARIP3. Arrows depict LXXLL motifs starting form amino acids 19 and 304, and related LXXLI motifs starting from amino acids 157 and 399. The SAP motif, the putative RING finger-like structure, the SUMO-1 binding motif, and the region found to interact with AR in a yeast two-hybrid screen are indicated.

1.2. Structure of PIAS proteins

PIAS proteins share a high degree of sequence homology with the most conserved regions being the first 60 amino acids at their N terminus and the central domain containing a putative zinc-binding structure. The serine-rich C terminus of PIAS proteins is the least conserved part. In the N terminus, PIAS proteins have a SAP (SAF-A/B, Acinus and PIAS) motif predicted to be involved in sequence- or structure-specific DNA binding (Fig. 1, Aravind and Koonin 2000). SAP motif is found in the sequences of many chromatin-associated proteins, such as the scaffold attachment factors A and B (SAF-A and -B), plant poly(ADP-ribose) polymerase (PARP) and human Acinus (Romig et al. 1992, Renz and Fackelmayer 1996, Sahara et al. 1999). SAF-A and -B are nuclear proteins that bind to AT-rich chromosomal regions known as scaffold- or matrix-attachment regions (SAR/MAR) (Romig et al. 1992, Renz and Fackelmayer 1996). Mutations of SAF-A SAP motif result in

a complete loss of the DNA-binding activity (Göhring et al. 1997). By targeting proteins to specific chromosomal locations, SAP motif may contribute to the coupling of transcription with splicing and repair and to apoptotic degradation of chromatin (Aravind and Koonin 2000).

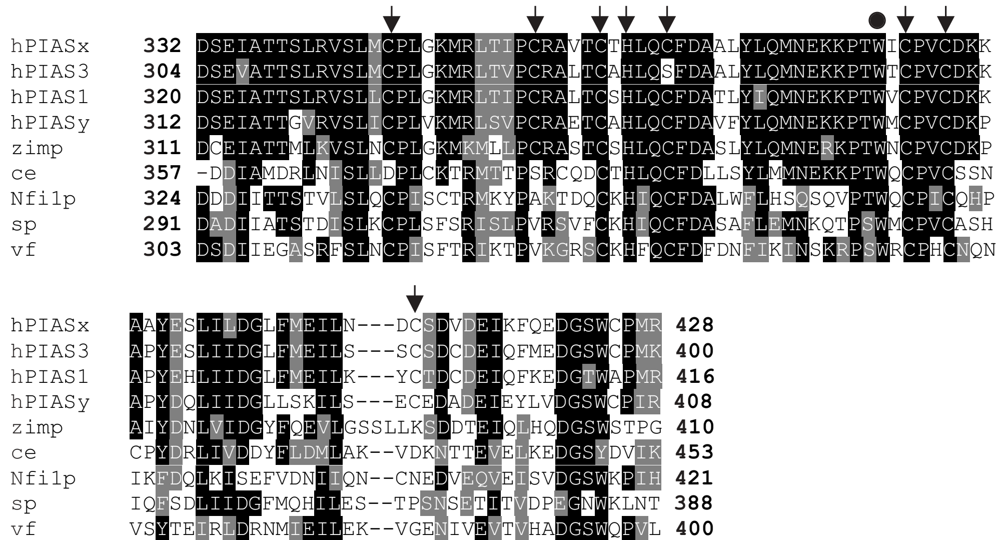

Fig. 2. Sequence alignment of the potential zinc finger region of PIASx and related proteins. The region of human (h) PIASxα containing the zinc finger structure (protein database sequence identification number GI:3643113; amino acids 332–428) was aligned with the same region of related proteins. hPIAS3 (4996563, amino acids 304–400); hPIAS1 (3643107, amino acids 320–416); hPIASy (7706230, amino acids 312–408); zimp = *Drosophila* Zimp-A (4761232, amino acids 311–410); ce = a predicted *C. elegans* protein (7509196, amino acids 357–453); Nfi1p = *S. cerevisiae* Nfi1 protein (2498628, amino acids 324–421); sp = a predicted protein from *S. pombe* (7493364, amino acids 291–388); vf = *V. faba* protein (2104683, amino acids 303–400). Black boxes and gray shadings depict amino acids that are identical or conserved among the sequences, respectively. Conserved cysteines (at least in four family members) and histidine are indicated by arrows, and the circle depicts the conserved Trp residue.

The central part of PIAS proteins is predicted to form a zinc-binding structure, termed Miz Zn-finger (Wu et al. 1997). All PIAS proteins except PIAS3, harbor seven cysteine residues and a histidine residue which are mandatory for a C_3HC_4-type RING finger motif (Fig. 2, Joazeiro and Weissman 2000). Threading analysis of this PIAS region suggests that its three-dimensional structure is similar to a C_3HC_4 RING finger fold. However, the spacing between potential zinc-coordinating residues and the amino acid composition of mammalian PIAS RING-like structure differ substantially from the C_3HC_4 RING finger. Interestingly, PIAS3 does not have a cysteine residue at the fourth position of the putative C_3HC_4 motif.

The LXXLL motif is a short amino acid sequence involved in interactions of coactivators with the ligand-binding domain (LBD) of nuclear receptors (Heery et al. 1997). PIAS proteins have two LXXLL motifs starting at residues 19 and 304 in the ARIP3 sequence. The N-terminal LXXLL motif of PIASy is not involved in binding to

Stat1 or AR, but it is required for its inhibitory activity (Gross et al. 2001, Liu et al. 2001). PIAS proteins harbor a motif containing Ser and Glu/Asp residue-rich stretches preceded by hydrophobic residues C-terminal to the zinc-binding structure of PIAS proteins (amino acids 472-482 in ARIP3 sequence). A similar motif from the PM-Scl75 protein is suggested to act as a SUMO-1-interacting motif since it is sufficient for the interaction of PM-Scl75 with SUMO-1 in yeast (Minty et al. 2000).

1.3. Expression of PIAS proteins

The genes encoding PIAS proteins are located in distinct chromosomes in the human genome. The PIASy gene has been mapped to chromosomal location 19p13.3 (Sturm et al. 2001), the PIAS3 gene was localized to chromosome 1 (1q21) (Ueki et al. 1999) and the PIAS1 gene to chromosome sub-band 15q22 (Weiskirchen et al. 2001). BLAST search localized the PIASx gene to chromosome 18. ARIP3/PIASxα, PIAS1/GBP and PIASy are mainly expressed in testis, but low levels of mRNAs are also detected in other tissues (Valdez et al. 1997, Moilanen et al. 1999, Tan et al. 2000, Gross et al. 2001). In addition, EST database search with PIAS sequences revealed distribution of PIAS proteins in various tissues. Immunostaining of adult rat testis localized ARIP3 protein in the nuclei of Sertoli cells, spermatogonia, and primary spermatocytes (Moilanen et al. 1999). Likewise, PIAS1 protein is expressed in Sertoli cells, Leydig cells and spermatogenic cells including spermatocytes and round spermatids (Tan et al. 2000). Expression of PIAS1, PIASxα and PIASxβ mRNA is detected by *in situ* hybridization throughout the germinal epithelium, but there are some regional differences: expression of PIAS1 is higher near the central region associated with round spermatids and PIASxα transcript is more abundant in the peripheral layers of cells such as Sertoli cells, spermatogonia and early spermatocytes (Tan et al. 2002). mRNA levels of PIAS1, PIASxα and PIASxβ in mouse testis increase during sexual development, which is consistent with the high expression in the developing spermatogenic cells (Tan et al. 2000 and 2002).

In contrast to PIAS1, PIASxα and PIASy, PIAS3 mRNA is widely expressed in various human tissues (Chung et al. 1997). Similarly, expression of KChAP/PIAS3β is detected by northern blotting in a variety of tissues with especially high levels in lung and kidney (Wible et al. 1998). In addition to adult tissues, PIAS proteins are expressed during the embryonic development. Expression of PIASγ (a mouse homologue of human PIASy), PIAS3 and GBP is detected in mouse embryos by RT-PCR as early as on embryonic day 7.5 (Sturm et al. 2000), and northern blot analysis of whole embryos reveals the presence of mouse Miz1 transcripts from embryonic day 9.5 onwards (Wu et al. 1997).

The factors regulating PIAS protein levels are unclear at the moment, but androgens have been shown to induce the expression of PIAS3 mRNA in LNCaP prostate carcinoma cells (Junicho et al. 2000). Estrogens induce the accumulation of PIAS3 mRNA

in multiple myeloma (MM) cells and increase the physical association of PIAS3 with Stat3, which is suggested to represent a possible mechanism for estrogen-mediated inhibition of IL-6-inducible MM cell proliferation (Wang et al. 2001b). Interestingly, downregulation or upregulation of PIAS protein expression that correlates with increased or decreased STAT activity, respectively, has been associated with many distinct clinical conditions, such as anaplastic lymphoma kinase-positive T/null-cell lymphoma, acute hepatic failure and cystic fibrosis (Kamohara et al. 2000, Kelley and Elmer 2000, Zhang et al. 2002), as well as with physiological processes such as macrophage maturation (Coccia et al. 2002). Down-regulation of PIASy expression has also been detected in association with stage progression in chronic myeloid leukemia (Ohmine et al. 2001), and GBP/PIAS1 is down-regulated in RAS-transformed cells (Zuber et al. 2000).

1.4. PIAS proteins in cytokine-signaling pathway

STAT proteins are transcription factors that are activated by various cytokines (Leonard and O'Shea 1998, Ihle 2001). Binding of the cytokine to its cell surface receptor results in docking of STAT proteins to the receptor and tyrosine phosphorylation of STATs. Phosphorylated STATs form dimers and translocate to the nucleus where they bind to the promoter regions of their target genes and induce transcription (Leonard and O'Shea 1998). PIAS1 interacts with Stat1 and inhibits transcriptional activation by blocking the DNA-binding activity of Stat1 (Liu et al. 1998). This interaction is specific, since PIAS1 is not able to associate with other STAT proteins (Liu et al. 1998). Likewise, PIAS3 binds selectively to Stat3 and inhibits its DNA-binding and transcriptional activities (Chung et al. 1997). PIASy has also been shown to interact with and repress the transcriptional activity of Stat1, but in contrast to PIAS1, without blocking the Stat1 DNA-binding activity (Liu et al. 2001).

The association of Stat1 and Stat3 with PIAS1 and PIAS3, respectively, is detected only when STAT proteins are activated by ligands (Chung et al. 1997, Liu et al. 1998). Tyrosine phosphorylation of Stat1 is required for the interaction between Stat1 and PIAS1 *in vivo* (Liu et al. 1998). A separate study demonstrated that the dimerization of Stat1 is crucial for Stat1-PIAS1 interaction, since PIAS1 is able to bind a Stat1 dimer, but not a tyrosine-phosphorylated or unphosphorylated Stat1 monomer (Liao et al. 2000). The nature of Stat1-PIAS1 interaction has been further defined by Mowen and colleagues, who found that Stat1 dimers are methylated and that methylation prevents PIAS1 from binding to the Stat1 dimer (Mowen et al. 2001). The importance of PIAS proteins in STAT-mediated signaling is supported by the finding that the amounts *Drosophila* PIAS protein (dPIAS) and STAT protein (STAT92E) have to be correctly balanced for the normal blood cell and eye development to occur (Betz et al. 2001).

1.5. Other interaction partners and functions of PIAS proteins

During the last few years, PIAS proteins have been reported to interact with various proteins distinct from their original interaction partners, and different functions have been suggested for the PIAS proteins (Table 1). ARIP3/PIASxα associates with DJ-1, a protein connected to male rat infertility (Takahashi et al. 2001a). Since ARIP3/PIASxα is able to repress AR-dependent transcription, DJ-1 was suggested to function as a positive regulator of AR by impairing the binding of ARIP3/PIASxα to the receptor (Takahashi et al. 2001a). ARIP3/PIASxα was also found to interact with the p67 isoform of mouse disabled 2 (mDab2) in differentiating F9 cells (Cho et al. 2000). The function of mDab2 is not clear, but it has been suggested to play a role in signal transduction. In addition to interaction with Stat3, PIAS3 has been found to bind to many different proteins in yeast two-hybrid screens. The zinc finger protein Gfi-1 interacts with PIAS3 and enhances Stat3 signaling by blocking the repressive activity of PIAS3 (Rödel et al. 2000). PIAS3 also associates with and represses the transcriptional activity of the microphthalmia transcription factor (MITF) that plays a role in mast cell and melanocyte development (Levy et al. 2002). Zentner et al. (2001) demonstrated that PIAS3 binds to high mobility group protein HMGI-C and corepresses GR and Stat3 signaling pathways with HMGI-C in salivary epithelial cells. PIAS1 was recently reported to be a binding partner of CRP2 (cysteine- and glycine-rich protein 2) (Weiskirchen et al. 2001).

Both PIAS1 and PIASy have been found to interact with p53 tumor suppressor protein in yeast two-hybrid assays (Nelson et al. 2001, Megidish et al. 2002). In contrast to PIASy that inhibits p53-dependent transactivation (Nelson et al. 2001), PIAS1 is capable of activating p53-mediated gene expression in variety of cell lines (Megidish et al. 2002). In line with the ability of PIAS1 to enhance the transcriptional activity of the proapoptotic protein p53, PIAS proteins have been demonstrated to possess proapoptotic activity (Liu and Shuai 2001). PIAS1 is able to induce apoptosis through activation of c-Jun N-terminal kinase (JNK), and the proapoptotic function of PIAS1 is independent of the inhibitory activity of PIAS1 in STAT-mediated gene activation (Liu and Shuai 2001). KChAP/PIAS3β that functions as a chaperone for specific Kv channels, is also able to induce K^+ efflux and apoptosis in prostate cancer cells (Wible et al. 1998, Kuryshev et al. 2000).

An important role of PIAS proteins in chromosome organization was demonstrated by studies showing that *Su(var)2-10* locus in the *Drosophila* genome encoding a PIAS protein is required for proper chromosome structure and chromosome inheritance (Hari et al. 2001). Further evidence for the role of PIAS proteins in chromosome organization is offered by Strunnikov et al. (2001), who showed that defects in chromosome condensation in *S. cerevisiae* caused by the loss of SMT4 gene are bypassed when the SIZ1 gene encoding a yeast homologue of mammalian PIAS proteins is overexpressed. This finding

links PIAS proteins to SUMO-1 modification processes, since SMT encodes an evolutionarily conserved protease exhibiting SUMO-cleaving isopeptidase activity.

Table 1. The interaction partners of PIAS proteins

PIAS protein	Interaction partner in the original yeast two-hybrid screen	other interactions	
ARIP3/PIASxα	AR (Moilanen et al. 1999)	GR, PR, ER	Kotaja et al. 2000
		DJ-1	Takahashi et al. 2001a
		mDab2	Cho et al. 2000
		p73	Minty et al. 2000
Miz1/PIASxβ	Msx2 (Wu et al. 1997)	AR, GR, PR, ER	Kotaja et al. 2000
		p73	Minty et al. 2000
PIAS1	Stat1 (Liu et al. 1998)	AR	Tan et al. 2000
		CRP2	Weiskirchen et al. 2001
		p53	Megidish et al. 2002
		TTF-1	Missero et al. 2001
GBP	Gu/RNA helicase II (Valdez et al. 1997)		
PIAS3		Stat3	Chung et al. 1997
		AR	Junicho et al. 2000
		Gfi-1	Rödel et al. 2000
		MITF	Levy et al. 2002
		HMGI-C	Zentner et al. 2001
		TTF-1	Missero et al. 2001
KChAP	Kvβ1.2 (Wible et al. 1998)		
PIASy		Stat1	Liu et al. 2001
		AR	Gross et al. 2001
		p53	Nelson et al. 2001
		LEF1	Sachdev et al. 2001

2. Steroid receptor-mediated signaling

Living cells have to respond to a high number of signals that they receive constantly from their environment. Signaling molecules can be secreted by distant endocrine organs and transported to target cells via circulation (endocrine signaling). On the other hand, signals can be produced by surrounding cells (paracrine signaling) or by the target cell itself (autocrine signaling). Many kinds of signaling molecules, such as lipid-soluble hormones, small peptides, polypeptides and lipids, exist. Signaling molecules are detected by

receptors located either on the cell surface or inside the cell. Binding of a ligand to a cell surface receptor usually activates a cascade of protein phosphorylation events finally leading to activation of transcription factors that can induce transcription of target genes. Nuclear receptors, in turn, have lipid-soluble ligands that can penetrate through the cell membrane and bind to their cognate intracellular receptors. Steroid hormone receptors belong to the nuclear receptor superfamily that also include receptors for thyroid hormones, vitamin D, and retinoids, and thus regulate a broad range of functions connected to growth, development and homeostasis. These receptors are ligand-regulated sequence-specific transcription factors that bind to specific hormone response elements usually located at promoter regions of their target genes, and thereby regulate transcription. In addition to the receptors and general transcription machinery, many other coregulator proteins are involved in the regulation of transcription. These coactivators and corepressors bind to receptors or other coregulators, and they either enhance or repress receptor-mediated transcription.

2.1. General transcription machinery

Promoters of mRNA-encoding genes contain three types of DNA elements. Basal promoter elements such as the TATA-box and the initiator motif (Inr) are located near the start site of transcription and bind proteins belonging to the general transcription machinery (Goodrich et al. 1996). Promoter proximal elements located upstream of the start site, and distal enhancer elements that can be located many thousands of base pairs from the start site, bind sequence-specific transcription factors. General transcription machinery containing RNA polymerase II and general transcription factors (GTFs) is sufficient to promote basal gene transcription *in vitro*. However, sequence-specific transcription factors as well as numerous different coregulator proteins, such as those with chromatin remodeling activities, are required for efficient transcription and specific regulation of gene expression *in vivo* (Orphanides et al. 1996, Woychik and Hampsey 2002).

Genes encoding mRNAs are transcribed by RNA polymerase II (RNAP II). General transcription factors needed for RNAP II-mediated basal transcription include TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Orphanides et al. 1996, Woychik and Hampsey 2002). TFIID is composed of TATA-binding protein (TBP) and TBP-associated factors (TAFs). The TBP subunit of TFIID binds to TATA box, which is a DNA element located 25-30 bp upstream of the transcription start site. The binding is followed by the recruitment of TFIIB, which, in turn, binds RNAP II and associated TFIIF. The preinitiation complex (PIC) is complete after the entry of TFIIE and TFIIH (Orphanides et al. 1996, Woychik and Hampsey 2002). TFIIH contains a kinase subunit that phosphorylates the C-terminal domain (CTD) of the largest subunit of RNAP II (Lu et al. 1992, Serizawa et al. 1992). The phosphorylation of RNAP II after initiation of

transcription is crucial for gene expression, since this modification is required for progression of transcription into the elongation phase (Dahmus 1996).

2.2. Nuclear receptor superfamily

The nuclear receptor superfamily comprises a large family of proteins (49 genes in the human genome), including the receptors for steroid hormones (androgens, estrogens, progestins, mineralocorticoids and glucocorticoids), retinoids, vitamin D and thyroid hormones (Beato et al. 1995, Mangelsdorf et al. 1995, Aranda and Pascual 2001). In addition, the superfamily includes so-called orphan receptors that share a similar structure with other receptors, but they are not activated by ligands, or their ligands are still unidentified (Giguere 1999). Identification of ligands for the orphan receptors has been a subject for intense studies, and novel ligands, both natural and synthetic compounds, have been characterized; PPAR (peroxisome proliferator activated receptor) is activated by prostaglandins and unsaturated fatty acids, PXR (pregnane X receptor) by pregnanes, CAR (constitutive androstane receptor) by androstanes, LXR (liver X receptor) by oxysterols, and FXR (farnesoid X receptor) by bile acids (Giguere 1999).

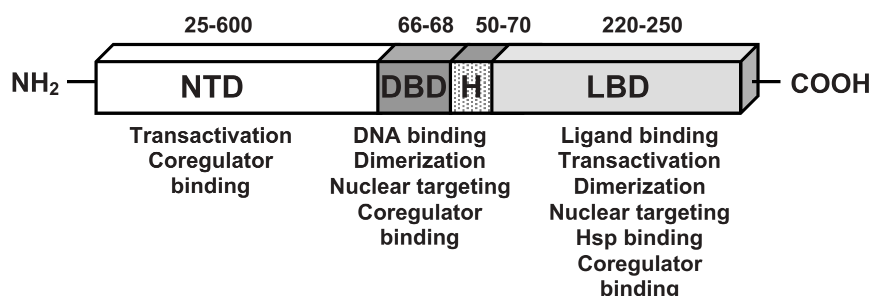

Fig. 3. Modular structure of nuclear receptors. NTD, the N-terminal transactivation domain; DBD, the DNA-binding domain; H, the hinge region; LBD, the ligand-binding domain. The main functions of each domain are shown. The numbers depict the number of amino acid residues in the four domains in different nuclear receptors.

Nuclear receptors share a conserved modular structure (Fig. 3). The three major functional domains are (i) the N-terminal transactivation domain (NTD), (ii) the DNA-binding domain (DBD), and (iii) the C-terminal ligand-binding domain (LBD). The short nonconserved region between the DBD and the LBD is called the hinge region. The most variable region of nuclear receptors both in sequence and size is the NTD. The three-dimensional structure of this domain has not been resolved. Most of the nuclear receptors, such as AR and glucocorticoid receptor (GR), have a ligand-independent transcriptional activation function (AF1) in their NTD (Hollenberg and Evans 1988, Tora et al. 1989, Simental et al. 1991). The NTD has been suggested to function by binding general

transcription factors (Beato and Sanchez-Pacheco 1996, Ford et al. 1997) and coregulator proteins (Ikonen et al. 1997, Onate et al. 1998, Alen et al. 1999, Bevan et al. 1999, Hittelman et al. 1999, Ma et al. 1999, Wallberg et al. 1999). In the case of AR, the NTD also participates in intramolecular interactions with the LBD of the receptor (Kraus et al. 1995, Ikonen et al. 1997).

The DBD located in the central part of the receptor is the most conserved region among nuclear receptors. The three-dimensional structures of the DBD of many nuclear receptors have been resolved by nuclear magnetic resonance or crystallographic studies (Härd et al. 1990, Schwabe et al. 1990 and 1993, Luisi et al. 1991). The DBD harbors two zinc fingers that are formed when four cysteine residues coordinately bind a zinc ion in each of the two motifs. The first zinc finger is critical for specific DNA contacts, and the second zinc finger interacts with the DNA phosphate backbone and is involved in receptor dimerization (Freedman 1992, Quigley et al. 1995). In addition to DNA binding and dimerization, the DBD of nuclear receptors is involved in interactions with coregulators (Moilanen et al. 1998a, 1998b and 1999, Blanco et al. 1998, Puigserver et al. 1998), and it is also required for cross-talk with other transcription factors such as NF-κB and AP-1 (Schüle et al. 1990, Ray and Prefontaine 1994, Aarnisalo et al. 1999).

The LBD contains the ligand-binding pocket, activation function 2 (AF2) and dimerization surface. The crystal structures of various nuclear receptors have revealed a common structure with 12 α helices and one β turn arranged as an antiparallel α-helical sandwich in a three-layer structure (Bourguet et al. 1995, Renaud et al. 1995, Wagner et al. 1995, Wurtz et al. 1996, Brzozowski et al. 1997, Nolte et al. 1998, Williams and Sigler 1998). Upon binding of the ligand, specific α helices are repositioned leading to sealing of the ligand-binding cavity with helix H12. The ligand-induced repositioning of H12 provides the surface(s) for coactivator interactions and generates the active AF2. The crystal structure of estrogen receptor α (ERα) LBD complexed with the antagonist raloxifen shows that the antagonist induces a conformation distinct from the agonist-induced conformation; in the antagonist-induced conformation, H12 is placed in an "antagonist position" (Brzozowski et al. 1997). This conformation blocks the surface formed by helices H12, H3, H4 and H5 normally used for the binding of an LXXLL motif of TIF2/GRIP1, a member of SRC family of nuclear receptor coactivators (Shiau et al. 1998). The crystal structure of PPARα LBD bound to an antagonist and a SMRT co-repressor motif shows that the antagonist-bound receptor adopts a conformation, which facilitates the binding of corepressors (Xu et al. 2002).

2.3. Mode of steroid receptor action

In an inactive state, steroid receptors are associated with large multiprotein complexes, including heat shock proteins Hsp90, Hsp56, Hsp70 and p23 (Beato et al. 1996, Pratt and

Toft 1997). In these complexes, the receptors are maintained in a high-affinity ligand binding form. Binding of a ligand induces a conformational change in the receptor structure that leads to dissociation of heat shock proteins and allows dimerization of the receptors (Fig. 4, Pratt and Toft 1997, Moras and Gronemeyer 1998). To activate gene transcription, nuclear receptors have to be transported to the nucleus. The cellular localization prior to ligand binding varies among different steroid receptors. GR is predominantly located in the cytoplasm, and ligand induces its nuclear transport (Picard and Yamamoto 1987, Sackey et al. 1996). In contrast to GR, unliganded ER and progesterone receptor (PR) seem to be mostly nuclear (Htun et al. 1999, Lim et al. 1999). AR has been reported to be both nuclear and cytoplasmic depending on the experimental conditions used (Jenster et al. 1993, Zhou et al. 1994, Karvonen et al. 1997).

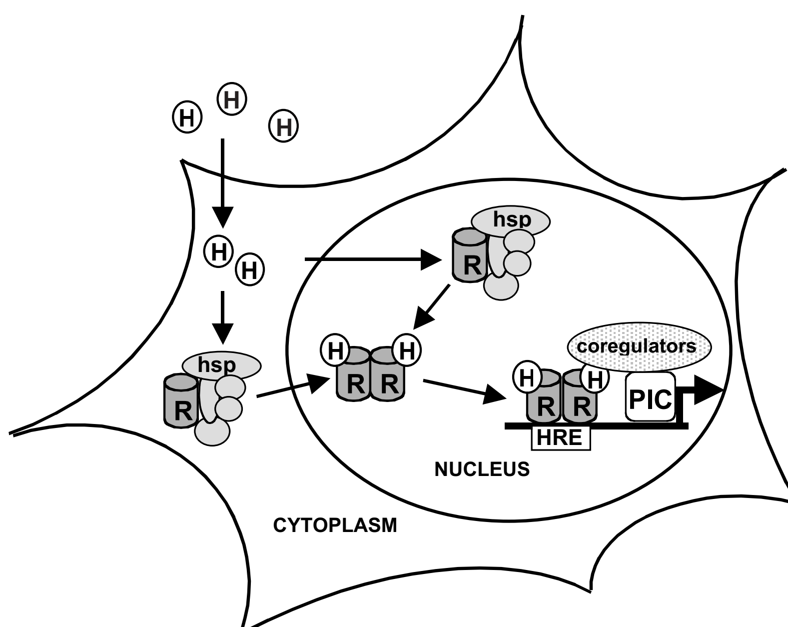

Fig. 4. Mode of steroid receptor action. R, steroid receptor; H, hormone; hsp, heat shock protein; HRE, hormone response element; PIC, preinitiation complex. Upon hormone binding, receptors dimerize, bind to the hormone response element and thereby regulate the gene transcription. Coregulator proteins bind to the receptor dimer and modulate steroid receptor-dependent transcription.

Nuclear receptors are grouped into four classes according to their ligand-binding, DNA-binding and dimerization properties (Mangelsdorf et al. 1995). Class I nuclear receptors including steroid receptors form homodimers, but some members of this class are also suggested to form heterodimers (Trapp et al. 1994, Liu et al. 1995, Cowley et al. 1997, Lee et al. 1999). Class II receptors, such as vitamin D receptor (VDR), retinoic acid receptor (RAR), PPARs and thyroid hormone receptor (TR), heterodimerize with retinoid X receptor (RXR). Class III contains some orphan receptors that bind DNA as

homodimers, and class IV orphan receptors bind DNA as monomers (Mangelsdorf et al. 1995). AR, GR, PR and mineralocorticoid receptor (MR) homodimers are capable of binding to the palindromes of the sequence AGAACA separated by three nucleotides (5'-AGAACAnnnTGTTCT-3'), whereas ER recognizes the palindromic consensus sequence 5'-AGGTCAnnnTGACCT-3'. Class II nuclear receptors bind preferentially to direct repeats separated by one to five nucleotides (DR1-5), but they can also recognize response elements in palindromic or inverted palindromic configurations (Glass 1994, Aranda and Pascual 2001). In addition to the positive response elements that mediate receptor-dependent activation of gene expression, so-called negative response elements that bind the receptors and mediate negative regulation by the ligand have been reported (Sakai et al. 1988, Drouin et al. 1989, Zhang et al. 1997).

Most of the steroid receptors excluding ER are able to recognize the same response element, and they also have overlapping expression patterns. This raises a question of how the receptor specific actions are achieved *in vivo*. One possibility is that the binding of other transcription factors to the same promoter region modulate the specificity of the receptor. In addition, the promoter regions of many natural target genes of steroid receptors, especially those of AR, contain "modified" response elements with enhanced affinity to specific receptors (Claessens et al. 1996, Zhou et al. 1997, Schoenmakers et al. 1999 and 2000, Verrijdt et al. 2000). These findings are, however, unlikely to be the only explanations for the specificity of target gene activation. A new aspect to the regulation of steroid receptor-dependent transcription was introduced with the discovery of an increasing number of coregulator proteins (Fig. 5).

2.3.1. Coactivators

Coactivators do not usually possess DNA-binding activity, but they interact with the DNA-bound receptor or with other receptor-bound coregulators, and enhance the steroid receptor-dependent transcription. Coactivators form large coactivator complexes, and it is suggested that the nuclear receptor-mediated transcription requires several different protein complexes that may act sequentially, combinatorially or in parallel (Fig. 5, Glass and Rosenfeld 2000, Hermanson et al. 2002, McKenna and O'Malley 2002). The coactivator complexes with histone acetyltransferase (HAT) activity promote transcription by acetylating histone tails which is known to "loosen" nucleosome structures making genes more accessible for transcription. The complexes with ATP-dependent chromatin-remodeling activities also enhance transcription by opening up tightly packed chromatin. In addition, the multiprotein TRAP/DRIP/Mediator complex interacts with both receptor dimers and general transcription machinery and thus functions as a bridging factor between them. Steroid receptors and coactivators have been suggested to bind to the promoters in a cyclic fashion so that the binding of the receptor dimer is followed by the recruitment of

different coactivator complexes, after which dissociation of proteins takes place and the cycle is repeated (Shang et al. 2000, Burakov et al. 2002).

In addition to the above-described proteins that function as acetyltransferases, ATPases and mediators, many other coactivators with different functions have been reported (McKenna et al. 1999). The E3 ubiquitin-protein ligase E6-AP interacts with and coactivates steroid receptors in a fashion that is independent of its ligase activity (Nawaz et al. 1999a, Smith et al. 2002). The small nuclear RING finger protein SNURF that is able to bind steroid receptors and modulate steroid receptor-dependent transcription is also a potential ubiquitin E3 ligase (Moilanen et al. 1998a, our unpublished results). The high-mobility group proteins 1 and 2 (HMG-1 and -2) – chromatin non-histone proteins that bind DNA and induce bends in DNA – increase the sequence-specific DNA-binding and transcriptional activity of steroid receptors (Boonyaratanakornkit et al. 1998). Furthermore, the androgen receptor-interacting nuclear protein kinase (ANPK/HIPK3) is a Ser/Thr protein kinase capable of enhancing AR-mediated transcription (Moilanen et al. 1998b). Interestingly, the steroid receptor RNA activator (SRA) that selectively coactivates AF1 of steroid receptors, differs from other coactivators in that the functional form of SRA is not a protein but a RNA transcript (Lanz et al. 1999).

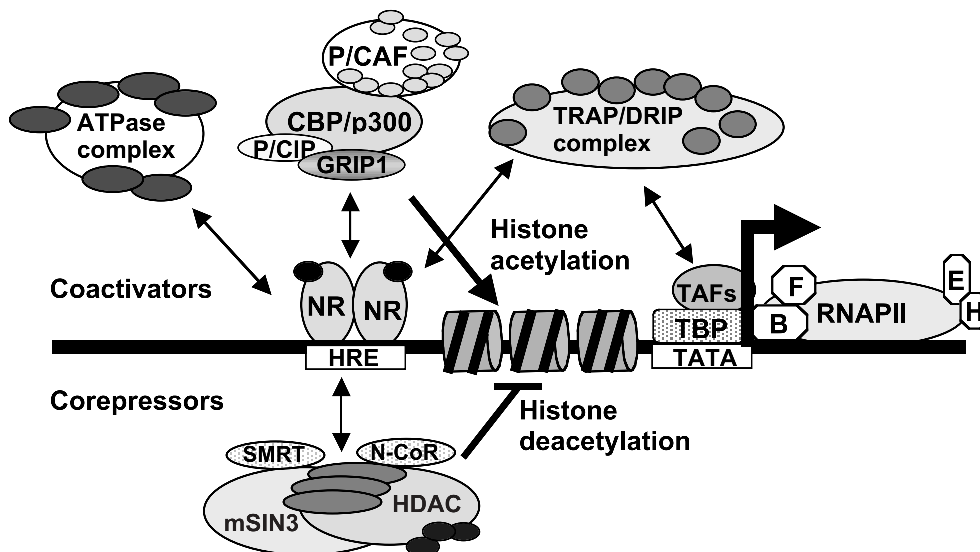

Fig. 5. Coactivator and corepressor complexes modulate the activity of nuclear receptors (NR). Coactivators form distinct complexes with different functional properties: ATPase activity-containing complexes are able to remodel chromatin, HAT-containing complexes acetylate histones and TRAP/DRIP complexes mediate interaction between receptors and general transcription machinery. Corepressor complexes contain histone deacetylase activities (HDAC). Double-headed arrows show the interactions between coregulators and receptors. RNAPII: RNA polymerase II; B, E, F and H: TFIIB, TFIIE, TFIIF and TFIIH, respectively (adapted from Glass and Rosenfeld 2000).

Complexes with histone acetyltransferase activity

The best-characterized coactivator family, the SRC family (the p160 family), includes SRC-1/NCoA-1 (Oñate et al. 1995, Kamei et al. 1996), GRIP1/TIF2/SRC-2 (Hong et al. 1996, Voegel et al. 1996), pCIP/TRAM-1/RAC3/ACTR/AIB-1/SRC-3 (Anzick et al. 1997, Chen et al. 1997, Li et al. 1997, Takeshita et al. 1997, Torchia et al. 1997). The nuclear receptor interaction domain (NID) of the SRC family members contain three LXXLL signature motifs, where L represents leucine residue and X represents any amino acid (Heery et al. 1997). The LXXLL motifs have a well-characterized role in the binding of the coactivator to the LBD of the receptor (Darimont et al. 1998, Shiau et al. 1998). The LXXLL-LBD interaction is ligand-dependent and in the absence of ligand, or in the presence of antagonist, the surface for the binding of the LXXLL peptide is blocked (Shiau et al. 1998). The NID that is located in the central part of SRC proteins is not, however, the only region binding to nuclear receptors, since the C-terminal region of SRC-2/GRIP1 has been shown to mediate the binding to the AF1 of AR (Alen et al. 1999, Ma et al. 1999). The physiological importance of the SRC proteins has been demonstrated by gene targeting in mice (Xu et al. 1998 and 2000). Disruption of the SRC-1 gene caused partial resistance to steroid hormones (Xu et al. 1998). The expression of another family member, SRC-2/GRIP1, was increased in the SRC-1 null mice suggesting that SRC-2/GRIP1 might compensate partially for the loss of SRC-1 (Xu et al. 1998). Disruption of the SRC-3 gene had more severe effects resulting in dwarfism, delayed puberty, reduced female reproductive function, and blunted mammary gland development (Xu et al. 2000). SRC-1 and SRC-3 harbor HAT activities (Chen et al. 1997, Spencer et al. 1997), but a more important function of these proteins seems to be the recruitment of other HAT coactivators to the promoter-bound receptor dimer. The SRC family members interact with CBP (CREB-binding protein) and a highly related p300 (Kamei et al. 1996, Torchia et al. 1997, Voegel et al. 1998). CBP and p300 have been demonstrated to play important roles in the nuclear receptor function (Chakravarti et al. 1996, Hanstein et al. 1996, Kamei et al. 1996, Yao et al. 1998). CBP/p300 exhibits a strong HAT activity (Ogryzko et al. 1996), and it also recruits another HAT activity, P/CAF, to the coactivator complex (Blanco et al. 1998). The SRC family members interact also with other enzymatic activities, such as the arginine methyltransferase CARM1 (Chen et al. 1999a).

Complexes with ATP-dependent chromatin remodeling activity

The yeast SWI/SNF complex possesses ATP-dependent chromatin remodeling activity, and it is able to cause local changes in chromatin structure and thus facilitate the binding of sequence-specific transcription factors to the nucleosomal DNA (McKenna et al. 1999, Glass and Rosenfeld 2000). The ATPase activity-containing component of the yeast SWI/SNF complex is encoded by *swi2* and *snf2* genes. Importance of these factors in

nuclear receptor function has been demonstrated. Mammalian homologues of yeast SWI2 and SNF2, hBRM and BRG-1, interact with ER in a ligand-dependent manner (Ichinose et al. 1997). In addition, the activity of GR in yeast requires SWI/SNF factors (Yoshinaga et al. 1992), and interaction with BRG-1-containing complexes is required for GR function on a stably integrated MMTV promoter (Fryer and Archer 1998).

TRAP/DRIP complexes

TRAP (TR-associated protein) and DRIP (VDR-interacting protein) complexes are homologous multiprotein complexes that were originally found to associate with TR or VDR, respectively (Fondell et al. 1996, Rachez et al. 1998). TRAP and DRIP complexes are able to enhance transcriptional activity of TR and VDR in cell-free systems, and DRIP complex stimulates hormone-dependent transactivation by VDR also on chromatin templates (Fondell et al. 1996, Rachez et al. 1998, Rachez et al. 1999). Many components of the TRAP/DRIP complex are present in a mammalian complex corresponding to the yeast mediator, and the TRAP/DRIP complex also shares similar components with SMCC (SRB/mediator coactivator complex), NAT (negative regulator of activated transcription), and CRSP (cofactor required for Sp1 activation) complexes (Hampsey and Reinberg 1999, Glass and Rosenfeld 2000). The TRAP/DRIP complex has been suggested to function by mediating the interaction between nuclear receptors and general transcription machinery (McKenna et al. 1999, Glass and Rosenfeld 2000). TRAP220 protein in the TRAP/DRIP complex mediates the interaction with the nuclear receptors by binding directly to the LBD of the receptors (Yuan et al. 1998). TRAP220 is essential for embryonic development since mice lacking the *trap220* gene die during an early gestational stage (Ito et al. 2000). Primary embryonic fibroblasts of TRAP220 null mutants show impaired TR function (Ito et al. 2000).

2.3.2. Corepressors

Class II NRs can bind DNA in the absence of a ligand and repress transcription of the target genes. Repression may be passive resulting from competition for DNA binding sites with active transcription factors or formation of inactive heterodimers with other receptors. On the other hand, unliganded NRs bound to DNA may also actively repress the initiation of transcription, either directly or indirectly with the help of other factors (McKenna et al. 1999, Glass and Rosenfeld 2000). N-CoR (NR corepressor) and highly related SMRT (silencing mediator for RAR and TR) are corepressor proteins that bind unliganded NRs and inhibit transcription (Chen and Evans 1995, Hörlein et al. 1995). N-CoR and SMRT interact with Sin3 protein, which, in turn, recruits multiprotein complexes containing histone deacetylase (HDAC) activities (Heinzel et al. 1997, Nagy et al. 1997). By this

means, repression of gene transcription by NRs can be mediated through recruitment of HDAC complexes and deacetylation of histones.

The steroid receptors do not show DNA-binding activity in the absence of hormone. However, an antagonist-bound steroid receptor is able to dimerize and bind to DNA, but the resultant receptor dimer is unable to stimulate transcription. SMRT and N-CoR have been shown to interact with antagonist-bound steroid receptors, and the recruitment of these corepressors seems to be essential for the activity of ER and PR antagonists (Jackson et al. 1997, Smith et al. 1997, Wagner et al. 1998, Zhang et al. 1998). Additionally, many different proteins have been reported to interact with ligand-activated NRs and negatively regulate their transcriptional activity. For example, RIP140 is able to repress transcriptional activity of various NRs (Treuter et al. 1998, Subramaniam et al. 1999), and the members of PIAS protein family can either enhance or repress steroid receptor-dependent transcription depending the cell line and the promoter studied (Kotaja et al. 2000, Tan et al. 2000, Gross et al. 2001).

2.4. Androgen receptor

Androgens play a crucial role in the male sexual development and in the maintenance of the male phenotype. Two main physiological androgens are testosterone and 5α-dihydrotestosterone. The latter one is converted from testosterone by the 5α-reductase enzyme. The effects of androgens are mediated by AR that was cloned and sequenced in 1988 (Chang et al. 1988, Lubahn et al. 1988). The AR gene is located in X chromosome. The AR protein is expressed in a wide variety of genital and nongenital tissues (Quigley et al. 1995). A number of AR target genes have been identified, but the physiological functions of some of these genes remain to be established. Examples of the AR responsive genes include probasin, C3(1) gene of prostatic binding protein, prostate-specific antigen (PSA), human glandular kallikrein-1 (hKLK2) and sex-limited protein (Slp) (Claessens et al. 1989, Adler et al. 1992, Murtha et al. 1993, Rennie et al. 1993, Cleutjens et al. 1996). Androgens have also been reported to regulate the expressions of some cell cycle regulatory proteins such as the cyclin-dependent kinase 2 (CDK2), the CDK4, and the cyclin-dependent kinase inhibitors p16 and p21 (Lu et al. 1997 and 1999).

Various diseases are associated with defects in the function of AR protein, and a wide spectrum AR gene mutations have been characterized (Quigley et al. 1995). Diseases associated with the mutated AR include androgen insensitivity syndrome (AIS), prostate cancer and Kennedy's disease. Complete absence of AR function results in the complete AIS (also termed complete testicular feminization) with a failure in the development of both the internal and external male structures. Less severe mutations in AR lead to a range of intermediate phenotypes. The AR mutations that cause AIS can be single point mutations, larger deletions or insertions that affect binding of ligand or DNA, create

premature stop codons or alter mRNA splicing (Quigley et al. 1995, McPhaul 1999). Kennedy's disease that is also known as spinal and bulbar muscular atrophy (SBMA), is an X-linked motor neuron disorder associated with an increased length of the polymorphic glutamine repeat (CAG repeat) in the N terminus of AR (Quigley et al. 1995). Androgens and AR have an important role in the development of prostate, and thus a lot of attention has been focused on their potential role in the development and progression of prostate cancer – the most common malignancy of men in many industrialized countries. Blocking of androgen action is often used as a treatment for inoperable prostate cancers. Unfortunately, most of the prostate cancers become eventually resistant to the androgen deprivation therapy. Hence, mutations or amplifications of the AR gene may be involved in the progression of the disease (Lopez-Otin and Diamandis 1998).

3. Covalent modifications in nuclear receptor function

Expression of proteins can be regulated practically at any step in a pathway starting from initiation of gene transcription and leading to synthesis of a functional protein (Orphanides and Reinberg 2002). Even though the initiation of transcription is a crucial regulatory step, also mRNA processing, mRNA transport, translation and folding of proteins are subjected to important regulation. The activities of proteins are further controlled by regulating their localization and rate of turnover. In addition, posttranslational modifications serve a quick way to modulate the functions of proteins. Proteins are subjected to various covalent modifications, such as glycosylation, acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination or sumoylation. Covalent modifications also regulate nuclear receptor-dependent transcription by modifying activities of transcription factors and by remodeling the chromatin structural proteins.

3.1. Modification of structural proteins of chromatin

DNA is packed into a hierarchy of structures, the basic repeating structural unit being the nucleosome. The nucleosome core consists of a histone octamer (H3-H4 tetramer and two H2A-H2B dimers) wrapped with 146 bp of DNA. Histone H1 binds to the core histones and to the linker DNA. Because of the tight packing, genes and their promoter regions are not accessible to the binding of transcription factors. Thus, chromatin structure has to be loosened to enable efficient transcription. The N-terminal tails of histones are susceptible to many covalent modifications, such as acetylation, methylation, phosphorylation and ubiquitination, and the modifications have a key role in altering chromatin structure and function (Spencer and Davie 1999). Acetylation of the core histones is connected to transcriptionally active chromatin, whereas transcriptionally silent genes are typically hypoacetylated (Struhl 1998). Acetylation of the specific lysine residues of histone tails

loosens the nucleosome packing and facilitates the interaction of transcription factors with nucleosomal DNA (Struhl 1998, Spencer and Davie 1999). Mechanistic connection between histone acetylation and gene expression has been clarified by the finding that some coactivators, such as SRC-1, SRC-3, CBP/p300 and P/CAF that are recruited to the promoter region by nuclear receptors, possess histone acetyltransferase activity (Ogryzko et al. 1996, Chen et al. 1997, Spencer et al. 1997, Blanco et al. 1998, McKenna et al. 1999, Hermanson et al. 2002). Corepressor complexes, on the other hand, contain histone deacetylases that repress transcription by deacetylating histone tails (Struhl 1998, McKenna et al. 1999).

DNA methylation has a well-characterized role in the silencing of gene expression (Bird and Wolffe 1999), but methylation of proteins also contributes to chromatin remodeling and gene transcription (Stallcup 2001). Like acetylation, methylation of the core histone tails is associated with the active chromatin (Stallcup 2001). Interestingly, two proteins with methyltransferase activities, coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1), have been found to interact with the nuclear receptor coactivator GRIP1 and cooperate with GRIP1 in the nuclear receptor-dependent transcription (Chen et al. 1999a, Koh et al. 2000). CARM1 methylates specific arginine residues of histone H3 (Schurter et al. 2001), and PRMT1 preferentially modifies H4 (Chen et al. 1999a, Strahl et al. 2001). The core histones and histone H1 are phosphorylated at specific serine and threonine residues, and these modifications have also been shown to be involved in the regulation of transcription (Spencer and Davie 1999).

3.2. Modification of transcription factors

In addition to modifying chromatin structure, covalent modifications participate in the regulation of transcription by modulating activities of proteins involved in transcription, such as RNAP II, sequence specific transcription factors and coregulators. Ligands play an important role in regulating the function of the steroid receptors, but some receptors appear also to be activated in a ligand-independent fashion (Beato et al. 1996). Both ligand-dependent and ligand-independent activities of the receptors are modulated by cross-talk with other cellular signaling pathways that start from the cell surface receptors and are carried to the nucleus via phosphorylation cascades mediated by various kinases (Weigel 1996, Weigel and Zhang 1998). Phosphorylation has been demonstrated to regulate many functions of NRs, including transcriptional activity, DNA binding, binding to coactivators, dimerization and stability (Arnold et al. 1995, Kato et al. 1995, Chen et al. 1999b, Tremblay et al. 1999, Lange et al. 2000). In addition to the NRs, coregulator proteins, such as SRC family members and CBP/p300, are phosphorylated, and the modification

modulates their function as transcriptional coregulators (Font de Mora and Brown 2000, Rowan et al. 2000, Lopez et al. 2001).

Acetylation and methylation have not been studied as extensively as phosphorylation, but transcription factors have also been reported to be modulated by these modifications. The coactivators CBP/p300 and P/CAF are able to acetylate AR, and also ERα is subject to acetylation by CBP/p300 (Fu et al. 2000, Wang et al. 2001a). Interestingly, mutation of the AR acetylation site abrogates the ligand-dependent function of the receptor and coactivation by coactivators, correlating with increased binding to N-CoR corepressor (Fu et al. 2000 and 2002). Acetylation of ACTR/SRC-3 by CBP/p300 disrupts the association of ACTR with the NRs, suggesting that acetylation of coregulators acts as a regulatory mechanism in hormonal signaling (Chen et al. 1999c). Regulation of transcription factors by methylation is supported by the study demonstrating that Stat1 is methylated by PRMT1 and that methylation is required for transcriptional activation (Mowen et al. 2001). Interestingly, inhibition of methylation facilitates the binding of PIAS1 to Stat1, leading to decreased DNA binding and inhibition of STAT signaling (Mowen et al. 2001). In addition, the coactivator CBP/p300 is a target for methylation by CARM1, and methylation blocks the interaction of CBP/p300 with the transcription factor CREB (cAMP responsive element binding protein) (Xu et al. 2001)

3.2.1. Ubiquitination and proteasome-mediated degradation

Ubiquitin is a small (8.5 kDa) protein that can be covalently attached to lysine residues of target proteins (Weissman 2001). Target protein can be modified at one or more lysines with a single ubiquitin (monoubiquitination), or with ubiquitin chains formed by attachment of ubiquitin molecules to lysine residues of the previous ubiquitins (polyubiquitination) (Hicke 2001, Weissman 2001). Ubiquitination is mediated by at least three types of enzymes (Weissman 2001). First, the E1 ubiquitin-activating enzyme forms a thiolester bond with ubiquitin. Subsequently, ubiquitin is transferred to the E2 ubiquitin-conjugating enzyme, and finally, the E3 ubiquitin ligases mediate the transfer of ubiquitin to the lysine residue of the target protein. There are two major families of ubiquitin E3 enzymes: the HECT (homologous to E6-AP carboxyl terminus) domain E3s form thiolester intermediates with ubiquitin, whereas the RING finger E3s mediate the transfer of ubiquitin to substrate without thiolester bond formation (Weissman 2001).

The specificity of the action of ubiquitination on target proteins is generated largely by the enzymes that recognize the substrates, and also by the types of ubiquitin conjugates formed (Chan and Hill 2001, Weissman 2001). The best-characterized function of protein ubiquitination is the targeting of proteins to proteasome-mediated degradation (Hochstrasser 1996). Interestingly, monoubiquitination does not lead to protein degradation, but attachment of four or more ubiquitins does (Thrower et al. 2000). In addition to protein degradation, polyubiquitination controls other cellular functions, such

as regulation of translation and DNA repair (Weissman 2001). The choice of lysine in ubiquitin sequence that is used for multiubiquitin chain formation affects the fate of target protein; polyubiquitin chains linked through lysine 48 are potent targeting signals for degradation, whereas lysine 63 linkages seem to be important for DNA repair (Hicke 2001, Weissman 2001). Monoubiquitination, on the other hand, has been demonstrated to regulate totally different events, such as functions of histones, endocytosis of proteins and budding of retroviruses (Hicke 2001).

Transcription factors are targets for ubiquitin-mediated proteasomal degradation, and there is positive correlation between the potency and instability of a given transcriptional activator (Thomas and Tyers 2000, Salghetti et al. 2001). Involvement of ubiquitination in transcriptional regulation is supported by a study that identified a ubiquitin-protein ligase subunit within the CCR4-NOT transcription repressor complex (Albert et al. 2002). Interestingly, ubiquitination was recently shown to mediate cofactor exchange on DNA-bound LIM homeodomain transcription factors (Ostendorff et al. 2002). Steroid receptors are also targets for ubiquitin-dependent degradation (Nawaz et al. 1999b, Wallace and Cidlowski 2001). Stabilization of GR by proteasome inhibitor MG132 results in enhanced transcriptional activity (Wallace and Cidlowski 2001, Deroo et al. 2002). In contrast to GR, treatment of cells with MG132 was shown to inhibit the activity of ERα, suggesting that ubiquitination and proteasome function is required for ERα-mediated transcription (Lonard et al. 2000). Since steroid receptor coregulators are also targets for degradation via the proteasome, the proteasome-dependent degradation may play an important regulatory role in the receptor and coactivator turnover and coactivator complex exchange in the steroid receptor-mediated transcription (Lonard et al. 2000, Hermanson et al. 2002).

4. SUMO-1 MODIFICATION

SUMO modification (sumoylation) is a recently characterized covalent modification that leads to attachment of SUMO (small ubiquitin-related modifier) protein to specific lysine residues of target proteins (Melchior 2000, Yeh et al. 2000). SUMO is a small polypeptide that shows a significant structural homology to ubiquitin. SUMO and ubiquitin are only 18% identical, but they share a similar three-dimensional structure, the ββαββαβ ubiquitin-fold (Fig. 6, Bayer et al. 1998, Jin et al. 2001). Members of the SUMO protein family are present in protozoa, yeast, plants and metazoa. SUMO family in metazoa consist of three related proteins, SUMO-1 (also known as PIC1, Ubl1, sentrin, GMP1, Smt3c or hSmt3), SUMO-2 (sentrin2 or Smt3a) and SUMO-3 (sentrin3 or Smt3b) (Melchior 2000). SUMO-2 and SUMO-3 are 95% identical, but SUMO-1 shares only about 50% sequence identity with SUMO-2/3. The least conserved region of SUMO proteins is the N terminus.

It is highly flexible, protrudes from the core of the proteins, and is absent in the ubiquitin (Bayer et al. 1998).

Both ubiquitin and SUMO have the two conserved Gly residues in their C termini that are essential for the conjugation (Bayer et al. 1998). Before conjugation, the last four, eleven or two amino acids of SUMO-1, -2 or -3, respectively, have to be proteolytically cleaved to expose the Gly residues. Lysines in ubiquitin sequence that are used to generate ubiquitin polymers are absent in SUMO-1 sequence, which is in line with the finding that only one SUMO-1 molecule is attached to a given lysine residue of target protein. In contrast, SUMO-2/3 is capable of forming polymers by using lysine 11 as an attachment site for another SUMO-2/3 (Tatham et al. 2001). The corresponding lysine is not conserved in SUMO-1, and SUMO-1 was demonstrated to be unable to form polySUMO chains *in vitro* (Tatham et al. 2001). Sumoylation is mechanistically similar to ubiquitination, but the function of sumoylation is less well understood than that of ubiquitination. SUMO-1 and SUMO-2/3 probably have distinct regulatory roles, and SUMO-2/3 conjugation has been suggested to play a role in the cellular response to environmental stress (Saitoh and Hinchey 2000).

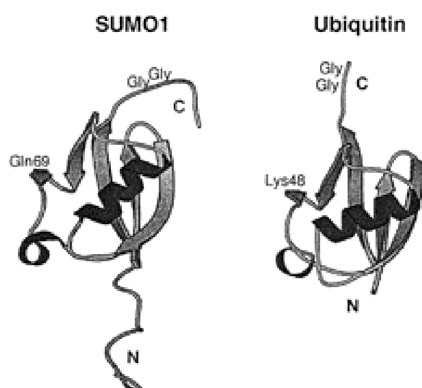

Fig. 6. Comparison of the three-dimensional structures of SUMO-1 and ubiquitin. SUMO-1 contains the characteristic ββαββαβ ubiquitin-fold common to ubiquitin-like proteins. The Gly-Gly motif is shown at the C terminus of the proteins. The most prominent difference between the proteins is the flexible N-terminal tail of SUMO-1 (Melchior 2000).

4.1. Enzymes involved in SUMO modification pathway

The general mechanism of sumoylation and ubiquitination are highly related, but the enzymes involved in the two processes are distinct (Melchior 2000). After the C-terminal processing, SUMO is activated in an ATP-dependent reaction by formation of a thiolester bond with the E1 enzyme. Subsequently, SUMO is transferred to the E2 SUMO conjugating enzyme. In ubiquitination, the E3 ubiquitin ligases are required to ensure substrate specificity (Weissman 2001), and recently, the E3 ligases for sumoylation have

also been characterized. Like ubiquitination, sumoylation is a reversible process, and SUMO is cleaved from target proteins by isopeptidases (Fig. 7, Melchior 2000).

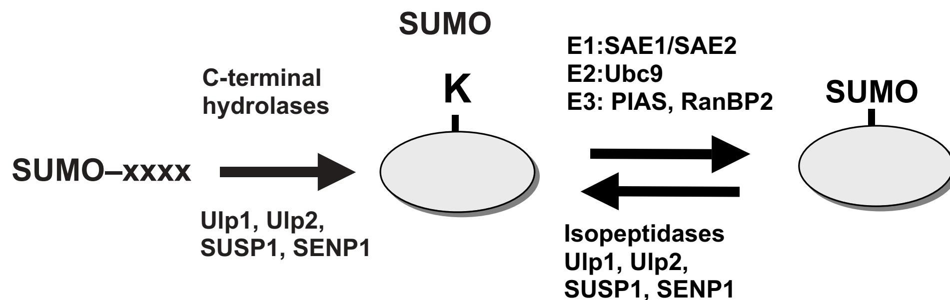

Fig. 7. The SUMO modification pathway. The C-terminal amino acids have to be cleaved by C-terminal hydrolases to expose the Gly-Gly motif for conjugation. Attachment of SUMO to lysine residues of target proteins is catalyzed by E1, E2 and E3 enzymes related to the enzymes in ubiquitination pathway. Sumoylation is reversible, and SUMO can be removed from target proteins by isopeptidases (adapted from Melchior 2000).

4.1.1. E1, E2 and E3 activities

The cDNAs for the E1 enzyme activity in sumoylation pathway has been cloned from both yeast and human. The E1 is a heterodimer composed of Aos1/SAE1/Sua1 and Uba2/SAE2 (Johnson et al. 1997, Desterro et al. 1999, Gong et al. 1999, Okuma et al. 1999). Aos1 is homologous to the N-terminal region of the ubiquitin E1 Uba1, whereas Uba2 shares homology with the C-terminal half of Uba1. Uba2 bears the cysteine residue required for the formation of SUMO-E1 enzyme thiolesters, but both Uba2 and Aos1 are required for the activation of SUMO *in vitro* and *in vivo* (Johnson et al. 1997, Desterro et al. 1999, Gong et al. 1999). Human Aos1/Uba2 heterodimer is able to form thiolester bonds with all members of the SUMO family *in vitro*, thus SUMO-1, SUMO-2 and SUMO-3 can be activated by the same E1 enzyme (Gong et al. 1999).

In contrast to large number of E2 conjugating enzymes in ubiquitin pathway, only one SUMO conjugating enzyme, Ubc9, has been identified (Desterro et al. 1997, Gong et al. 1997, Johnson and Blobel 1997, Schwarz et al. 1998, Müller et al. 2001). Ubc9 is highly conserved from yeast to human, and it shares a striking similarity with the large family of ubiquitin-conjugating enzymes. However, the surface charge distributions of Ubc9 and ubiquitin E2s are very different, and the Ubc9 surface involved in interaction with SUMO-1 is highly complementary in its electrostatic potential and hydrophobicity to the surface of SUMO-1 (Liu et al. 1999). In line with this notion, Ubc9 is specific for SUMO and does not conjugate ubiquitin (Desterro et al. 1997, Gong et al. 1997, Johnson and Blobel 1997, Schwarz et al. 1998). Ubc9 is capable of interacting with almost all SUMO substrates. SUMO-1 consensus attachment sequence, ΨKXE (Ψ is a large hydrophobic residue and X represents any amino acid), has been demonstrated to be a

major determinant of Ubc9 binding and SUMO-1 modification (Sampson et al. 2001). The peptides corresponding to the SUMO-1 conjugation sites of p53 and c-Jun were shown by NMR spectroscopy to bind to a surface adjacent to the active site Cys93 of human Ubc9 (Lin et al. 2002). In addition, crystallographic analysis of a complex between mammalian Ubc9 and the C-terminal domain of RanGAP1 revealed the structural determinants for recognition of consensus SUMO sequence by Ubc9 (Bernier-Villamor et al. 2002).

In ubiquitination, the E3 ubiquitin ligases are needed to ensure substrate specificity (Weissman et al. 2001). They are defined as proteins that bind specific protein substrates and promote the transfer of ubiquitin from a thiolester intermediate to amide linkages with protein or polyubiquitin chains (Melchior 2000). The requirement for similar SUMO E3 ligase activity was doubted in the past because, first, the SUMO E2 enzyme Ubc9 is able to bind almost all known SUMO substrates, and second, E1 and E2 enzymes are sufficient for sumoylation of specific substrates *in vitro*. However, very recently the PIAS proteins were demonstrated to act as E3 SUMO ligases for various substrates both in yeast and in mammalian cells (Hochstrasser 2001, Jackson 2001, Kotaja et al. 2002). Yeast PIAS homologue Siz1 promotes SUMO conjugation to the yeast septins both *in vitro* and *in vivo* (Johnson and Gupta, 2001, Takahashi et al. 2001b and 2001c). In addition, PIASy stimulates sumoylation of the Wnt-responsive transcription factor LEF1, and PIAS1 and PIASxβ act as SUMO ligases for c-Jun and p53 (Kahyo et al. 2001, Sachdev et al. 2001, Schmidt and Müller 2002). The E3 activity of PIAS proteins is dependent on their RING finger-like structure. Interestingly, nucleoporin RanBP2 that is not related to PIAS proteins was also identified as a SUMO E3 ligase (Pichler et al. 2002). RanBP2 does not contain a RING finger structure, and in contrast to PIAS proteins that are nuclear, RanBP2 is located in nuclear pores (Pichler et al. 2002).

4.1.2. SUMO C-terminal hydrolases and isopeptidases

The C-terminal hydrolases are responsible for cleaving the C terminus of SUMO before conjugation to target proteins (Melchior 2000, Müller et al. 2001). Sumoylation is a reversible process, and SUMO-protein conjugates are highly susceptible to deconjugation by isopeptidases (Melchior 2000). C-terminal hydrolases and isopeptidases are cysteine proteases that share some homology with viral cysteine proteases, but are unrelated in sequence to deubiquitinating enzymes (Melchior 2000, Müller et al. 2001). In yeast, two SUMO-specific proteases, Ulp1 (ubiquitin-like protease 1) and Ulp2, have been identified (Li and Hochstrasser 1999 and 2000). These proteases are specific for SUMO, in that they do not cleave ubiquitinated proteins. A 30-kDa ubiquitin-related SUMO-1 hydrolase has been isolated from bovine brain, and two human enzymes, SENP1 (sentrin-specific protease) and SUSP1 (SUMO-1-specific protease), have been cloned (Suzuki et al. 1999, Gong et al. 2000, Kim et al. 2000). In addition, an expressed sequence tag (EST) databank search, based on the conserved ULP domain from yeast Ulp1, have identified additional

homologous sequences in human, suggesting the existence of a larger family of SUMO-specific proteases (Yeh et al. 2000). Most of the identified SUMO processing enzymes are usually able to function as both C-terminal hydrolases and isopeptidases. The substrate specificity of different proteases might be partly determined by their intracellular localization; for example, Ulp2 and SENP1 are intranuclear, SUSP1 is found in the cytoplasm and Ulp1 appears to be concentrated around the nuclear envelope (Gong et al. 2000, Kim et al. 2000, Li and Hochstrasser 2000).

4.2. SUMO target proteins and possible functions of sumoylation

Genetic studies have demonstrated that the sequences encoding E1 and E2 enzymes, SUMO and Ulp1 protease are essential for the viability of *S. cerevisiae* (Dohmen et al. 1995, Seufert et al. 1995, Johnson et al. 1997, Li and Hochstrasser 1999). In contrast, SUMO and Ubc9 in *S. pombe* are not essential for the viability, but inactivating mutations of these genes cause severe defects in growth and chromosome segregation (Al-Khodairy et al. 1995, Tanaka et al. 1999). Temperature-sensitive mutants in the *S. cerevisiae* genes coding for Uba2, Ubc9 and Ulp1 also show strong cell-cycle defects, and the mutant cells arrest predominantly at the G2/M boundary of the cell cycle (Seufert et al. 1995, Johnson et al. 1997, Li and Hochstrasser 1999). These results suggest an essential role for sumoylation in cell cycle progression. The major targets of SUMO in *S. cerevisiae* are the septins, which function in the regulation of cytokinesis. Sumoylation plays a role in controlling dynamics of so-called septin ring formation at the yeast bud neck during the cell cycle (Johnson and Blobel 1999). In addition to yeast, the importance of sumoylation pathway has been demonstrated in *Drosophila* and *C. elegans*. *Drosophila* Ubc9 was recently shown to mediate the dissociation of chromosomes at the end of meiotic prophase I (Apionishev et al. 2001), and *C. elegans* Ubc9 was demonstrated to be essential for embryogenesis (Jones et al. 2001).

The proteins involved in sumoylation pathway (SAE1, SAE2, Ubc9 and SUMO-1) are predominantly located in the nucleus (Azuma et al. 2001, Rodriguez et al. 2001), and most of the sumoylated proteins identified thus far are nuclear (Kamitani et al. 1997). However, there are also very well characterized, efficiently sumoylated, substrates, such as RanGAP1 and yeast septins, that are restricted to the cytoplasmic compartment (Matunis et al. 1996, Mahajan et al. 1997, Johnson and Blobel 1999). The nuclear localization signal (NLS) is required for SUMO-1 conjugation of some substrates *in vivo*, suggesting that in these cases proteins must be targeted to the nucleus to undergo SUMO-1 modification (Rodriguez et al. 2001). The finding that RanBP2, which is localized to the cytoplasmic filaments of nuclear pore complexes (NPCs), has SUMO-1 E3 ligase activity suggest that NLS-containing targets of SUMO-1 modification can be modified on their way to the nucleus (Pichler et al. 2002).

Several proteins are modified by SUMO-1 (Melchior 200, Yeh et al. 2000, Müller et al. 2001). SUMO is attached to lysine residues of the target proteins that are embedded in the consensus sumoylation sequence ΨKXE (Melchior 2000, Yeh et al. 2000, Müller et al. 2001). Even though SUMO-1 and SUMO-1-conjugating machinery is essential for the normal growth of yeast, the role of sumoylation in mammalian cells is not well established. However, the modification has been shown to be involved in the regulation of subcellular and subnuclear localization of proteins as well as protein-protein interactions, stabilization of proteins by preventing ubiquitination, and regulation of the activities of transcription factors (Melchior 2000, Yeh et al. 2000, Müller et al. 2001).

4.2.1. Regulation of protein targeting and protein–protein interactions

The Ran GTPase-activating protein RanGAP1 was the first protein identified as a target for SUMO modification (Matunis et al. 1996, Mahajan et al. 1997). RanGAP1 is a key regulator of Ran GTPase, which controls the nucleo-cytoplasmic transport through the nuclear pore complex (NPC). The SUMO-1 modification of RanGAP1 is required for the interaction with RanBP2 that is localized at the cytoplasmic filaments of the NPC (Mahajan et al. 1997). SUMO-1 is not directly mediating the interaction, but modification has been suggested to induce a structural change in RanGAP1 that allows its binding to RanBP2 (Matunis et al. 1996, Mahajan et al. 1997, Saitoh et al. 1997). RanBP2 is also sumoylated, but the role of sumoylation in the function of RanBP2 is not known (Saitoh et al. 1998). During mitosis, the localization of RanGAP1 to mitotic spindles and kinetochores is also dependent upon the SUMO-1 conjugation of RanGAP1 (Joseph et al. 2002).

The PML nuclear bodies are nuclear speckles of unknown function containing several different proteins (Maul et al. 2000). The PML bodies seem to be preferential accumulation sites for sumoylated proteins (Yeh et al. 2000, Müller et al. 2001). The defining component of the PML bodies, the PML protein, is sumoylated at three distinct lysine residues, and sumoylation of PML is known to regulate the assembly and/or stability of the PML bodies (Sternsdorf et al. 1997, Kamitani et al. 1998a and 1998b, Müller et al. 1998, Duprez et al. 1999, Zhong et al. 2000). The SUMO-1-modified PML is able to recruit other proteins to the PML bodies. A transcriptional repressor Daxx is relocalized and stored in an inactive state in the PML bodies upon sumoylation of PML (Ishov et al. 1999, Li et al. 2000). Daxx represses the transcriptional activity of Pax3 transcription factor, and sumoylation of PML induces the activity of Pax3 through sequestration of Daxx into the PML bodies (Lehembre et al. 2001).

Another component of the PML bodies, Sp100, is also a subject to covalent modification by SUMO-1, but sumoylation is not needed for the localization of Sp100 in the PML bodies (Sternsdorf et al. 1999). Instead, Sp100 interacts with chromosomal non-histone proteins of the HP1 and HMG1/2 families (Lehming et al. 1998, Seeler et al.

1998), and sumoylated Sp100 has a higher affinity for the HP1 protein (Seeler et al. 2001). Further evidence for the role of sumoylation in the regulation of subnuclear localization is provided by studies demonstrating that the SUMO-1-modified HIPK2, a transcriptional corepressor of homeodomain transcription factors, is targeted to detergent-insoluble subnuclear complexes (Kim et al. 1999), and that the ETS-related transcriptional repressor TEL is found in cell-cycle-specific nuclear dots in a sumoylation-dependent manner (Chakrabarti et al. 2000).

4.2.2. Sumoylation of transcription factors

Various transcription factors are targets for SUMO modifications, but the role of sumoylation in the regulation of the functions of these proteins is still unclear. AR is sumoylated in an androgen-enhanced fashion (Poukka et al. 2000). Mutation of SUMO-1 attachment sites in the N-terminal domain (lysines 386 and 520) of AR enhances transcriptional activity of the receptor on minimal promoters (Poukka et al. 2000). The transcriptional activities of c-Jun and c-Myb are also negatively regulated by SUMO-1 modifications, in that disruption of major sumoylation target lysines in these proteins enhances their activity (Müller et al. 2000, Bies et al. 2002). p53 is also sumoylated, and overexpression of SUMO-1 has been suggested to enhance the transcriptional activity of wild-type p53, but not the sumoylation deficient p53 (Gostissa et al. 1999, Rodriquez et al. 1999). However, opposite results have also been reported (Kwek et al. 2001). p53 is recruited into PML bodies by a specific PML isoform or by coexpression of SUMO-1, but the recruitment is not dependent on the SUMO-1 modification of p53 itself (Fogal et al. 2000).

SUMO-1 modifications also regulate activities of heat shock transcription factor 1 (HSF1) and HSF2 that mediate the induction of heat shock protein gene expression in cells exposed to elevated temperature and other stress conditions (Goodson et al. 2001, Hong et al. 2001). In contrast to HSF2, which is constitutively modified, HSF1 is only modified after stress treatment (Goodson et al. 2001, Hong et al. 2001). Mutation of the principal sumoylation site of HSF1 was demonstrated to result in a decrease in its stress-induced transcriptional activity (Hong et al. 2001). Sumoylation was suggested to convert both HSF1 and HSF2 to active, DNA-binding forms (Goodson et al. 2001, Hong et al. 2001). Interestingly, histone deacetylase 1 (HDAC1) is also a target for SUMO-1 modification, and sumoylation appears to affect the activity of the enzyme, since mutation of the SUMO-1 attachment site reduces HDAC1-mediated transcriptional repression (David et al. 2002).

In addition to transcription, sumoylation is connected to other DNA-dependent processes, such as DNA repair. For example, topoisomerase I and II (TOP1 and II) are substrates for SUMO-1 attachment, and the SUMO pathway has been suggested to be involved in the repair of TOP1-mediated DNA damage (Mao et al. 2000a and 2000b). SUMO-1 modification of human thymine-DNA glycosylase (TDG), an enzyme that

initiates base excision repair and removes the aberrant bases, dramatically reduces DNA substrate binding affinity of TDG, which is associated with a significant increase in enzymatic turnover (Hardeland et al. 2002).

4.2.3. Regulation of protein stability

If SUMO and ubiquitin compete for the same lysine residues in the target protein, sumoylation may lead to stabilization of the protein by blocking ubiquitination and subsequent degradation, as exemplified by the inhibitor protein IκBα (Desterro et al. 1998). IκBα is a regulatory protein that binds the transcription factor NF-κB and keeps NF-κB inactive in the cytoplasm. Upon stimulation, IκBα becomes phosphorylated, which acts as a signal for ubiquitination and degradation of IκBα (Israel 2000). Degradation of IκBα releases NF-κB to the nucleus, where it regulates target gene transcription. IκBα is SUMO-1 modified at the same lysine residue used for ubiquitination, and sumoylation thereby stabilizes IκBα leading to inhibition of NF-κB activation (Desterro et al. 1998). A similar role of sumoylation has been suggested in the regulation of Mdm2 (murine double minute clone 2), an E3 ubiquitin ligase for p53 (Buschmann et al. 2000). However, a note of caution has been raised, since all available data do not support this model (Melchior and Hengst 2000). Thus, the role of sumoylation in the regulation of E3 ubiquitin ligase activity of Mdm2 remains to be solved.

4.3. Future perspectives

Genetic studies in yeast have demonstrated the important role of sumoylation in cell growth and division, but the biological significance of SUMO modifications in mammalian cells is still elusive. To find out the physiological importance of sumoylation, gene targeting in mice is needed to specifically inactivate the different *sumo* genes or the genes encoding components of SUMO-conjugating machinery. In addition, selective inhibitors that block either sumoylation or SUMO deconjugation would be useful tools in characterization of the function of sumoylation. Analogous inhibitors of proteasome-mediated degradation, such as MG132, have had a marked impact on the study of protein turnover and understanding the role of protein ubiquitination. At the same time, identification of novel sumoylation substrates, as well as characterization of the effects of sumoylation on their activities has to be continued to understand better the role of SUMO modifications in the regulation of cellular functions.

AIMS OF THE STUDY

The PIAS protein family is a small group of highly conserved nuclear proteins that interact with many different transcription factors. Because of the high degree of sequence homology, it is likely that PIAS proteins also share similar functions. Since ARIP3/PIASxα was originally characterized as an androgen receptor coregulator, and since two PIAS proteins, ARIP3/PIASxα and PIAS1, are highly expressed in testis, PIAS proteins were assumed to play a role in androgen receptor-dependent signaling. The domain structure of PIAS proteins and the mechanism underlying their function were, however, largely unsolved. The following specific aims were addressed in this study:

- To study the functions of different PIAS proteins in steroid receptor-dependent transcription and to compare the effects of PIAS proteins in steroid receptor- and STAT-dependent signaling
- To characterize the domains important for the function of ARIP3
- To elucidate the mechanisms underlying PIAS protein action by searching for interaction partners
- To examine the role of PIAS proteins in SUMO-1 modification pathway
- To study sumoylation of the steroid receptor coactivator GRIP1 and to elucidate the effects of this modification on the GRIP1 function

METHODS

More detailed descriptions of the methods and materials used in this study are found in the original publications (I-IV) as indicated in the table below.

Table 2. Methods used in this study.

Method	***original publication***
Plasmid construction and recombinant DNA technology	I, II, III, IV
Cell culture	I, II, III, IV
Transfections and reporter gene assay	I, II, III, IV
Mammalian two-hybrid assay	I, II
Immunoprecipitation	II, III
Production of recombinant proteins in bacteria	I, II, III
Protein–protein interactions *in vitro* (GST pull-down)	I, II
SDS-PAGE and immunoblotting	I, II, III, IV
Detection of SUMO-1 modifications in intact cells	III
In vitro SUMO-1 modification	III
Immunocytochemistry	III, IV
Confocal microscopy	III, IV

THE SEQUENCES OF PIAS PROTEINS USED IN THIS STUDY

The full-length rat ARIP3 cDNA (Gene bank accession number AF044058) was obtained by screening a rat testis cDNA library with the ARIP3 fragment found in the yeast two-hybrid screen as a probe (Moilanen et al. 1999). Mouse PIAS3 (AF034080) and mouse PIAS1 (AF077950) were generous gifts from Dr. K. Shuai (Los Angeles, CA). N-terminally truncated mouse Miz1 (AF039567) cDNA lacking the sequence encoding the first 131 amino acids was a kind gift from Dr. R. Maxson (Los Angeles, CA). ARIP3/PIASxα and Miz1/PIASxβ are splice variants encoded by the same gene, and their first 550 amino acids are identical. Full-length Miz1 was constructed by ligating nucleotides encoding amino acids 1-337 of ARIP3 to the C terminus of Miz1 encoding amino acids 338-621 (I).

RESULTS AND DISCUSSION

1. PIAS PROTEINS FUNCTION AS STEROID RECEPTOR COREGULATORS (I, II)

ARIP3 (androgen receptor-interacting protein 3) was originally found in a yeast two-hybrid screen using the AR DBD and part of the hinge region as bait (Moilanen et al. 1999). ARIP3 also interacts with full-length AR and modulates AR-dependent transcription (Moilanen et al. 1999). Other PIAS proteins have been shown to interact with and to regulate activities of very dissimilar transcription factors (Chung et al. 1997, Valdez et al. 1997, Wu et al. 1997, Liu et al. 1998). However, the high degree of sequence homology among PIAS proteins predicts similar functions. The abilities of four PIAS proteins – ARIP3/PIASxα, Miz1/PIASxβ, PIAS1 and PIAS3 – to modulate steroid receptor-dependent transcription were studied by using transient transfections and reporter gene assays. The fifth PIAS family member, PIASy, was not investigated in this study. Different cell lines and different reporter constructs were used. All studied PIAS proteins were able to modulate AR, GR, ER and PR-mediated signaling, but their effects differed depending on the receptor, the promoter, and the cell type. On the minimal ARE_2TATA promoter, PIAS proteins enhanced both AR and GR-dependent transcription, but the degree of enhancement varied according to the PIAS protein used. In HeLa cells, Miz1 and PIAS1 were more potent coactivators of AR than ARIP3 and PIAS3. In contrast, ARIP3 enhanced GR-dependent transactivation from the minimal promoter more efficiently than other PIAS proteins. The effects of PIAS proteins on complex promoters were more diverse. Miz1 and PIAS1 activated AR-dependent transcription from the natural probasin promoter, whereas ARIP3 repressed AR activity under the same conditions. Likewise, ARIP3 repressed GR-dependent transcription from the mouse mammary tumor virus (MMTV) promoter despite the fact that ARIP3 is very strong coactivator of GR on the minimal promoter.

The differences among the effects of PIAS protein on AR and GR-mediated transactivation on the minimal promoter were diminished in HepG2 cells, and all PIAS proteins activated the function of AR and GR to a similar degree. Moreover, ARIP3 was not able to repress the activity of AR from the probasin promoter in HepG2 cells. These interesting differences in the cell line-specific functions of PIAS proteins may derive from the differences in the expression of endogenous PIAS proteins or factors regulating the function of PIAS proteins in these cells lines. Interestingly, PIAS proteins are able to interact directly with other family members (Tan et al. 2002, our unpublished results). Tan et al. (2002) also demonstrated that the coregulatory effects of PIAS1 on AR are modulated by coexpression of PIASxα, PIASxβ or PIASy, suggesting that the net effect of PIAS proteins is dependent on the relative amounts of different family members. Miz1 and PIAS1, but not ARIP3 and PIAS3, exhibit some intrinsic transcription-activating function

both in HeLa and HepG2 cells. This is in line with the ability of Miz1 and PIAS1 to activate AR-mediated transcription from both minimal and complex promoters, but not with the results obtained in HepG2 cells. Thus, the transactivation functions of Miz1 and PIAS1 cannot be the only explanation for the differences in the actions of PIAS proteins.

In summary, PIAS proteins are efficient modulators of steroid receptor signaling, and they function in a receptor-, promoter- and cell line-dependent fashion. The effects of PIAS proteins were prominent even with low amounts of expression plasmids transfected, and under similar conditions, the effects of PIAS proteins on STAT-mediated transcription were modest. Thus, under ectopic expression conditions, the action of PIAS proteins is clearly not restricted to inhibition of STAT signaling, but they also function as coregulators for steroid receptors. The role of PIAS proteins in steroid receptor-dependent signaling has subsequently been addressed by other laboratories. The interaction of PIAS1 and AR was discovered in a yeast two-hybrid assay, when a HeLa cell cDNA library was screened using an AR region containing the DBD and the LBD as bait (Tan et al. 2000). PIAS1 has been demonstrated to modulate the activities of AR, GR and PR (Tan et al. 2000, Gross et al. 2001). Similarly, PIASxα, PIASxβ, PIAS3 and PIASy interact with AR and function as AR coregulators (Junicho et al. 2000, Gross et al. 2001, Tan et al. 2002). These results further confirm the involvement of PIAS proteins in steroid receptor-dependent transcription.

2. Domains important for coregulator function of PIAS proteins (I, II, III)

To modulate the steroid receptor-dependent transcription, PIAS proteins have to interact with the receptors, either directly or indirectly. Direct interactions of full-length ARIP3 and Miz1 with AR, GR, PR and ER was demonstrated *in vitro* in GST pull-down assays. The interactions in this system were independent of the presence of the ligand. All four PIAS proteins were also able to interact with AR in a ligand-enhanced fashion in mammalian two-hybrid assay. In the original yeast two-hybrid screen, the C-terminal region (amino acids 443-547) of ARIP3 was found as an fragment interacting with the AR DBD. The C terminus of ARIP3 also interacted with full-length AR in mammalian two-hybrid assay (Moilanen et al. 1999). Likewise, the C terminus of PIAS1 has been shown to mediate the interaction with STAT1 (Liao et al. 2000). However, in coimmunoprecipitation experiments, the C-terminally truncated ARIP3 (ARIP3Δ467-547) interacted with AR as efficiently as wild-type ARIP3, indicating that this region is not alone responsible for binding to AR. Instead, the potential zinc-binding domain of ARIP3 (amino acids 347-418) was mandatory for the interaction with full-length AR. Other groups have demonstrated that the N terminus of PIAS1 (amino acids 1-318) and PIASy (amino acids 1-406) are capable of interacting with the AR DBD *in vitro* (Tan et al. 2000, Gross et al. 2001). Similarly, the DBD of AR is not the only region reported to be involved in the binding to

PIAS proteins, since Junicho et al. (2000) showed that an intact N terminus of AR is required for the interaction with PIAS3. In coimmunoprecipitation assays, deletion of either the LBD or the DBD of AR weakened the interaction with ARIP3, but N-terminal deletions of AR did not have marked effects on the interaction (unpublished results). In conclusion, more than one functional domains in PIAS proteins and steroid receptors mediates the interactions between these proteins *in vivo*. All the interactions are not necessarily direct, but may be bridged by other proteins present in the same complex.

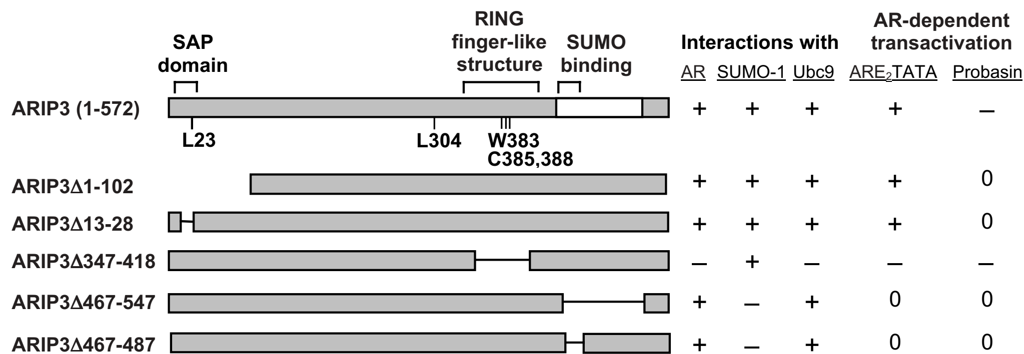

Fig. 8. The ARIP3 mutants used in this study and the summary of the properties of the mutants. The SAP motif, the RING finger-like structure and the SUMO-1-binding motif are shown. Amino acids that were mutated to study the functions of LXXLL motifs (Leu 23 and 304) and the RING finger-like structure (Trp 383, Cys 385 and Cys 388) are depicted. Interactions of ARIP3 and ARIP3 mutatants with AR (in coimmunoprecipitation experiments), SUMO-1 (in GST pull-down) and Ubc9 (in GST pull-down) are indicated with + (interacts) and – (does not interact or weakened interaction) signs. The capability of different ARIP3 forms to modulate AR-dependent transcription on ARE_2TATA and probasin promoters are also shown: +, enhancement; –, repression; 0, no effect.

To find out which regions of ARIP3 are important for its function as a steroid receptor coregulator, different ARIP3 deletion mutants were studied in transient transfections (Fig. 8). In line with the finding that amino acids 443-547 interacted with AR in the yeast two-hybrid screen, deletion of amino acids 467-547 abolished the ability of ARIP3 to modulate AR-dependent transcription. This region also contains a serine-rich acidic domain (amino acids 457-483) which is involved in the binding to SUMO-1. Since deletion of the amino acids 467-547 did not abolish the interaction of AR with ARIP3 in coimmunoprecipitations, it is possible that the inability of ARIP3Δ467-547 to modulate AR activity was due to the loss of some other interaction, such as binding to SUMO-1. PIAS proteins harbor a putative DNA-binding motif, the SAP module (Aravind and Koonin, 2000), at their N termini (residues 11-45 in ARIP3). Interestingly, when the first 100 amino acids of ARIP3, or amino acids 13-28 of the SAP motif were deleted, the ability of ARIP3 to repress the AR-dependent transcription from the probasin promoter was abolished (unpublished). These ARIP3 mutants were still able to interact with AR in coimmunoprecipitation experiments. The SAP domain is thus important for the coregulator

function of ARIP3, maybe through the SAP motif-mediated DNA binding. Our initial *in vitro* DNA-binding assays using GST-ARIP3 have failed to show high-affinity DNA-binding, but the N-terminally fused GST may have impaired the function of the SAP module (unpublished results). Tan et al. (2002) have recently demonstrated that PIAS1 binds double-stranded A-T rich DNA, and the SAP module is needed for this binding. In addition, Sachdev et al. (2001) showed that PIASy binds to nuclear matrix attachment region (MAR) DNA, and the association with MARs and the nuclear matrix is mediated via the N terminus of PIASy. Mutation of the two LXXLL motifs had only a modest effect on the coregulator function of ARIP3, suggesting that the interaction between ARIP3 and AR or GR do not rely on these motifs.

Seven cysteine residues and a histidine residue in the highly conserved central region of PIAS proteins may potentially form a zinc-binding C_3HC_4-type RING finger motif. Zinc fingers typically form interfaces for interactions with DNA and for protein-protein contacts. Interestingly, the deletion of the ARIP3 zinc-binding structure had a dramatic effect on the ability of ARIP3 to modulate transcription from the minimal ARE-driven promoter, in that the deletion converted ARIP3 to a strong negative regulator of the AR function. Point mutations of the conserved cysteines 385 and 388 to serines caused similar effects, suggesting that this region in PIAS proteins indeed forms a zinc-coordinated structure. ARIP3Δ347-418 devoid of the zinc-binding structure did not interact with AR in coimmunoprecipitation assay. However, the ARIP3(C385,388S) mutant that behaved in a fashion similar to ARIP3Δ347-418 in transactivation assays, was able to interact AR. Hence, the effects of the zinc-binding structure mutants of ARIP3 cannot be merely explained by the loss of interaction with AR. Most probably other domains of ARIP3 are also involved in the binding to AR, or alternatively, some other, currently unknown proteins that exist in the AR-ARIP3 complexes mediate the interaction *in vivo*. One interesting possibility is that the SAP domain of ARIP3 and DNA binding are involved in ARIP3 function, and ARIP3 with the deleted zinc-binding domain is still able to bind DNA and in this way repress the activity of AR. However, when both the SAP domain and the zinc-binding region of ARIP3 were deleted, the mutant ARIP3 still repressed AR-mediated transcription, arguing against the role of SAP domain in the repressive effect of ARIP3Δ347-418 (unpublished results). In conclusion, the C terminus, the SAP motif and the zinc-binding structure of ARIP3 all play roles in the coregulator activity of ARIP3, but further studies are required to clarify the specific functions of these domains.

3. PIAS PROTEINS ARE MODIFIED BY SUMO-1 (III)

SUMO-1 was found to interact with ARIP3 in a yeast two-hybrid screen using the full-length ARIP3 as bait (unpublished results). In GST pull-down assay, ARIP3 bound

strongly to SUMO-1 and also to the SUMO-1 conjugating E2 enzyme Ubc9. Deletion of the amino acids 467-487 of ARIP3 abolished the interaction with SUMO-1. This region contains a Ser-rich and Glu/Asp-rich stretches preceded by hydrophobic residues, and a similar motif in PM-Scl75 has been shown to be sufficient for the interaction with SUMO-1 in yeast (Minty et al. 2000). In addition to the PIAS family members and PM-Scl75, many other proteins, such as PML, SAE2, HIPK2, HIPK3 and SNURF contain a motif corresponding to the sequence of the SUMO-1-binding motif (Minty et al. 2000). Thus, interaction with SUMO-1 may play a more general role in the function of these proteins. The zinc-binding region of ARIP3 (amino acids 347-418), on the other hand, is involved in the binding of Ubc9. Because of the interaction with SUMO-1 and the E2 enzyme of sumoylation pathway, it was pertinent to test weather ARIP3 and other PIAS proteins are subjected to covalent modification by SUMO-1. Coexpression of SUMO-1 with ARIP3, Miz1 or PIAS1 in COS-1 cells resulted in the formation of higher molecular mass bands that were identified to be SUMO-1-modified PIAS proteins. In contrast, the sumoylation of PIAS3 was very weak. The migration of the higher molecular mass bands of ARIP3 on SDS-PAGE suggests that up to three SUMO-1 moieties can be attached to ARIP3 and that the form containing two SUMO-1 moieties is the major sumoylated product in COS-1 cells.

Even though PIAS proteins are efficiently sumoylated, their sequences do not contain consensus SUMO-1 attachment sites. Deletion of the zinc-finger region (amino acids 347-418) dramatically weakened the sumoylation, and when the amino acids 346-475 were deleted, modification was completely abolished. Mutation of the lysine pairs 324 and 326, 379 and 380, 390 and 391, and 430 and 431 to arginines failed to alter the pattern of ARIP3 SUMO-1 modification. Thus, it is still unclear which lysine residues of PIAS proteins are used for attachment of SUMO-1. Since deletion of the central zinc-binding region may cause improper folding of the protein and thereby lead to the loss of SUMO-1 modification, a converse experiment was performed in COS-1 cells to study the sumoylation of ARIP3 regions 341-418 and 341-490 fused to green fluorescent protein (GFP). GFP-ARIP3(341-490) was sumoylated, whereas no sumoylation of GFP-ARIP3(341-418) was detected. This confirms that the central region of ARIP3 between amino acids 419 and 490 contains at least one SUMO-1 attachment site. To study weather PIAS proteins can also be modified under cell-free conditions, an *in vitro* SUMO-1 modification system using purified GST-fusions of SAE1, SAE2, Ubc9 and SUMO-1 was established. PIAS proteins were translated *in vitro* and incubated with recombinant SUMO-1, SAE1/SAE2 and Ubc9 in the presence of ATP. ARIP3, Miz1 and PIAS1 were modified by at least three GST-SUMO-1 molecules, but sumoylation of PIAS3 was undetectable under these conditions.

In analogy to the SUMO-1-binding motif of ARIP3, many cellular proteins contain short ubiquitin-binding motifs: ubiquitin-associated domains (UBAs) and ubiquitin-

interacting motifs (UIMs) (Hofmann and Falquet 2001, Buchberger 2002). UIM motifs of endocytic proteins Eps15 and Eps15R have recently been confirmed to be responsible for ubiquitin binding, and interestingly, also for monoubiquitination of these proteins (Klapisz et al. 2002, Polo et al. 2002). In contrast, deletion of the SUMO-1-binding motif (amino acids 467-487) did not abolish ARIP3 sumoylation, indicating that this region is not needed for SUMO-1 modification of ARIP3. UBA motif-containing proteins have also been shown to inhibit multiubiquitination by binding to substrate-anchored ubiquitin (Buchberger 2002). Likewise, UIM domain has been suggested to have similar role in blocking polyubiquitination of UIM-containing proteins by formation of an intramolecular interaction between the UIM and the substrate-bound ubiquitin (Polo et al. 2002). ARIP3 devoid of the SUMO-1-binding motif was sumoylated to a greater extent than the wild-type protein, and this mutant also promoted sumoylation of COS-1 cell proteins more efficiently that wild-type ARIP3, suggesting that the motif could indeed have some regulatory role in the function of ARIP3.

4. PIAS proteins interact with SUMO-1-modified proteins and colocalize with SUMO-1 in nuclei (III)

When a FLAG-tagged ARIP3 coexpressed with SUMO-1 in COS-1 cells was immunoprecipitated with the anti-FLAG antibody and subsequently immunoblotted with SUMO-1 antibody, many additional high molecular mass SUMO-1 immunoreactive bands were detected. Since they were not detected with anti-FLAG antibody, it was assumed that they represented other SUMO-1-modified proteins coimmunoprecipitating with ARIP3. This hypothesis was confirmed by repeating the immunoprecipitation experiment after denaturating treatment to break protein–protein interactions. When immunoprecipitated samples were immunoblotted with anti-SUMO-1 antibody, only the bands corresponding to SUMO-1-modified ARIP3 were detected. PIASy was also demonstrated to coimmunoprecipitate SUMO-2-modified proteins from cell lysates (Sachdev et al. 2001). Sumoylation and thiol groups seem also to be somehow involved in the interaction of ARIP3 with AR, since AR coimmunoprecipitates with ARIP3 only when N-ethylmaleimide (NEM) is present in the buffers. NEM is a cysteine protease inhibitor that blocks the action of SUMO-1 deconjugating enzymes (Li and Hochstrasser 1999, Suzuki et al. 1999). However, both unmodified and SUMO-1-modified AR was coimmunoprecipitated with ARIP3, indicating that AR sumoylation is not needed for the interaction. The role of ARIP3 sumoylation in the interaction remains to be characterized. As NEM modifies free thiol groups, its effects may also be mediated by modification of proteins that are involved in the ARIP3-AR interaction.

Since ARIP3 binds to SUMO-1 *in vitro*, it was tempting to speculate that recruitment of sumoylated proteins is mediated by the SUMO-1-binding motif of ARIP3

(amino acids 467-487). However, deletion of this region of ARIP3 did not abolish the interaction with other sumoylated proteins, but the recruitment was affected, in that the pattern of the recruited proteins was shifted to a lower molecular mass range. Deletion of amino acids 347-418 of ARIP3, on the other hand, eliminated the recruitment of other SUMO-1 modified proteins totally, indicating that the zinc-binding region of ARIP3 is required for this function. However, the zinc-binding region in isolation was not sufficient for the recruitment of sumoylated proteins, since no sumoylated proteins were immunoprecipitated with GFP-ARIP3(341-418), whereas GFP-ARIP3(341-490), including both the zinc-binding region and the SUMO-1-binding motif, was capable of tethering sumoylated proteins.

Nuclear distribution of ARIP3 and other PIAS proteins display speckled pattern when transfected into COS-1 cells and visualized by confocal microscopy using a monoclonal antibody against the FLAG epitope and a rhodamine-conjugated secondary antibody. The PIAS-containing granules are distinct from PML nuclear bodies as detected by double staining of cells with anti-FLAG and anti-PML antibodies (Ulla Karvonen, personal communications). GFP-SUMO-1 expressed alone is typically diffusely distributed in the nucleoplasm, but forms occasionally granular structures that differ in size from those formed by PIAS proteins. Interestingly, coexpression of PIAS proteins with GFP-SUMO-1 resulted in perfect colocalization of the two proteins in the same granules. Deletion of the SUMO-1-binding motif of ARIP3 partially disrupted the colocalization, leading to fewer, but bigger, granules and to a considerable amount of diffuse nuclear SUMO-1. The zinc-binding region of ARIP3 was crucial for the targeting of ARIP3 and SUMO-1 to the nuclear granules, since coexpression of SUMO-1 with ARIP3Δ347-418 resulted in a diffuse nuclear distribution of both proteins.

These results demonstrated that PIAS proteins are not only subject to covalent modification by SUMO-1, but they are also able to bind other sumoylated proteins in cells and to colocalize with SUMO-1 in nuclei. Even though the free SUMO-1-binding motif of ARIP3 is an obvious candidate to mediate these functions, the deletion of the zinc-binding structure had a more dramatic effect on the ability of ARIP3 to bind other sumoylated proteins than that of the SUMO-1-binding motif. The zinc-finger region-deficient ARIP3 was not targeted to the nuclear granules, suggesting that incorrect targeting of this mutant could be one explanation for its inability to recruit SUMO-1-modified proteins. ARIP3Δ467-487 was targeted to the nuclear granules, but the colocalization with SUMO-1 was affected. Since ARIP3Δ467-487 is still efficiently sumoylated, the remaining granules containing colocalized ARIP3 and SUMO-1 probably represent SUMO-1 covalently attached to ARIP3. In conclusion, the regions of ARIP3 that are important for the binding of SUMO-1 and Ubc9 *in vitro* and for the coregulator function of ARIP3 in AR-dependent transcription, also influence intranuclear targeting of ARIP3 and its association with SUMO-1 and SUMO-1-modified proteins.

5. PIAS proteins modulate transcription factors by functioning as SUMO-1 E3 ligases (III, IV)

The fact that ARIP3, Miz1 and PIAS1 interact with other sumoylated proteins and possess a peculiar sumoylation feature, i.e., they lack consensus SUMO-1 acceptor sites, suggest that these proteins participate in the sumoylation process itself. Many ubiquitin E3 ligases are able to ubiquitinate themselves, and autoubiquitination has been suggested to play a role in the regulation of their activities (Joazeiro and Weissman 2000). For example, the activity of Mdm2 is controlled by autoubiquitination and subsequent degradation (Fang et al. 2000, Honda and Yasuda, 2000). Interestingly, one group of ubiquitin E3 ligases contains RING fingers that are required for their ligase activity (Lorick et al. 1999, Joazeiro and Weissman 2000), and the zinc-binding structure of PIAS proteins resembles the C_3HC_4 RING finger fold of ubiquitin ligases.

The involvement of PIAS proteins in sumoylation pathway was investigated by coexpressing ARIP3 with SUMO-1 in COS-1 cells and immunoblotting COS-1 cell lysates with the antibody against SUMO-1. ARIP3 increased markedly the overall sumoylation of COS-1 proteins. The zinc-binding region of ARIP3 was mandatory for this function, because ARIP3Δ347-418 was not able to enhance protein sumoylation. SUMO-1 modification of AR occurs at two lysine residues located in the N-terminal region (Poukka et al. 2000). Coexpression of ARIP3 or PIAS1 in HeLa cells stimulated the attachment of both endogenous and ectopically expressed SUMO-1 to AR. In line with these results, the AR mutant lacking the LBD showed an impaired interaction with ARIP3 and had also weakened sumoylation compared to the wild-type AR in intact cells (unpublished results). Purified GST-ARIP3 was also able to enhance SUMO-1 attachment to AR *in vitro*. Additionally, sumoylation of the purified recombinant c-Jun was promoted by GST-ARIP3. c-Jun has previously been demonstrated to be SUMO-1 modified at the lysine residue 229 (Müller et al. 2000). Interesting differences in the substrate-specificity of PIAS proteins were observed, when SUMO-1 modification of the nuclear receptor coactivator protein GRIP1 was studied. GRIP1 was sumoylated when coexpressed with SUMO-1 in COS-1 cells. Cotransfection of PIAS proteins resulted in enhanced SUMO-1 modification of GRIP1 in a PIAS protein-selective fashion. ARIP3 had only a modest effect, but Miz1 and especially PIAS1 strongly promoted the attachment of SUMO-1 to GRIP1. Thus, different PIAS proteins seem to act as E3 SUMO ligases with dissimilar substrate preferences for target proteins.

Deletion of the zinc-binding structure of ARIP3 abolished its ability to enhance sumoylation of both AR and c-Jun *in vitro*, whereas the SUMO-1-binding motif of ARIP3 was not required for this function. c-Cbl and a number of other RING-type ubiquitin ligases contain a conserved tryptophan residue in the RING domain, and this residue is critical for the E3 ligase activity of c-Cbl (Joazeiro et al. 1999). PIAS proteins also harbor a conserved tryptophan in the middle of their RING finger-like structure (Fig. 2), and

mutation of this tryptophan to alanine abrogated the ability of ARIP3 to enhance sumoylation of c-Jun and AR *in vitro*. Similarly, the RING-like domain of PIAS1 was required for its E3 activity towards GRIP1, since the deletion of this region (amino acids 310-407) or mutation of tryptophan 372 to alanine converted PIAS1 inactive.

These results suggest that PIAS proteins function as E3-like ligases in the SUMO-1 modification pathway. They fulfill the criteria of E3 ligases, since they interact with specific substrates and the E2 conjugating enzyme and promote sumoylation in a fashion that is dependent on the RING finger-like domain. Contemporaneous studies from other laboratories reported that Siz1 and a related protein Siz2 (Nfi1), yeast homologs of mammalian PIAS proteins, promote sumoylation in yeast, and Siz1 was also shown to enhance sumoylation of septins *in vitro* (Johnson and Gupta 2001, Takahashi et al. 2001b and 2001c). In addition, PIAS1 and PIASy are able to enhance SUMO-1 modification of p53 and LEF1, respectively (Kahyo et al. 2001, Sachdev et al. 2001), and Schmidt and Müller (2002) demonstrated that PIAS1 and PIASxβ act as SUMO E3 ligases for c-Jun and p53. In conclusion, the PIAS family members have been identified as the first representatives of E3 SUMO ligases, underlying the importance of the PIAS protein family in the SUMO-1 modification pathway (Fig. 9).

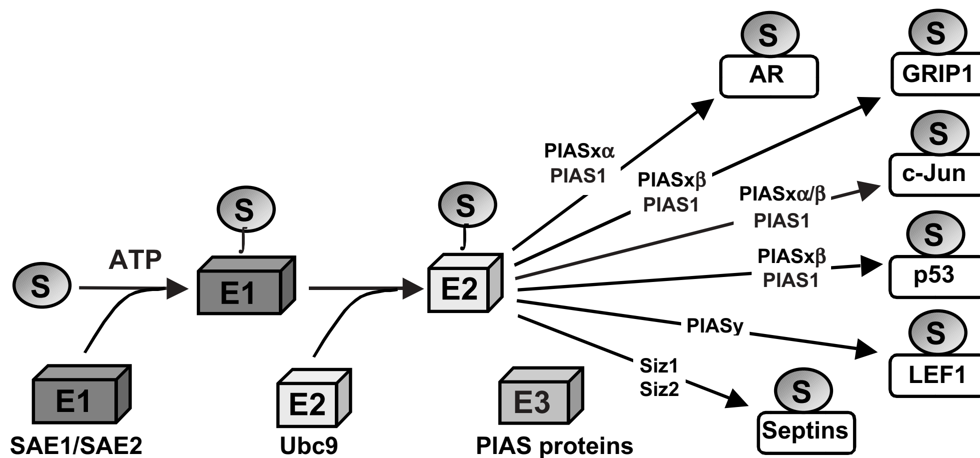

Fig. 9. PIAS proteins function as E3 SUMO ligases in the sumoylation pathway. S, SUMO-1. PIAS proteins have been reported to enhance sumoylation of AR, GRIP1, c-Jun, p53, LEF1 and yeast septins. For more details, see text.

As mentioned before, disruption of the zinc-binding structure of ARIP3 influenced markedly the function of ARIP3 as a steroid receptor coregulator. Similarly, mutation of Trp383, that abolishes the E3 SUMO ligase activity of ARIP3, converted ARIP3 from an activator to a repressor of AR-dependent transcription on the minimal ARE_2TATA promoter. Deletion of the zinc-binding region of PIAS1, or mutation of the conserved Trp

in this region, changed also PIAS1 function dramatically. It was somewhat surprising that ARIP3 devoid of the SUMO ligase activity repressed AR function, since SUMO-1 modifications are shown to regulate negatively the activity of AR (Poukka et al. 2000). In addition, the activity of the sumoylation-deficient AR mutant (K386R,K520R) on the ARE_2TATA promoter was repressed by ARIP3, in contrast to activation of wild-type AR under the same conditions. However, the effects of PIAS proteins on the AR-dependent transcription cannot be merely mediated through sumoylation of AR. PIAS proteins have multiple interaction partners, and they are also able to influence the sumoylation of other proteins involved in transcription, such as the coactivator GRIP1. Therefore, activation or repression of transcription by PIAS proteins most likely reflects their combinatorial effects on different factors regulating a given promoter.

Even though the SUMO-1-binding motif of ARIP3 was not required for either its own sumoylation or the E3 ligase activity, the motif was critical for the coregulator function of ARIP3 on AR-dependent transcription: deletion of this motif (amino acids 467-487) abolished the ability of ARIP3 to modulate the activity of AR. Thus, the SUMO-1-binding motif seems to have a role in the function of ARIP3 that is distinct from the SUMO-1 ligase activity. Endocytic proteins with ubiquitin-interacting motifs play a role in targeting of monoubiquitinated membrane proteins to endocytic pathway (Polo et al. 2002, Raiborg et al. 2002, Shih et al. 2002). Ubiquitinated proteins and ubiquitin-binding motif-containing proteins have been proposed to form an extensive network of interactions that could influence many aspects of cellular physiology (Polo et al. 2002). Likewise, the SUMO-1-binding motifs of cellular proteins may have a role in formation of interactions with sumoylated proteins and in targeting of proteins to specific cellular locations. Neither the sumoylation of AR nor the SUMO-1-binding motif of ARIP3 was required for the AR-ARIP3 interaction in coimmunoprecipitation experiments. However, the SUMO-1-binding motif may mediate the interaction of ARIP3 with other sumoylated proteins involved in the AR-dependent transcription. Taken together, these results suggest that the modulatory effects of PIAS proteins on steroid receptor-dependent transcription are in most cases exerted through their SUMO-1-binding and SUMO-1 ligase activities.

6. SUMO-1 MODIFICATIONS REGULATE THE FUNCTION OF GRIP1 (III, IV)

GRIP1 (Hong et al. 1997, Leo and Chen 2000) was demonstrated to be subject to covalent modifications by SUMO-1. GRIP1 was sumoylated at several lysine residues, since coexpression of SUMO-1 with GRIP1 in COS-1 cells resulted in the formation of at least four higher molecular mass bands, which were confirmed to be SUMO-1-modified GRIP1. The possibility that the latter GRIP1 forms could result from the attachment of polySUMO-1 chain was also considered, because Pichler et al. (2002) recently reported that the nucleoporin RanBP2 is very efficiently modified by up to 25 molecules of SUMO-

1, and polySUMO-1 chains are formed on RanBP2. Digestion of SUMO-1 modified GRIP1 with the recombinant isopeptidase Ulp1 resulted only one digestion species corresponding to one SUMO-1 molecule, suggesting that a single SUMO-1 monomer is attached to more than one lysine residue in GRIP1 (unpublished results).

GRIP1 sequence contains four lysine residues (lysines 239, 731,788 and 1452) that fulfill the consensus sumoylation sequence (Fig. 10). Mutation of lysine 1452 to arginine did not affect the sumoylation of GRIP1, but lysine 239 and central lysines 731 and 788 were used as SUMO-1 attachment sites. Additional conjugation sites may exist, since combined mutation of lysines 239, 731 and 788 did not abolish sumoylation totally. Lysine 66 is located in an incomplete consensus SUMO-1 attachment sequence, FKPD, and the possibility that it is used as a residual SUMO-1 attachment site was considered. However, combination of K66R mutation to other mutations did not reduce the sumoylation (unpublished results). Mutation of the major sumoylation sites K239, K731 and K788 may also lead to sumoylation of K1452 that is not otherwise subjected to modification.

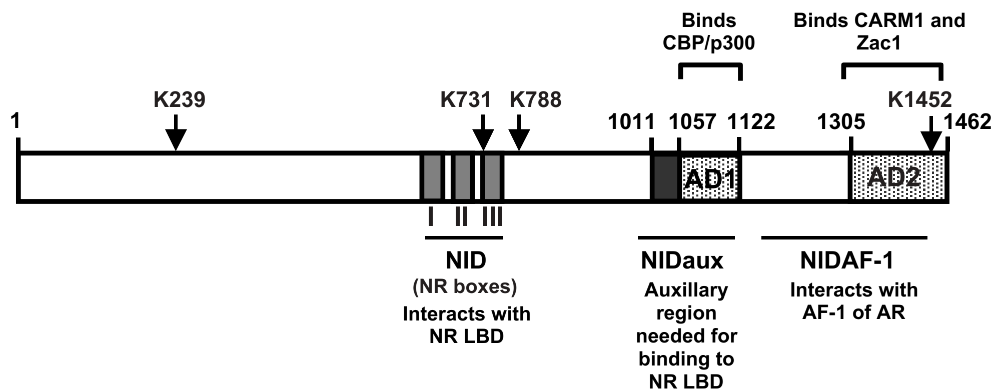

Fig. 10. Structure of GRIP1. NID, nuclear receptor-interaction domain; AD1 and AD2, activation domains 1 and 2; I, II and III, LXXLL motifs I, II and III. The roles of different NID regions in nuclear receptor binding are shown. The regions interacting with different coregulators are indicated. Arrows depict the consensus sumoylation sites (Lys 239, 731, 788 and 1452) (adapted from Ma et al. 1999).

The localization of GRIP1(K731,788R) in COS-1 cells did not differ from that of wild-type GRIP1, as demonstrated by indirect immunocytochemistry and fluorescence microscopy. However, the resolution of the confocal fluorescence microscopy may not be good enough to detect subtle differences in localization. In addition, other methods such as microscopy of living cells and photobleaching techniques are needed to visualize possible kinetic differences in protein targeting. Lysines 731 and 788 are located in the nuclear receptor interaction domain (NID) of GRIP1 containing the three LXXLL motifs used for the interaction with NR LBDs (Ding et al. 1998, Leo and Chen 2000). Interestingly, mutation of lysines 731 and 788 impaired the nuclear colocalization of GRIP1 with AR,

indicating the importance of these sumoylation sites in targeting or recruitment of GRIP1 to AR-containing subnuclear sites.

SUMO-1 modifications are involved in subcellular and subnuclear targeting of proteins (Melchior 2000, Yeh et al. 2000). Sumoylation of PML is required for its targeting to nuclear PML bodies (Müller et al. 1998), and SUMO-1 modification of RanGAP1 directs the protein to cytoplasmic filaments of the nuclear pore complex (Mahajan et al. 1997). In addition, attachment of SUMO-1 to PML is needed for PML-mediated recruitment of other proteins, indicating an important role of sumoylation in protein complex formation (Ishov et al. 1999). SUMO-1 modification of GRIP1 could direct this coactivator to transcriptionally active sites. It is currently unclear why colocalization of GRIP1(K731,788R) with AR varied among the cells; the two proteins localized in separate nuclear compartments in about half of the unsynchronized cells, whereas the other half displayed colocalization pattern indistinguishable from that with the wild-type GRIP1. Potential explanations include cell cycle related factors that influence nuclear architecture. GRIP1 has been shown to localize to nuclear foci, a subset of which associates with the PML bodies (Baumann et al. 2000), and it is plausible that sumoylation of GRIP1 influences the tethering of the coactivator to these bodies. Interestingly, sumoylation of PML and association of certain proteins with the PML bodies are indeed regulated with the cell cycle (Everett et al. 1999, Grobelny et al. 2000, Wu et al. 2000).

In agreement with impaired colocalization of GRIP1(K731,788R) with AR, mutation of lysines 731 and 788 blunted the transcriptional activity of GRIP1 on AR-dependent transcription. Disruption of lysines 731 and 788 also attenuated the ability of GRIP1 to activate the function of the AR LBD in isolation. However, the NID mutations did not influence the capacity of GRIP1 to modulate the activity of AR AF1. Thus, lysines 731 and 788 seem to be important for the function of GRIP1 on the AR LBD. It is possible that SUMO-1 modifications alter the binding of GRIP1 to AR. However, since LXXLL motifs interact directly and specifically with NR LBDs, it is unlikely that SUMO-1 molecule attached to the lysine residue in the immediate vicinity of the LXXLL motifs could strengthen the interaction. Thus, it is more likely that the effects on the transcription are reflected from the targeting and colocalization of GRIP1 and AR.

It was somewhat unexpected that mutations of the SUMO-1 acceptor sites in GRIP1 attenuated its ability to enhance the AR-dependent transcription, since elimination of sumoylation sites from many DNA-binding transcription factors, such as AR, p53, c-Jun and c-Myb, increases transcriptional activity (Gostissa et al. 1999, Rodriguez et al. 1999, Müller et al. 2000, Poukka et al. 2000, Bies et al. 2002). However, it is possible that SUMO-1 modifications of AR and GRIP1 occur at different stages of the AR transcription complex formation to serve distinct roles in gene activation. A possible model, as illustrated in Fig. 11, is that sumoylation of GRIP1 by the PIAS proteins recruits the coactivator to AR-containing subnuclear sites. Subsequently, AR-bound GRIP1 recruits

CBP/p300 and other coactivators to the promoter, and the target gene transcription is activated. Sumoylation of AR may lead to displacement of the receptor from DNA after the first cycle of transcription has commenced. In view of this model, sumoylation may also regulate the function of other coactivators, such as CBP/p300, involved in the regulation of transcription. Further experiments, such as chromatin immunoprecipitation assays, are needed to confirm this model.

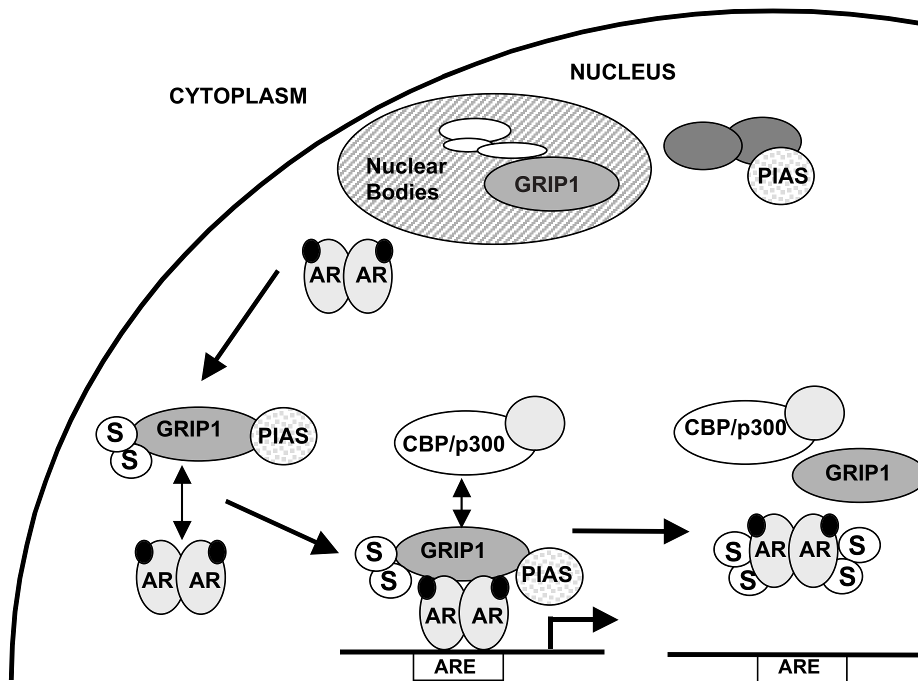

Fig. 11. A model representing the possible roles of SUMO-1 modifications in the AR-dependent transcription. Unmodified GRIP1 localizes in subnuclear lumps that are distinct from AR-containing granules. Sumoylation of GRIP1 by PIAS proteins recruits GRIP1 to AR-containing subnuclear sites. Subsequently, AR-bound GRIP1 recruits CBP/p300 and other coactivators to the promoter and AR-dependent transcription is stimulated. The specific effects of SUMO-1 modifications on AR function are still unclear but sumoylation might lead for example to displacement of the receptor from DNA.

7. PIAS PROTEINS COOPERATE WITH GRIP1 IN STEROID RECEPTOR-DEPENDENT TRANSCRIPTION (II, IV)

The effects of PIAS proteins on steroid receptor-dependent transcription can be mediated by the interactions with other coregulator(s) involved in the regulation of transcription. Thus, it is important to identify interaction partners of PIAS proteins to better understand their function. It was demonstrated that ARIP3, as well as Miz1, PIAS1 and PIAS3, interact with the nuclear receptor coactivator GRIP1. In mammalian two-hybrid assay, the C terminus of GRIP1 exhibited the strongest interaction with ARIP3. However, ARIP3 was also able to interact with the N terminus and the middle part of GRIP1, but to a lesser

extent than with the C terminus. The C terminus of GRIP1 contains activation domain 2 (AD2) that has been demonstrated to bind the Zac1 protein and the arginine methyltransferase CARM1 (Chen et al. 1999a, Huang and Stallcup 2000). The zinc-binding structure of ARIP3 forms the interaction interface for GRIP1, since deletion of this region, or mutation of two conserved cysteines of the zinc-binding structure, abolished the interaction of ARIP3 with GIRP1, as demonstrated by mammalian two-hybrid assays and immunoprecipitation experiments.

Coexpression of PIAS proteins and GRIP1 in HeLa cells resulted in a synergistic enhancement of AR-dependent transcription. Miz1 and PIAS1 were more potent AR coactivators than ARIP3 and PIAS3, and they also showed a more pronounced synergism with GRIP1 on the AR-dependent transcription. These results correlate with the abilities of PIAS proteins to promote sumoylation of GRIP1. Unlike other PIAS proteins, PIAS3 did not act in a synergistic fashion with GRIP1. As discussed above, sumoylation of PIAS3 was also weaker than that of other PIAS proteins, but the possible connection between these results requires further investigation. ARIP3Δ347-418 and ARIP3(C385,388S) mutants did not cooperate with GRIP1, attesting to the importance of the zinc-binding region for ARIP3 function. ARIP3Δ347-418 did not interact with either AR or GRIP1 in coimmunoprecipitation experiments. However, it repressed the activity of AR, and when coexpressed with GRIP1, it also blunted the GRIP1 coactivator function on AR-dependent transcription. It is likely that in intact cells, ARIP3Δ347-418 is still able to interact with both AR and the central domain of GRIP1 through its other regions. These interactions would, in turn, block the activity of AR and the ability of GRIP1 to activate the receptor action.

The interactions of ARIP3 with AR and GRIP1 *in vivo* may be also stabilized by some currently unknown proteins existing in the same complex. Good candidates are free SUMO-1, other SUMO-1-containing proteins, or proteins of the SUMO-1 modification pathway. The presence of the SUMO-1-binding motif in the ARIP3 sequence suggests that this motif may play a role in the complex formation. The nature of ARIP3-AR-GRIP1 interactions and other proteins involved in the complex formation remain to be characterized. The involvement of sumoylation in the interaction and cooperation of PIAS proteins and GRIP1 is supported by the fact that the same region of ARIP3 needed for the cooperation with GRIP1 is also required for ARIP3 to function as an E3-type SUMO-1 ligase. In addition, PIAS proteins – PIAS1 to the greatest extent – are able to promote SUMO-1 modification of GRIP1. In keeping with these results, the synergistic effect of PIAS proteins and GRIP1 was abolished when the main sumoylation sites of GRIP1 were eliminated, indicating the crucial role that SUMO-1 modifications play in transcriptional cooperation between GRIP1 and PIAS proteins.

In summary, this study has clarified the function of PIAS proteins by elucidating their novel role as SUMO E3 ligases and SUMO-1-binding proteins. These activities were

found out to be important for the ability of PIAS proteins to act as steroid receptor coregulators. Furthermore, sumoylation of GRIP1 was described as a new mechanism to regulate the function of this coactivator. Together these results suggest an emerging role of sumoylation in the regulation of transcription. However, genetic studies using null mutants are required to confirm the role that sumoylation and PIAS proteins play *in vivo* in steroid receptor-dependent transcription. Specific inhibitors of sumoylation would also help to elucidate the biological significance of SUMO-1 modifications in the transcriptional regulation. Finally, the role of sumoylation in the assembly and/or disassembly of transcriptionally active protein complexes on gene promoters remains to be assessed.

CONCLUSIONS

The members of the conserved PIAS protein family modulate the signaling mediated by AR and other steroid receptors. AR is covalently modified by SUMO-1, and in addition, SUMO-1 modifications of other transcription factors and cellular proteins are emerging as important factors in the regulation of protein-protein interactions and cellular targeting of proteins. This study characterizes the function of PIAS proteins in steroid receptor-dependent transcription and the role of PIAS proteins in the SUMO-1 modification pathway. The results are summarized as follows:

- Ectopically expressed PIAS1, PIAS3, ARIP3/PIASxα and Miz1/PIASxβ modulate the transcription mediated by AR, GR, ER and PR. These effects are dependent on the receptor, the promoter and the cell line, and PIAS proteins can function as both coactivators and corepressors. PIAS1 and Miz1, but not ARIP3 and PIAS3, contain an intrinsic transcription activation function.

- PIAS proteins interact with both SUMO-1 and Ubc9. They colocalize with SUMO-1 in the same nuclear granules and are able to recruit other sumoylated proteins in a non-covalent fashion. PIAS proteins are themselves covalently modified by SUMO-1, and importantly, they also act as E3 SUMO-1 ligases capable of enhancing sumoylation of different proteins, such as AR, the steroid receptor coactivator GRIP1 and c-Jun. The E3 SUMO-1 ligase activity is dependent on the conserved RING finger-like structure of PIAS proteins.

- GRIP1 is sumoylated at multiple lysine residues. Mutation of the major sumoylation sites of GRIP1 blunts its coactivator function in steroid receptor-mediated transcription and impairs nuclear colocalization of GRIP1 with AR.

- PIAS proteins and GRIP1 interact with each other, and they are able to cooperate in steroid receptor-dependent transcription. The cooperation of PIAS proteins and GRIP1 is dependent on the PIAS RING finger-like structure and the sumoylation sites of GRIP1.

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