dence of cleft palate in the Diemerzeedijk district of the Netherlands, probably due to steroids. In both of these cases, the U.S. Environmental Protection Agency (USEPA) did not affirm a causal relationship, and classified them as cases requiring special attention.

No consensus has been reached concerning the relationship of hypothyroidism observed in the inhabitants along Lake Michigan to the ingestion of PBB- (polybrominated biphenyls-) contaminated fish.

Effects on mature females (e.g., increased incidence of breast cancer)

No reports affirm the effects of dioxins on mature human females (e.g., effects on breast cancer or endometriosis as discussed below). There are many unresolved questions on this topic. However, none of the studies conducted in mature experimental animals revealed data endorsing the plausibility of occurrence of such effects. On the other hand, it is known that the age at menarche is lower and the incidence of breast cancer higher in females exposed to dioxins. Some investigators cite these data when discussing the health hazards of dioxins. It is also known that females exposed to dioxins are often taller.

In European countries, a height increase of about 3.5 mm per year and an approximately one-year decrease in the age at menarche have been reported during the past 30 years. It is difficult to identify the influence of extrinsic endocrine factors on these changes, and no studies addressing this issue have been reported to date. Although several studies have been published concerning the effects of female hormone preparations, including pills used for contraception and hormone replacement therapy in post-menopausal women, no studies have provided data that establish the effects of EDCs.

Endometriosis

Endometriosis is a disease of unexplained origin that is seen in primates with sexual cycles. It has been pointed out that this disease tends to be more severe in individuals exposed to dioxins (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] and to PCBs). Data yielded from experiments in rhesus monkeys are used as evidence to corroborate the causal relationship between dioxins and endometriosis. Thus, we cannot rule out the biological plausibility of these effects. However, no reports affirming the causal relationship in humans have been published.

Possibility of other effects on humans

Biological plausibility has also been considered for the following effects of hormone-mimics on humans: qualitative dysfunction of human sperm, effects on neurobehavior of neonates, and immune functions. The effects on immune functions have been suggested by reports of cases with Yu-sho (PCB intoxication).

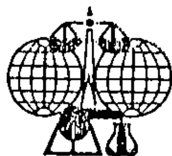
CONCLUSION

The International Program of Chemical Safety (IPCS), a section of the World Health Organization, has released a Web site publication "Global Assessment of the State-of-the Science of Endocrine Disruptors" (GAED), June 2002 (URL: <http://ehp.niehs.hih.gov/who/>). WHO/IPCS started the GAED program in March 1998 after the publication of *Our Stolen Future* (Theo Colbone et al., 1996). The publication took three years to edit; covering a policy to document all the published pertinent literatures, to summarize them as descriptive manner solely based on those published literatures. Twenty-seven expert scientists and 20 independent peer-reviewers participated in editing the GAED.

Other reports on nonylphenol and octylphenol, released by the Japanese Ministry of Environment (MoE), revealed an "ovotestes" formation that was observed in the assay of the laboratory experimen-

tal fish (*Medaka*) exposed to doses close to those recorded in the monitoring fields in the MoE surveillance. Further, phthalates, such as di-(2-ethylhexyl)phthalate, di-cyclohexylphthalate, and butylbenzylphthalate, as selected and prioritized chemicals by the MoE, showed some unique data in different endpoints, including mRNA expression, in dose ranges lower than those no observed effect levels (NOELs) and/or no observed adverse effect levels (NOAELs) reported previously.

The effects of EDCs on human health are unknown at this moment. However, due to the biologically plausible data currently accumulated, the existence of endocrine disruptions under certain circumstances seems to be a reality. Thus, by the time of the SCOPE/IUPAC symposium, the EDC research for the next stage may shift from plausibility to possibility, and put forward further mechanistic research.



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Pure and Applied Chemistry

Implications of Endocrine Active Substances for Humans and Wildlife—a SCOPE/IUPAC Project

- Molecular Mode of Action of Nuclear Receptors: Fundamentals for Understanding the Action of Endocrine Active Substances
- Environmental Fate and Metabolism of Endocrine Active Substances
- Effects of Endocrine Active Chemicals in Rodents and Humans, and Risk Assessments for Humans
- Effects of Endocrine Active Substances in Wildlife Species
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Special Topic Issue

Health Hazards of Endocrine-Disrupting Chemicals on Humans as Examined from the Standpoints of Their Mechanisms of Action

JMAJ 46(3): 97–102, 2003

Tohru INOUE*, Katsuhide IGARASHI and Jun SEKIZAWA

Chairperson, Biological Safety Research Center, National Institute of Health Sciences*

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Chairperson, Biological Safety Research Center, National Institute of Health Sciences*

Abstract: Hormonally active compounds were first recognized in "*Silent Spring*" by Rachel Carson in 1962, which implicated pesticides, such as DDT and derivatives. Nearly four decades later, the book "*Our Stolen Figure*," by Theo Colborn *et al.*, and other pertinent publications have revisited and broadened the issue to a variety of chemicals and areas exposed. Translations of these books have just become available in Japan in the past three or four years, and since then Japan has started to join the debate and/or discussion of the global issue of endocrine disruptors—"Environmental Hormones." Although significant numbers of chemicals possessing a hormone-mimicking action have been recognized for many years and based on biological plausibility their receptor-mediating effects strongly suggest effects in humans similar to those seen in wildlife, little is known about the experimental evidence related to human adverse effects. The key issue in resolving the dilemmas posed by the biological plausibility and poor experimental evidence may be to clarify their mechanism of actions at low levels. In other words, the mechanisms of the possible low-dose effects may be resolved simultaneously by defining three major properties threshold, oscillation, and additive-synergism.

Key words: Receptor; Hormone mimics; Homeostasis; Effects at low dosage; Human hazards

Introduction

The objective of this paper is to summarize all the currently available information on the possible hazards of endocrine-disrupting chemicals (EDs) on human health from the stand-

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The Japanese text is a transcript of a lecture originally aired on October 12, 2001, by the Nihon Shortwave Broadcasting Co., Ltd., in its regular program "Special Course in Medicine".

points of the mechanisms of actions of these chemicals.

It is not uncommon to come across agrochemicals and industrial chemicals that have hormone-mimicking effects. These chemicals, the so-called "environmental hormones," often accumulate at detectable levels in the environment, and it has been feared that they may have adverse effects on living beings. Following reports of feminization and decreased colony size of wild creatures, and reports suggesting a possible association of these chemicals with abnormalities of reproductive organs and oncogenesis in human, attention has been focused on the possibility that these occurrences may be associated with exposure to EDs. In this connection, a Japanese translation of the book entitled "*Our Stolen Future*," written by Theo Colborn *et al.*, was published some time ago.

This paper will review the problems related to EDs, the courses of arguments regarding the harmful effects of these chemicals, and current medical topics pertaining to them.

Chemicals with Hormone-Mimicking Actions

Substances with hormone-mimicking effects can be divided into four groups: (1) hormones found *in vivo*, (2) medicines with hormone-mimic actions manufactured for use in hormonal therapy, etc., (3) plant hormones known to exert phytoestrogen-like actions, and (4) chemicals found in environments that can interact with hormone receptors.

In addition, substances which do not interact with hormone receptors but exert effects on gonads by their modifying effects on steroid metabolism may be deemed as hormone-mimics in the broader sense of the term. In this paper, however, emphasis shall be placed on the hormone-mimicking actions mediated by receptors which play essential roles in the mechanism of actions of hormone-mimics.

Characteristics of the Receptor-Mediated Actions of Hormone-Mimics

The receptor-mediated actions of hormone-mimics are fundamentally characterized by the similarity in the structures of the receptors involved, crossing the barrier of species. This characteristic allows us to estimate the possibility of the actions of these chemicals exerted in nature also occurring in humans.

Secondly, since similarities in the structure to various sex steroids and hormones are also known, it is possible that each individual hormone-mimic exerts diverse effects by acting on male hormone receptors, female hormone receptors, receptors in the nuclei (including some unknown receptors), etc.

Thirdly, many of these chemicals are eliminated from the living body in the form of conjugated inactive substances instead of as degraded metabolites. They may also be eliminated in the unchanged form. Therefore, if feces and urine containing these substances are eliminated into river water, it is plausible to imagine that even inactivated hormones can sometimes become active and exert hormone-mimic actions in the environment. This is one of the characteristics unique to this class of chemicals.

Receptor-mediated responses involve many unresolved questions. Various undefined elements may be involved, including the relationship between receptor binding and signals, the relationship between receptor-ligand binding (ligand: substances that can bind to receptors) and the dissociation of ligands from receptors, signal cross-talks, involvement of unknown nuclear receptors, etc.

The actions of these chemicals add to the effects of intrinsic hormones. For this reason, these chemicals may exert their actions in a way different from that known for other chemicals which do not have structural or functional counterparts *in vivo*. For example, stimulation of hormone receptors by these extrinsic chemicals may modify homeostasis *in vivo*, leading

to weakening of the physiological stimulation of these receptors by the intrinsic substances. Therefore, the influence of the continued effects of environmental hormones needs special study.

Pitfall in the Effects of Hormone-Mimics

We must distinguish the interactions of endocrine hormone-mimics with hormone receptors from the hazards caused to endocrine tissue. Bearing this in mind, let us now summarize the problems related to the effects of hormone-mimics.

1. Antagonistic effects on the maintenance of homeostasis

The endocrine system is regulated by homeostatic mechanisms. It is not uncommon for the effects of small amounts of hormone-mimics to interfere slightly with these mechanisms, often with no adverse influence; this is well-known. However, this is not always the case. There seems to be a group of genes that act antagonistically to each other in the maintenance of homeostasis.

With the uterus growth test, which is used to check for estrogenic activity, the ovary is removed in advance and the blood level of the intrinsic female hormone is reduced to the minimum. Under the thus-created extremely undeveloped state of the uterus, the test substance (a chemical or hormone) is administered to check for its effects on the growth of the uterus. This test (checking for growth of the uterus in ovariectomized animals) is designed to evaluate the hormone activity and effects of hormone-mimics under conditions of blockade of homeostasis.

This test method itself is valid. However, there is no sufficient rational evidence that indicates that the responses observed under such indirect control conditions of the living body can serve as an indicator of the health hazards of hormone mimics. Although the ovotestes seen in lower vertebrates may be used if the effects observed were to be valid as such an indicator, there is no consensus on what is valid as an indicator of the health hazards of ED's when mammals are used as experimental animals.

2. Down-regulation of the expression of receptors

It is known that the expression of genes encoding receptors is down-regulated by stimuli, leading to reduced receptor sensitivity. This can lead to a paradoxical outcome wherein the effects observed in the presence of low levels of a substance are not seen at high levels of the same substance. If this phenomenon occurred in individual organisms, the dose-response relationship will be non-linear.

This means that extrapolation of results obtained at high levels of the chemicals to conditions where low levels of the same substance are present would be difficult. It is needed to test the validity of this hypothesis, and analysis of the mechanisms underlying this phenomenon if the hypothesis were indeed valid, are thus important. Studies to resolve these questions are now under way.

3. Data gap concerning the effects of female hormones

In mature women, there are high levels of physiological hormones *in vivo*, and these are subject to cyclic control. It has been proposed that girls with inadequate physical growth begin menstruation at lower ages and undergo sexual maturation earlier than usual, and that hormone-mimics in these subjects can precipitate breast cancer.

The weak links in this hypothesis have been pointed out, and it has been shown experimentally that estrogen by itself may be teratogenic, although this tendency has been shown to be weak. It is known that organisms are programmed such that excessive exposure to estrogens during the intrauterine period or other developmental stages is avoided.

There are many open questions as to the

mechanism by which mature females remain physiologically stable, even when exposed daily to high levels of estrogen (400 pM/l). Some dramatic effects are probably needed to disturb this physiology.

4. Multi-generation tests and effects on fetuses

It has been shown that exposure to hormones or hormone-mimics during intrauterine or early neonatal periods can lead to irreversible changes in the pattern of development. This susceptibility period is short, extending from the 13th gestational day to about one week after birth. These effects are the so-called "intrauterine window effects."

In animal studies involving observation of experimental animals for two or more generations, no effects of EDs have been demonstrated. The question therefore arises as to why window effects are observed during the short period mentioned above. It is unknown whether or not these effects really do occur, and if they do, how are they produced.

Delayed growth of the thalamic nucleus specific to males (called sexual type II nucleus) is seen in male rats treated with female hormones. We may say that under conditions of homeostasis of the physiological hormones in mature individuals, exposure to dose levels that usually cause only reversible changes can lead to irreversible changes, if the exposure occurs during genesis, morphogenesis or functional development. However, there are no ample data endorsing this view in humans.

Considering the biological plausibility inferred from the experimental data accumulated to date,[*1] we may say that there are no sufficient data that clearly rule out this view. Close attention has therefore been paid to these effects in children.

New theories of methodology, focusing on the effects in fetuses and children, are now being developed, primarily in the United States, within the framework of children's program, etc.

Health Hazards at Low Levels of Exposure

Chemicals used for agriculture or industrial purposes are marketed, in general, only after their effects on living beings have been investigated. We may therefore understand that they are used on the premise that the possibility of these chemicals exerting hazardous effects on health at relatively high dose levels has been almost ruled out. Nevertheless, problems with EDs have begun to be highlighted. These problems may be not confined to those related to the accumulation of these substances through food chains in the ecosystem, but also to the possibility additionally that these chemicals may exert effects at low dose levels even if they have been declared safe at high dose levels. The latter possibility may apply, however, only to some cases and not to others.

We may say that a major issue pertaining to EDs that must be resolved urgently is whether or not they pose health hazards at low dose levels. This issue can be summarized into the following three questions: (1) presence/absence of threshold level, (2) presence/absence of synergistic or additive effects, and (3) possibility of extrapolation of high-dose effects to low-dose levels (i.e., presence/absence of a linear dose-response relationship). No clear-cut answers have as yet emerged to these questions. Considering the above-mentioned characteristics of the effects of hormones, it is plausible to imagine how difficult it may be to resolve these questions.

To determine if these chemicals exerted hazardous effects on health at low dose levels, the following basic questions may need to be considered; their biological plausibility is hardly denied.

*1 Biological plausibility: Likelihood of a phenomenon as judged by considering the difference or similarity of elements of reactions in individual organisms, on the basis of the results of a series of a related biological experiments. (cf. probability)

(1) Regarding the presence or absence of threshold levels, it seems likely that many chemicals suspected of being EDs can easily permeate across the cell membrane, which is composed of phospholipids. Therefore, assuming that one receptor molecule reacts with one chemical molecule, the lower limit of the dose level exerting the chemical's effects would be very low.

   Of course, since the probability of the binding of a ligand to the receptor will be low if the dose level is low, we cannot say that there is no threshold level for the effects seen in the low dose level range. In fact, for bisfenol A, which has been attracting close attention because of its hazardous effects on health at low dose levels, the presence/absence of a threshold level has not yet been reported. It seems rational, therefore, to assume that these health hazards occur in a very low dose level range.

(2) If we consider not only the affinity of each substance for the receptor, but also the non-linearity of responses (e.g., waveform responses as a result of reduced receptor expression following an increase in dose level), it is possible to assume that there are U-shaped or reverse U-shaped reactions or oscillational dose-response curves. *Interim* data endorsing such a view are being accumulated.

(3) Regarding the possibility of synergistic or additive effects, the observation of additive effects among different nuclear receptors has been reported. Data yielded by analysis of interactions between receptor signals also suggest such a possibility. In fact, the dose-response curves for some composite materials were reported to be additive, but not synergistic.

Thus, the questions on health hazards at low dose levels have several aspects: (1) the type of receptor-mediated actions of the hormone mimics, (2) diverse reactive characteristics on the part of the receptors, (3) diverse modification during expression of intracellular signals, and (4) factors involved in irreversible changes related to morphogenesis and functional development. Resolution of all these aspects of the question will lead to clarification of the mechanism of actions of the substances from each of the aforementioned standpoints. While these questions are among the hottest research themes at present, they are certainly unlikely to be resolved easily.

At a workshop held in North Carolina, USA, in October 2000, health hazards of chemicals at low dose levels were discussed. Investigators for and against the possibility of these substances posing health hazards at low dose levels gave detailed accounts of their studies, and no definitive conclusions could be reached, as the arguments of both sides appeared to be tenable.

This means that reports affirming the plausibility of these substances posing health hazards at low dose levels in animal experiments cannot be immediately rejected. The workshop concluded by pointing out the necessity of paying attention to the possible hazards on fetuses and neonates.

Health Hazards of Hormone-Mimics on Humans

The possibility of health hazards of hormone-mimics on humans have not been supported by adequate epidemiological data, and the number of cases for which the data clearly endorse such effects is quite small. The US National Research Council emphasizes the necessity of conducting further epidemiological studies on this topic (National Research Council, 1999).

In conclusion, this paper summarizes the current knowledge concerning the health hazards of hormone-mimics on humans. Reports dealing with the effects of these substances on humans are confined to those pertaining to the effects of dioxins and PCB, and the validity and usefulness of these results have not yet been established.

The following are based on case studies conducted to date.

1. Health hazards of dioxins

Regarding health hazards of dioxins, two-year dosing studies revealed weight loss and liver damage, and three-generation reproductive studies in rats disclosed intrauterine death and a decrease in litter size. Onset of endometriosis in rhesus monkeys has also been reported.

A causal relationship of EDs to the following episodes in humans has been suggested: biased male-to-female ratio in children born in the dioxin-exposed Seveso area of Italy, and increased incidence of cleft palate in the Diemerzeedijk district of the Netherlands, probably due to steroids. In both of these cases, the Environmental Protection Agency (EPA) of the United States did not affirm a causal relationship, and treated classified them as cases requiring special attention.

No consensus has been reached concerning the relationship of hypothyroidism observed in the inhabitants along Lake Michigan to the ingestion of PBB (polybrominated biphenyls)-contaminated fish.

2. Effects on mature females, e.g., increased incidence of breast cancer

No reports affirming the effects of dioxins on mature human females (e.g., effects on breast cancer or endometriosis as discussed below). There are many unresolved questions on this topic. However, none of the studies conducted in mature experimental animals revealed data endorsing the plausibility of occurrence of such effects. On the other hand, it is known that the age at menarche is lower and the incidence of breast cancer higher in females exposed to dioxins. Some investigators cite these data when discussing the health hazards of dioxins. It is also known that females exposed to dioxins are often taller.

In European countries, a height increase of about 3.5 mm per year and an approximately one-year decrease in the age at menarche have been reported during the past 30 years. It is difficult to identify the influence of extrinsic endocrine factors on these changes, and no studies addressing this issue have been reported to date. Although a number of studies have been published concerning the effects of female hormone preparations, including pills used for contraception and hormone replacement therapy in postmenopausal women, no studies have provided data that establish the effects of EDs.

3. Endometriosis

Endometriosis is a disease of unexplained origin that is seen in primates with sexual cycles. It has been pointed out that this disease tends to be more severe in individuals exposed to dioxins (TCDD/PCBs). Data yielded from experiments in rhesus monkeys are used as evidence to corroborate the causal relationship between dioxins and endometriosis. We cannot thus rule out the biological plausibility of these effects. However, no reports affirming the causal relationship in humans have been published.

4. Possibility of other effects on humans

Biological plausibility has been pointed out also on the following effects of hormone-mimics on humans: qualitative dysfunction of human sperm, effects on neurobehavior of neonates, and immune functions. The effects on immune functions have been suggested by reports of cases with Yu-sho (PCB intoxication).

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The Aryl Hydrocarbon Receptor Nuclear Transporter Is Modulated by the SUMO-1 Conjugation System*

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Masahide Tojo‡§¶, Kazuhito Matsuzaki§, Takeshi Minami‡, Yoshiomi Honda§, Hideyo Yasuda‖, Tsutomu Chiba¶, Hideyuki Saya§, Yoshiaki Fujii-Kuriyama, and Mitsuyoshi Nakao‡ ‡‡**

*From the ‡Department of Regeneration Medicine, Institute of Molecular Embryology and Genetics, Kumamoto University, and the §Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, the ¶Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Shogoinkawara-cho, Sakyo-ku, Kyoto 606-8507, the ‖Tokyo University of Pharmacy and Life Science, School of Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, and the **Department of Chemistry, Graduate School of Science, Tohoku University, Aoba-ku, Sendai 980-8578, Japan*

The aryl hydrocarbon receptor nuclear transporter (ARNT) is a member of the basic helix-loop-helix/PAS (Per-ARNT-Sim) family of transcription factors, which are important for cell regulation in response to environmental conditions. ARNT is an indispensable partner of the aryl hydrocarbon receptor (AHR) or hypoxia-inducible factor-1α. This protein is also able to form homodimers such as ARNT/ARNT. However, the molecular mechanism that regulates the transcriptional activity of ARNT remains to be elucidated. Here, we report that ARNT is modified by SUMO-1 chiefly at Lys^{245} within the PAS domain of this protein, both *in vivo* and *in vitro*. Substitution of the target lysine with alanine enhanced the transcriptional potential of ARNT *per se*. Furthermore, green fluorescent protein-fused ARNT tended to form nuclear foci in ~20% of the transfected cells, and the foci partly colocalized with PML nuclear bodies. PML, one of the well known substrates for sumoylation, was found to augment the transcriptional activities of ARNT. ARNT bound AHR or PML, whereas the sumoylated form of ARNT associated with AHR, but not with PML, resulting in a reduced effect of PML on transactivation by ARNT. Our data suggest that the sumoylation of ARNT modulates its transcriptional role through affecting the ability of ARNT to interact with cooperative molecules such as PML. This exemplifies a crucial role of protein sumoylation in modulating protein-protein interactions.

The aryl hydrocarbon receptor nuclear transporter (ARNT)[1] belongs to the basic helix-loop-helix (bHLH)/PAS (Per-ARNT-Sim) family of proteins. These transcription factors are required for cell regulation to respond to various environmental conditions (1, 2). The bHLH/PAS proteins include the aryl hydrocarbon receptor (AHR) and hypoxia-inducible factor-1α (HIF1α). ARNT is an indispensable partner of these proteins for the formation of heterodimers such as AHR/ARNT and HIF1α/ARNT. Both transcription pathways are not only biologically significant, but represent remarkable regulatory mechanisms *in vivo*. First, polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene are exogenous ligands for AHR and induce the formation of the AHR·ARNT complex (3). In the absence of ligands, AHR is generally found in the cytoplasm in association with hsp90 and other molecules (3, 4). Upon the binding of a ligand, AHR is converted to a functional DNA-binding species in a multistep process involving nuclear translocation, dissociation from the hsp90-containing complex, and dimerization with ARNT. The resulting AHR·ARNT complex binds a specific *cis*-acting regulatory DNA sequence, termed the xenobiotic-responsive element (XRE), upstream of its target genes encoding drug-metabolizing enzymes such as cytochrome P450 (CYP1A1 and others), quinone reductase, and the glutathione *S*-transferase (GST) Ya subunit. Xenobiotic-activated AHR is then degraded by the ubiquitin/proteasome system after being exported from the nucleus to the cytoplasm (5). Mice deficient in the AHR gene demonstrate dioxin-induced cytotoxicities, including developmental defects and tumorigenesis, depending on the AHR/ARNT pathways (6, 7). Second, ARNT, also known as HIF1β, forms a HIF1α/ARNT heterodimer in response to oxygen tension in the cells. Under hypoxic conditions, it activates the transcription of a number of target genes whose promoters contain the binding motif termed the hypoxia-responsive element (HRE) (8). These include genes encoding erythropoietin, vascular endothelial growth factor, glycolytic enzymes, tyrosine hydroxylase, inducible nitric-oxide synthase, and heme oxygenase-1, all of which allow the cells to cope with lower oxygen levels. In addition, HIF1α/ARNT controls the gene expression involved in iron metabolism, pH regulation, cell proliferation and apoptosis, and tumorigenesis. The HIF1α protein is rapidly degraded by the ubiquitin/proteasome pathway under normoxic conditions (9, 10). In a hypoxic state, it becomes stable and translocates into the nucleus. It then dimerizes with ARNT to activate transcription. Thus, bHLH/PAS family members have significant features of their regulatory mechanisms, and ARNT is implicated in many signaling pathways mediated by AHR or HIF1α as its partner.

Increasing numbers of cellular proteins have been found to be covalently conjugated with SUMO-1 (small ubiquitin-related modifier-1). Sumoylation is thought to control the function of substrate proteins through affecting their interaction

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‡‡ To whom correspondence should be addressed. Tel.: 81-96-373-6800; Fax: 81-96-373-6804; E-mail: mnakao@gpo.kumamoto-u.ac.jp.

[1] The abbreviations used are: ARNT, aryl hydrocarbon receptor nuclear transporter; bHLH, basic helix-loop-helix; AHR, aryl hydrocarbon receptor; HIF1α, hypoxia-inducible factor-1α; XRE, xenobiotic-responsive element; GST, glutathione *S*-transferase; HRE, hypoxia-responsive element; E3, protein isopeptide ligase; NBs, nuclear bodies; HA, hemagglutinin; PBS, phosphate-buffered saline; WT, wild-type; DBD, DNA-binding domain; GFP, green fluorescent protein.

This paper is available on line at http://www.jbc.org

with cooperative molecules and subcellular localization (11–13). The conjugation by SUMO-1 in some cases antagonizes ubiquitination at the identical target lysine of substrates, resulting in the stabilization of sumoylated proteins (14, 15). Like the ubiquitin conjugation system, SUMO-1 is activated in an ATP-dependent manner by a Sua1/UBA2 heterodimer SUMO-1-activating enzyme); then transferred to a SUMO-1-conjugating enzyme, UBC9; and subsequently attached to the ε-amino group of target lysines of substrates (11–13). Sumoylation mostly targets the consensus sequence ΨKXE, where Ψ and K are a hydrophobic residue and the SUMO-1 acceptor lysine in substrate proteins, respectively (16–19). These include Ran GTPase-activating protein-1 (20, 21), Sp100 (16, 22), p53 (23–25), IκBα (14), c-Jun (26, 27), c-Myb (28), heat shock factor-1 (29), heat shock factor-2 (30), lymphoid enhancer factor-1 (31), and PML (32–34). Although it was unknown whether sumoylation requires E3 enzymes with SUMO-1 ligase activities, SUMO E3-like factors have recently been identified in mammals, including members of the PIAS (protein inhibitor of activated STAT, where STAT is signal transducer and activator of transcription) family of proteins and Ran-binding protein-2 (27, 31, 35, 36). Furthermore, some sumoylated proteins are found to localize to specified nuclear domains called nuclear bodies (NBs), also referred to as nuclear domain 10 or PML oncogenic domains (29–34). The SUMO-1 conjugation of PML enables it to target to NBs, and PML is indispensable in the formation of NBs. Proteins such as Sp100 and p53 are similarly modified by SUMO-1, but their translocation into NBs does not need SUMO-1 modifications (16, 25). Recently, some transcription factors and their co-regulators were identified to associate with PML, emphasizing the transcriptional roles of PML-NBs (37, 38). Thus, the SUMO-1 conjugation system may cooperate with PML-NBs in transcriptional control.

In addition to the heterodimerization with AHR or HIF1α, ARNT is likely to form a homodimer with itself to bind the E-box core sequence CACGTG with high specificity and affinity, suggesting a physiological role of the ARNT·ARNT complex (3, 39). Furthermore, the t(1,12)(q21;p13) translocation of human acute myeloblastic leukemia results in a fusion protein containing the amino-terminal part of TEL (translocated ETS leukemia, also known as ETV6) and almost all of ARNT (40). The activity of ARNT may directly contribute to leukemogenesis. It is therefore of considerable interest to study the function of ARNT and the mechanism that regulates the transcriptional role of this protein. During the investigation of ARNT in cultured mammalian cells, we discovered that ARNT is modified by SUMO-1 chiefly at Lys^{245} within the PAS domain, which is required for forming the complexes with bHLH/PAS and other molecules (3, 41, 42). ARNT associated with PML or PML-NBs, and the sumoylation of ARNT inhibited both the ability of ARNT to interact with PML and the positive effect of PML on the transactivation by ARNT. These data suggest the importance of the SUMO-1 conjugation system in modulating ARNT-mediated gene expression.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit anti-ARNT (H-172) and rabbit anti-SUMO-1 (FL-101) polyclonal antibodies (Santa Cruz Biotechnology), goat anti-GST polyclonal antibodies (Amersham Biosciences), mouse anti-ARNT (G-3) and mouse anti-PML (PG-M3) monoclonal antibodies (Santa Cruz Biotechnology), mouse anti-GMP-1 monoclonal antibodies (SUMO-1, Zymed Laboratories Inc.), mouse anti-FLAG monoclonal antibodies (M5, Sigma), mouse anti-Myc monoclonal antibodies (PL-14, Medical & Biological Laboratories), and mouse monoclonal antibody 9E10 (Roche Molecular Biochemicals) were used in this study.

Cell Lines and Cultures—MCF-7, HeLa, and Hepa-1 cells were grown in Dulbecco's modified Eagle's medium and Ham's F-12 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum.

Construction of Expression Plasmids—The full-length cDNA for murine ARNT was PCR-amplified from pBOS-ARNT (43) with a cloned *Pfu* DNA polymerase (Stratagene) using primers 5′-CCGGTGCCTCGAGCCGAATTCATGGCGGCGACTACAGCTAAC-3′ (forward, containing *Xho*I and *Eco*RI sites (underlined)) and 5′-CACAGGCTCGAGCTATTCGGAAAAGGGGGGAAAC-3′ (reverse, containing an *Xho*I site (underlined)). The PCR fragment was digested with *Eco*RI and *Xho*I, cloned into pcDNA3-FLAG, and inserted into the *Xho*I site of pEGFP-C1. To generate pcDNA3-Gal4-ARNT, the fragment of the Gal4 DNA-binding domain was amplified from pcDNA3-Gal4DBD (44) by primers 5′-CCGGTGGAATTCATGAAGCTACTGTCTTCTATCGAA-3′ (forward, containing an *Eco*RI site (underlined)) and 5′-CACAGGGAATTCGGATCCCGATACAGTCAACTGTCTTTGAC-3′ (reverse, containing *Eco*RI and *Bam*HI sites (underlined)) and cloned into the *Eco*RI site of pcDNA3-FLAG-ARNT. pcDNA3-His-FLAG-ARNT was constructed by inserting a fragment of FLAG-ARNT from pcDNA3-FLAG-ARNT into predigested pcDNA3-His-FLAG-SUMO-1. Next, to construct plasmids expressing ARNT K245A and ARNT K546A, site-directed mutagenesis was performed using oligonucleotides 5′-ACAGTGGCCAAGGAAGGCCAGCAGTCTTCC-3′ and 5′-CTGGCCTTCCTTGGCCACTGTTCCAGTCTT-3′ for K245A and oligonucleotides 5′-AAGCCCCTTGAGGCCTCAGAAGGTCTCTTT-3′ and 5′-GAGACCTTCTGAGGCCTCAAGGGGCTTGCT-3′ for K546A. To generate pcDNA3-FLAG-ARNT K245A/K546A, both pcDNA3-FLAG-ARNT K245A and pcDNA3-FLAG-ARNT K546A were digested with *Ssp*I, and the fragment containing FLAG and the region of amino acids 1–395 of ARNT K245A were ligated into predigested pcDNA3-FLAG-ARNT K546A.

For plasmids expressing GST-fused ARNT and ARNT K245A (amino acids 145–345), the cDNAs for ARNT and ARNT K245A were amplified primers 5′-TCCTTGGGATCCACTGGCAACACATCTACTGAT-3′ (forward, containing a *Bam*HI site (underlined)) and 5′-GGGAGAAAGCTTTACCTGCAGCCTGCCAATGGC-3′ (reverse, containing a *Hin*dIII site (underlined)). The amplified DNAs were cloned into pGEX-2T predigested with *Bam*HI and *Hin*dIII. To generate pcDNA3-Myc-AHR, the full-length cDNA for murine AHR was PCR-amplified from pBOS-AHR by primers 5′-CCGGTGCCTCGAG AATATTTAATGAGCAGCGGCGCCAACATC-3′ (forward, containing *Xho*I and *Ssp*I sites (underlined)) and 5′-CACAGGCTCGAGTCAACTCTGCACCTTGCTTAGGA-3′ (reverse, containing an *Xho*I site (underlined)). The PCR fragment was digested with *Ssp*I and *Xho*I and cloned into the *Eco*RV and *Xho*I sites of pcDNA3-Myc. To generate pcDNA3-Myc-PML, PML cDNA was amplified by primers 5′-AAGCTGGAATTCATGGAGCCTGCACCCGCCCGA-3′ (forward, containing an *Eco*RI site (underlined)) and 5′-CTTTTTCTCGAG AAGCTTCTAAATTAGAAAGGGGTGGGGGTA-3′ (reverse, containing *Xho*I and *Hin*dIII sites (underlined)). The cDNA for SUMO-1 (amino acids 1–97) was amplified from HeLa cDNAs by primers 5′-GCCACCCTCGAGATGTCTGACCAGGAGGCAAAA-3′ (forward, containing an *Xho*I site (underlined)) and 5′-GCCACCCTCGAG AAGCTTCTAACCCCCCGTTTGTTCCTGATA-3′ (reverse, containing *Xho*I and *Hin*dIII sites (underlined)) to construct pcDNA3-FLAG-SUMO-1. The cDNA for SUMO-1-(1–97) was also cloned into the *Xba*I site of pCGN-HA. To generate plasmids expressing His$_6$-fused FLAG-SUMO-1, the fragment of FLAG and SUMO-1 was obtained by digesting pcDNA3-FLAG-SUMO-1 with *Bam*HI and *Hin*dIII and cloned into pRSET, yielding pRSET-FLAG-SUMO-1. For expressing His$_6$-fused FLAG-SUMO-1, the cDNA for His$_6$-FLAG-SUMO-1 was amplified using primers 5′-GCCACCGGTACCGCCACCATGCGGGGTTCTCATCAT-3′ (forward, containing a *Kpn*I site (underlined)) and 5′-GCCACCTCTAGACTAACCCCCCGTTTGTTC-3′ (reverse, containing an *Xba*I site (underlined)). The PCR fragment was digested with *Kpn*I and *Xba*I and cloned into pcDNA3-FLAG, resulting in pcDNA3-His-FLAG-SUMO-1. The cDNA for human UBC9 was amplified from HeLa cDNAs and cloned into the *Xba*I site of pCGN-HA, yielding pCGN-HA-UBC9.

For pGL3-promoter-XRE, oligonucleotides containing six XREs (5′-CTAGATGCGTGTGCGTGTGCGTGTGCGTGTGCGTGTGCGTGT-3′ and 5′-CTAGACACGCACACGCACACGCACACGCACACGCACACGCAT-3′) were equally mixed, heated to 95 °C for 5 min, and then cooled to room temperature. The double-stranded oligonucleotides were ligated into the *Nhe*I site of the pGL3-promoter vector (Promega). For pGL3-basic-mCyp1A1, ~1.5 kb of the promoter sequence of the mouse *cyp1A1* gene was amplified from genomic DNA by primers 5′-TCCTGGGGTACCTCACAGAGCAGATATCAATGT-3′ (forward, containing a *Kpn*I site (underlined)) and 5′-TCTAGGAAGCTTGGGAGGATCGGGGAAGCTCCA-3′ (reverse, containing a *Hin*dIII site (underlined)). The PCR fragment was cloned into the *Kpn*I and *Hin*dIII sites of the pGL3-basic vector (Promega).

Western Blot Analysis—Cell lysates were prepared with SDS sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.005% brom-

phenol blue, and 100 mM dithiothreitol). Samples were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose filters with a constant current of 140 mA for 2 h. The filters were blocked with phosphate-buffered saline (PBS) containing 10% skim milk and then incubated with the appropriate antibodies in PBS containing 0.03% Tween 20 for 2 h and washed three times for 7 min with PBS containing 0.3% Tween 20. The filters were incubated with horseradish peroxidase-conjugated secondary antibodies for 40 min, and then specific proteins were detected using the ECL system (Amersham Biosciences).

Nickel Affinity Pull-down Assay—MCF-7 cells (30–50% confluent in six-well plates) were transfected with 1 μg of each of the expression vectors for His_6-FLAG-ARNT (wild-type (WT) or K245A) and HA-SUMO-1 per well using FuGENE 6 (Roche Molecular Biochemicals). At 48 h after transfection, the cells were washed with ice-cold PBS, harvested in 200 μl of Gua8 buffer (6 M guanidine HCl, 100 mM NaCl, 10 mM Tris, and 50 mM NaH_2PO_4 (pH 8.0)) (22) per well, briefly sonicated, and then centrifuged. The cell lysates were incubated for 2 h with 20 μl of ProBond nickel-chelating resin (Invitrogen). Bound proteins were washed twice with Gua8 buffer; three times with buffer containing 8 M urea, 100 mM NaCl, and 50 mM NaH_2PO_4 (pH 6.5)) (22) with 10 mM imidazole; and once with cold PBS before being eluted by boiling in SDS sample buffer. Transfected cells in one well were lysed directly with SDS sample buffer as an input sample. All samples were electrophoresed on an 8% SDS-polyacrylamide gel, followed by Western blot analysis.

In Vitro SUMO-1 Conjugation Assay—Sf9 insect cells expressing both Sua1 and human UBA2 (45) were lysed by sonication in buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 200 mM NaCl, 0.1% Nonidet P-40, and 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride. The lysates were centrifuged at 13,000 rpm for 15 min and used as a source of Sua1/human UBA2. His_6-UBC9 and His_6-FLAG-SUMO-1-(1–97) were expressed in *Escherichia coli* BL21(SI) and purified by affinity chromatography using ProBond. GST-ARNT WT and GST-ARNT K245A were expressed in BL21(star) and purified using glutathione-agarose beads (Sigma). GST-ARNT proteins were immobilized on glutathione-agarose beads and then incubated with Sua1/human UBA2, UBC9, and ATP with or without His_6-FLAG-SUMO-1 at 25 °C for 120 min in buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM ATP, 10 mM $MgCl_2$, and 2 mM dithiothreitol. After the reaction, the beads were washed with 0.2% Triton X-100 in PBS five times and then boiled in SDS sample buffer.

Luciferase Assay—MCF-7 cells were plated at a density of 6~8 × 10^5 cells/ml in a six-well plate and cultured for 24 h prior to transfection. The cells were introduced with 0.5 μg of reporter plasmid and 0.1 μg of pRL-CMV (Promega), which was used for monitoring the transfection efficiency, together with the indicated expression plasmids by TransFast (Promega). The transfected cells were treated with 10 μM 3-methylcholanthrene (Aldrich) and 200 μM cobalt chloride (Wako Bioproducts) or solvent alone for 12 h during the incubation. At 48 h after transfection, the cells were lysed in the lysis buffer provided by the manufacturer (Promega). The insertless pcDNA3 was used as a mock vector. The luciferase activities were determined with the dual-luciferase reporter assay system. Values are the means ± S.D. of the results from at least three independent experiments.

For Gal4 transactivation studies, 1 μg of Gal4-ARNT (WT or K245A) or the Gal4 DNA-binding domain (DBD) vector, 0.5 μg of the pG5luc reporter vector, and 0.1 μg of pRL-CMV were introduced into the cells in each well using TransFast. pCGN-HA-SUMO-1 (the indicated amount), pCGN-HA-UBC9 (1 μg), and pcDNA3-Myc-PML (1 μg) were cotransfected, and luciferase analysis was done at 48 h after transfection.

Confocal Laser Scanning Microscopic Analysis—For immunofluorescence analysis, MCF-7 and Hepa-1 cells were transfected with the indicated vectors using FuGENE 6. At 48 h after transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After washing with PBS, the cells were incubated with specific primary antibodies at room temperature for 60 min. The samples were incubated with fluorescein isothiocyanate (BIOSOURCE)- or Cy3 (Amersham Biosciences)-conjugated secondary antibodies for 60 min and visualized with an Olympus confocal laser scanning microscope. To avoid bleed-through effects in the double-staining experiments, each dye was independently excited, and images were electronically merged.

Immunoprecipitation—MCF-7 or HeLa cells were transfected with the indicated plasmids with FuGENE 6 and lysed at 48 h after transfection with hypotonic buffer (10 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.05% Nonidet P-40, 5% glycerol, 5 mM *N*-ethylmaleimide, 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 1.5 μM pepstatin, and 1 mM sodium orthovanadate) on ice for 30 min. After brief sonication and centrifugation (13,000 rpm) for 20 min, the supernatants were incubated with the indicated antibodies at 4 °C for 2 h and then with protein A/G beads (Calbiochem) at 4 °C for 2 h. For immunoprecipitation of FLAG-tagged proteins, anti-FLAG antibody M2-agarose affinity gel (Sigma) was used. The beads were washed five times with buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 0.5% Nonidet P-40. The immunoprecipitates were suspended in SDS sample buffer and separated by SDS-PAGE.

GST Pull-down Assay—The pcDNA3-FLAG-ARNT or mock vector was transfected into MCF-7 cells with or without pCGN-HA-SUMO-1 and pCGN-HA-UBC9 using FuGENE 6. At 48 h after transfection, the cells were lysed with binding buffer (10 mM HEPES (pH 7.5), 50 mM KCl, 2.5 nM $MgCl_2$, 50 μM $ZnCl_2$, 2.5 mM dithiothreitol, 0.025% Nonidet P-40, and 5% glycerol), briefly sonicated, and centrifuged. The supernatants were incubated with GST-PML or GST at 4 °C for 2 h. 25 μl of glutathione-agarose beads as a 50% slurry in binding buffer was added to the mixture. After incubation at 4 °C for 1 h, the beads (complexed with proteins) were washed five times with binding buffer and then boiled in SDS sample buffer.

RESULTS

Sumoylation of ARNT at Lysine 245 in Vivo and in Vitro—The fact that several transcription factors are modified by SUMO-1 prompted us to test whether ARNT possesses the consensus sequence for SUMO-1 conjugation. Because amino acids 244–247 and 545–548 of murine ARNT coexist with the minimal consensus sequence, Lys^{245} and Lys^{546} seemed to be putative SUMO-1 acceptor sites in ARNT (Fig. 1*A*). Both lysine residues of ARNT are perfectly conserved among species, including human, murine, and rat. To test whether ARNT can be conjugated with SUMO-1 and to determine the target site(s) of the SUMO-1 modification in ARNT, we generated three plasmids to express ARNT mutants in which Lys^{245} and/or Lys^{546} was substituted with alanine: ARNT K245A, ARNT K546A, and ARNT K245A/K546A. Wild-type ARNT and its mutants fused to FLAG were each expressed together with HA-tagged SUMO-1 in MCF-7 cells. Both MCF-7 and HeLa cells, used in this study, have been proven to maintain ARNT-related signaling pathways to target genes (Fig. 1*B*) (46). HA-SUMO-1 was efficiently utilized to modify substrate proteins in the cells (data not shown). Western blot analysis of whole cell extracts using anti-FLAG monoclonal antibodies revealed that a high molecular mass band of a covalently modified form (~150 kDa) was detected in both wild-type ARNT and ARNT K546A upon coexpression of HA-SUMO-1. In contrast, the ARNT K245A and ARNT K245A/K546A modifications were rarely found. Wild-type ARNT and its mutants were equally expressed as shown by the presence of their unconjugated form (~90 kDa). These data suggest that a fraction of ARNT is modified, probably by SUMO-1, chiefly at Lys^{245}, which exists in the PAS domain (Fig. 1*A*).

To confirm that the covalently modified band in Fig. 1*B* is a sumoylated form of ARNT, we expressed His-FLAG-tagged wild-type and mutant ARNT in the presence or absence of HA-SUMO-1 to examine the precipitates on nickel beads using anti-FLAG and anti-HA antibodies (Fig. 1*C*). The 150-kDa modified band of wild-type ARNT (but not of the K245A mutant) was seen in the cells expressing HA-SUMO-1 (*left panel*). Western blot analysis of the nickel bead precipitates using anti-HA antibodies indicated that the slowly migrating band originated in sumoylated ARNT (*right panel*). Therefore, these data suggest that Lys^{245} of ARNT is the major site of SUMO-1 modification in the cells. In addition, the size of the apparent SUMO-modified ARNT did not match that expected upon addition of a single SUMO protein. As described below, sumoylated ARNT in Fig. 1 (*B* and *C*) corresponded to *band b* in Fig. 2.

To further clarify the sumoylation of ARNT, we constituted

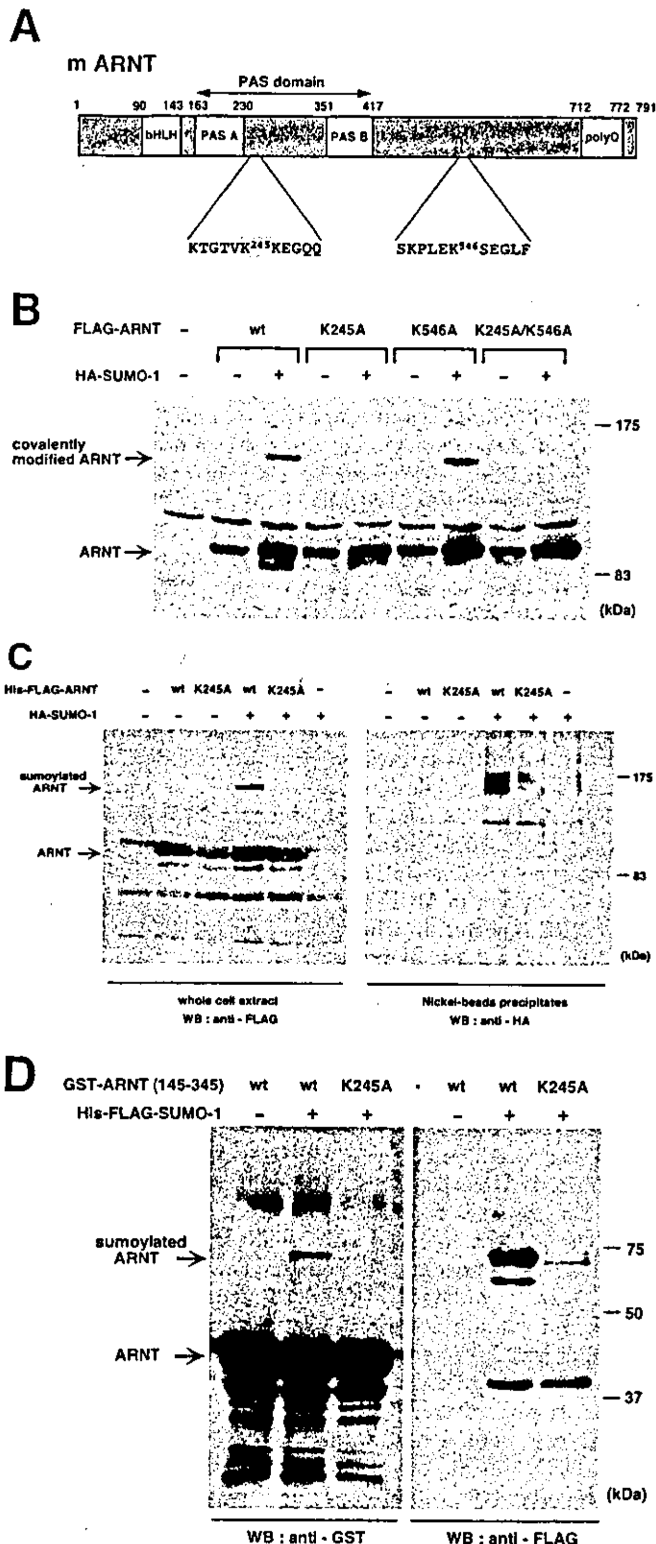

FIG. 1. **Sumoylation of ARNT at lysine 245 *in vivo* and *in vitro*.** *A*, schematic representation of murine (*m*) ARNT. Two putative SUMO-1 acceptor sites, lysines 245 and 546, are indicated below with the neighboring amino acid sequences. *B*, ARNT is covalently modified by SUMO-1 at Lys^{245} *in vivo*. Wild-type or mutant ARNT (K245A, K546A, or K245A/K546A) fused to FLAG was expressed with or without HA-tagged SUMO-1 in MCF-7 cells. *C*, evidence for SUMO-1 modification of ARNT *in vivo*. His-FLAG-tagged ARNT (WT or K245A) was expressed with or without HA-SUMO-1 in MCF-7 cells. Whole cell extracts (*left panel*) and the nickel bead precipitates (*right panel*) were probed with anti-FLAG and anti-HA antibodies, respectively. *D*, ARNT is sumoylated at Lys^{245} *in vitro*. Recombinant wild-type ARNT or ARNT K245A (amino acids 145–345) fused to GST was incubated with Sua1/UBA2, UBC9, and ATP in the presence or absence of His-FLAG-SUMO-1. Glutathione-agarose bead precipitates were analyzed by Western blot (*WB*) analysis with anti-GST (*left panel*) or anti-FLAG (*right panel*) antibodies.

in vitro the SUMO-1 transfer using recombinant proteins. Wild-type ARNT and ARNT K245A (amino acids 145–345) were prepared as GST fusion proteins. The reaction was performed in the presence of ATP using Sua1/UBA2, UBC9, GST-ARNT (WT or K245A), and His-FLAG-SUMO-1. GST pull-down assay was performed using glutathione-agarose beads (Fig. 1*D*). In addition to the unconjugated form, the sumoylated form of wild-type ARNT was detected by anti-GST antibodies (*left panel*). The use of anti-FLAG antibodies showed that wild-type ARNT formed an adduct with FLAG-SUMO-1 (*right panel*). The ARNT K245A mutant showed little acceptance for the SUMO-1 molecule, although unconjugated GST-ARNT WT and GST-ARNT K245A were comparably present. Collectively, these data provide evidence for SUMO-1 modification of ARNT at Lys^{245}.

ARNT Accepts at Least Three SUMO-1 Molecules in Vivo—As mentioned above, UBC9 and PIAS1 are believed to be key factors in the SUMO-1 conjugation system. To investigate the effect of these enzymes on the modification of ARNT, we expressed HA-tagged UBC9 and/or FLAG-fused PIAS1 in combination with FLAG-ARNT and HA-SUMO-1 in MCF-7 cells (Fig. 2*A*). The unmodified form of ARNT appeared as an ~90-kDa band (*lanes 2–8*). As was the case in Fig. 1, ARNT was covalently conjugated upon expression of SUMO-1 (*band b* (150 kDa) in *lane 3*). The fact that overexpressed ARNT alone did not induce the sumoylated form might be explained by the limited amount of free endogenous SUMO-1 (as described under "Discussion"). In agreement with this, the coexpression of UBC9 or PIAS1 did not induce the modified form of ARNT (*lanes 4* and *5*). Furthermore, combination of SUMO-1 with UBC9 or PIAS1 enhanced the SUMO modification of ARNT (*lanes 6* and *7*), although the coexistence of UBC9 and PIAS1 appeared not to produce a synergistic effect (*lane 8*). In addition to the modified band (*band b*), the presence of two minor species were observed in *lanes 6–8*, as shown by *bands a* (120 kDa) and *c* (180 kDa).

To test whether these bands (*bands a–c*) are associated with the sumoylation of ARNT, we performed an immunoprecipitation analysis in HeLa cells expressing FLAG-ARNT in combination with HA-SUMO-1 and HA-UBC9 (Fig. 2*B*). All species of FLAG-ARNT were collected by FLAG immunoprecipitation for subsequent Western blot analysis with anti-FLAG and anti-SUMO-1 antibodies. FLAG-ARNT was equally expressed in *lanes 2–4*. The expression of FLAG-ARNT and HA-SUMO-1 produced the faint FLAG-reactive band (*band a*) between the unconjugated and sumoylated forms (*band b*) of ARNT (*lane 3*). Moreover, the most slowly migrating band (*band c*) was seen in the cells cotransfected with FLAG-ARNT, HA-SUMO-1, and HA-UBC9 (*lane 4*). In addition, these three forms of ARNT (*bands a–c*) were clearly recognized by anti-SUMO-1 monoclonal antibodies (*lanes 8* and *9*). Although the calculated molecular mass of the SUMO-1 protein is ~11.5 kDa, SUMO-1-conjugated proteins run on SDS-polyacrylamide gel as if they have an additional 20–30 kDa for each covalently attached SUMO-1 molecule (20, 47). Therefore, *bands a–c* are most likely to be mono-, di-, and trisumoylated forms of ARNT, respectively. Unlike ubiquitin, SUMO-1 is thought to be conjugated to a single lysine in a monomeric form (11, 48). Collectively, our results suggest that 1) ARNT potentially accepts at least three SUMO-1 molecules; 2) at least three lysines in ARNT may be modified by covalent attachment of SUMO-1;

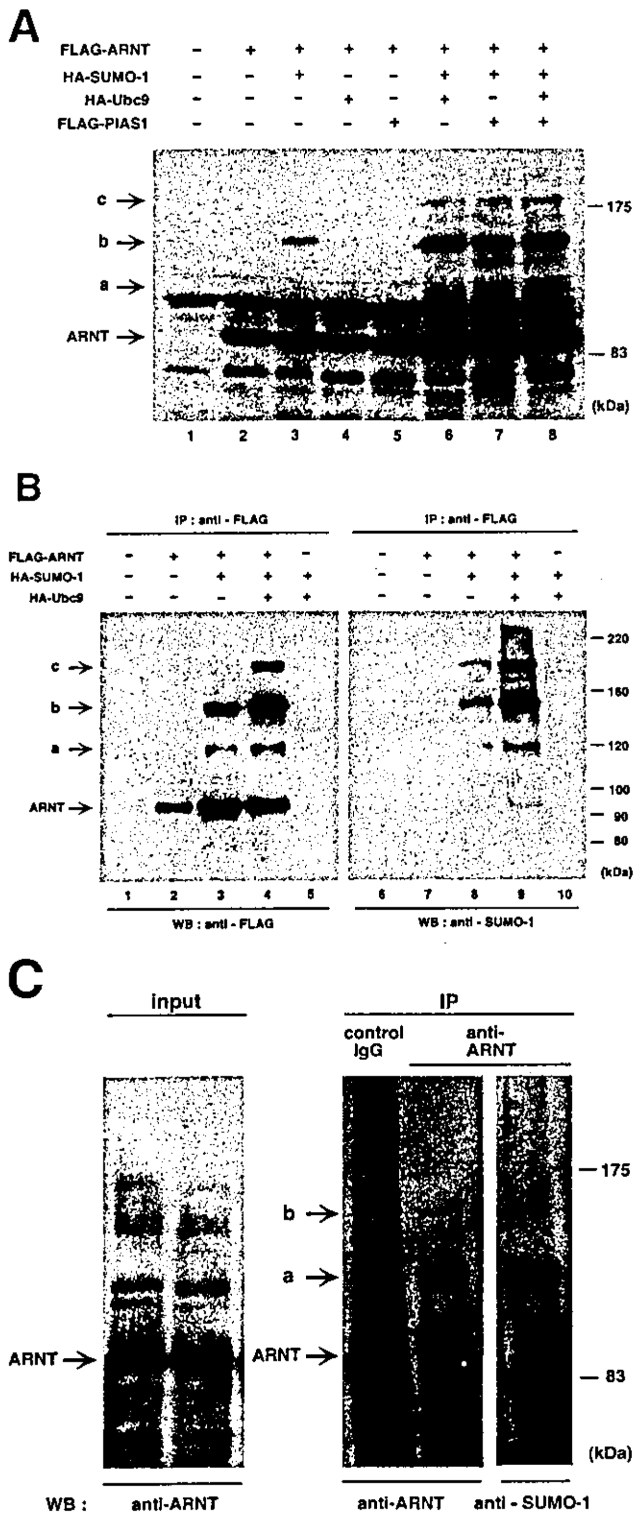

FIG. 2. **ARNT accepts at least three SUMO-1 molecules *in vivo*.** *A*, UBC9 and PIAS1 enhance SUMO-1 modification of ARNT. HA-tagged UBC9 and/or FLAG-fused PIAS1 was expressed in combination with FLAG-ARNT and HA-SUMO-1 in MCF-7 cells. Western blot analysis using anti-FLAG antibodies showed unmodified ARNT and covalently modified forms of ARNT (*bands a–c*). *B*, ARNT is potentially modified by three SUMO-1 molecules. FLAG-ARNT was expressed together with HA-SUMO-1 and/or HA-UBC9 in HeLa cells, and the FLAG immunoprecipitates (*IP*) were probed with anti-FLAG (*left panel*) and anti-SUMO-1 (*right panel*) antibodies. *C*, SUMO-1 modification of endogenous ARNT. Western blot (*WB*) analysis of whole cell lysates from MCF-7 cells was done with anti-ARNT antibodies (*left panel*). Immunoprecipitation of ARNT indicated that two high molecular mass bands (*bands a* and *b*) were detected by both anti-ARNT and anti-SUMO-1 antibodies (*right panel*).

and 3) Lys^{245} is primarily important as a SUMO-1 acceptor site. However, we did not exclude the other possibilities because these data were obtained from overexpression of ARNT, SUMO-1, and SUMO-conjugating enzymes.

We further examined the sumoylation status of endogenous ARNT in MCF-7 cells without any overexpression (Fig. 2*C*). The above-mentioned experiments suggested that a fraction of ARNT is modified by SUMO-1 *in vivo*. Western blot analysis with anti-ARNT monoclonal antibodies showed the presence of the unmodified form of ARNT (*input*). We then immunoprecipitated endogenous ARNT by anti-ARNT antibodies from the cell lysate and tested it by Western blot analysis with anti-ARNT and anti-SUMO-1 antibodies (*IP*). Two high molecular mass bands were immunoreactive to both antibodies, and they migrated as did *bands a* and *b* in Fig. 2 (*A* and *B*). Because the intensity of these sumoylated forms was much weaker than that of unmodified ARNT even after a long time exposure, the steady-state population of SUMO-modified ARNT seemed to be limited in the cells. In addition, the band corresponding to the trisumoylated form of ARNT was not found under this assay condition. Taken together, our data indicate that endogenous ARNT protein is modified by SUMO-1 *in vivo*.

Transcriptional Role of Sumoylation of ARNT—To investigate the significance of the SUMO-1 conjugation of ARNT, we examined whether sumoylation affects the transcriptional activity of ARNT. Because Lys^{245} is the major sumoylated site, we investigated the expression of wild-type ARNT and ARNT K245A. ARNT sumoylation was induced by a combination of SUMO-1 and UBC9, and appropriate luciferase reporter constructs were used to check the transactivation by ARNT. To test the effect of sumoylation of ARNT on AHR/ARNT-dependent transcription in MCF-7 cells, we employed the pGL3-promoter-XRE reporter, in which the luciferase gene is driven by six XREs and a minimal promoter, in the coexpression of AHR, ARNT (WT or K245A), SUMO-1, and UBC9 (Fig. 3*A*). Transfected cells were treated either with solvent or with 10 μM 3-methylcholanthrene for 12 h before cell lysis at 48 h after transfection. Upon 3-methylcholanthrene treatment (*black bars*), the expression of SUMO-1 augmented AHR/ARNT-mediated transcription, regardless of whether wild-type or mutant ARNT was used. In addition, UBC9 synergistically enhanced the effect of SUMO-1 on transcription by AHR/ARNT. To eliminate the influence of endogenous ARNT, similar experiments were carried out in Hepa-1-c4 cells, which are defective in ARNT; and the results showed that transcription by AHR/ARNT was stimulated by SUMO-1 and UBC9, despite the ARNT status (data not shown). We further utilized the pGL3-basic-Cyp1A1 reporter (containing ~1.5 kb of the promoter region of the mouse *Cyp1A1* gene), resulting in very similar data (data not shown).

Next, we examined the effect of SUMO-1 and UBC9 on HIF1α/ARNT-mediated transcription. The pGL3-promoter-HRE reporter (49), containing six HREs from the erythropoietin gene and a minimal promoter, was employed in combination with HIF1α, ARNT (WT or K245A), SUMO-1, and UBC9. Transfected cells were treated with 200 μM cobalt chloride, which is known to mimic hypoxia and to induce HIF1α/ARNT-mediated transcription (50), for 12 h before cell lysis (Fig. 3*B*). As was the case in Fig. 3*A*, SUMO-1 and UBC9 synergistically augmented hypoxia-induced transcription, and there was little difference between wild-type and mutant ARNT. Accordingly, these results suggest that the SUMO-1 conjugation system positively regulates both AHR/ARNT- and HIF1α/ARNT-mediated transcription, independent of the sumoylation of ARNT at Lys^{245}. This finding might be explained by the predominant roles of AHR and HIF1α in these transcription pathways, com-

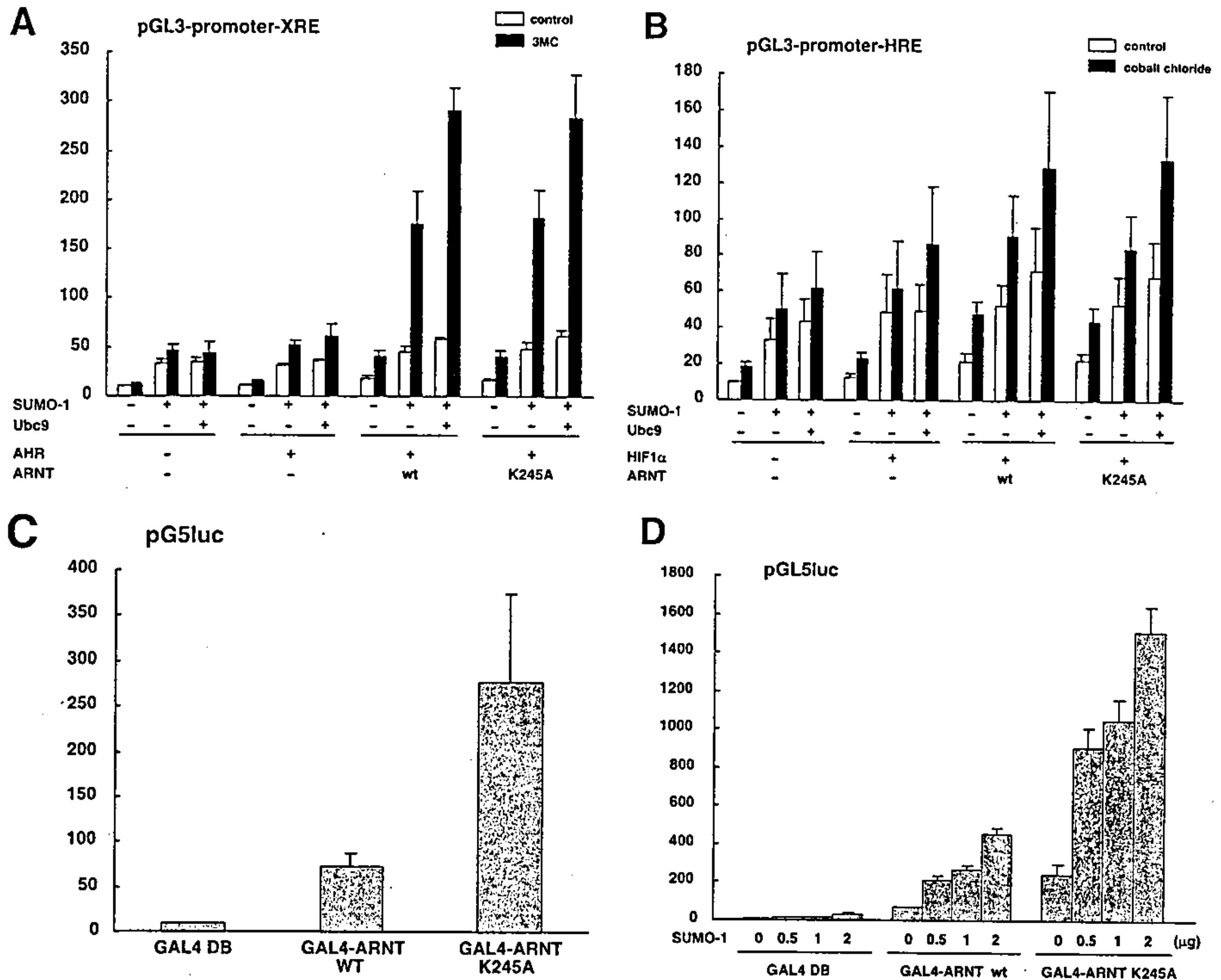

FIG. 3. **Transcriptional role of SUMO-1 conjugation of ARNT.** *A* and *B*, transcription by AHR/ARNT or HIF1α/ARNT is enhanced by SUMO-1 and UBC9, independent of the sumoylation of ARNT. MCF-7 cells were transfected with pGL3-promoter-XRE or pGL3-promoter-HRE (0.5 μg), in which the luciferase gene is driven by six XREs or six HREs and a minimal promoter, respectively, together with plasmids expressing AHR or HIF1α (0.5 μg), ARNT (WT or K245A; 0.5 μg each), SUMO-1 (1 μg), and UBC9 (1 μg). The transfected cells were treated with solvent alone (0.1% Me_2SO; *white bars*) or with 10 μM 3-methylcholanthrene (*3MC*; *black bars*) for 12 h (*A*) and with solvent alone (*white bars*) or with 200 μM cobalt chloride (*black bars*) for 12 h (*B*). At 48 h after transfection, luciferase activities were determined, and the relative luciferase activities with mock vectors in the absence of treatment were normalized to 10. *C*, the enhanced transcriptional capacity of the sumoylation-resistant ARNT mutant. The pG5luc reporter (0.5 μg), which contains Gal4-binding sites and a minimal promoter, was introduced into MCF-7 cells together with a plasmid expressing Gal4-ARNT (WT or K245A) or the Gal4 DBD (*DB*; 1 μg each). At 48 h after transfection, luciferase activities were determined. The relative luciferase activities of the Gal4 DBD were normalized to 10. *D*, the effect of SUMO-1 on the transcriptional activities of wild-type and mutant ARNT. Gal4-ARNT (WT or K245A) or the Gal4 DBD was expressed in MCF-7 cells together with increasing amounts of SUMO-1 (0–2 μg of the vector). The total amount of transfected DNAs was balanced by the addition of mock vector. Values represent the means ± S.D. from at least three independent experiments.

pared with the involvement of ARNT (51, 52). The sumoylated forms of AHR and HIF1α were not detected under our experimental conditions (data not shown).

To demonstrate whether SUMO-1 directly affects the transcriptional activity of ARNT, we used the luciferase reporter plasmid pG5luc, containing Gal4-binding sites and a minimal promoter, together with the expression of ARNT fused to the Gal4 DBD. This enabled us to elucidate the transcriptional activity of ARNT or probably the ARNT/ARNT homodimer without any influence of other proteins. The ability of Gal4-ARNT (WT or K245A) to activate transcription was first tested (Fig. 3*C*). Gal4-ARNT WT increased the luciferase activities by ~7-fold compared with the Gal4 DBD alone. In contrast, Gal4-ARNT K245A stimulated transcription over 3-fold in comparison with wild-type ARNT, suggesting that sumoylation inhibits the transcriptional capacity of ARNT itself. To further investigate the transcriptional role of the sumoylation of ARNT, Gal4-fused wild-type and mutant ARNT were expressed in MCF-7 cells with increasing amounts of SUMO-1 (Fig. 3*D*). The expression of SUMO-1 augmented transcription by wild-type ARNT and ARNT K245A in a dose-dependent manner. However, the transactivating activities of ARNT K245A were significantly higher than those of wild-type ARNT. Taken together, these data suggest that ARNT sumoylation *per se* represses its transcriptional ability and that certain cofactor(s) subjected to sumoylation may be involved in ARNT-related transactivation.

Localization of Part of ARNT in PML Nuclear Bodies—It has been shown recently that the sumoylation of some target proteins results in alteration of their intracellular distribution (11–13). We tested whether sumoylation influences the localization of ARNT in the nucleus. To address this question, we

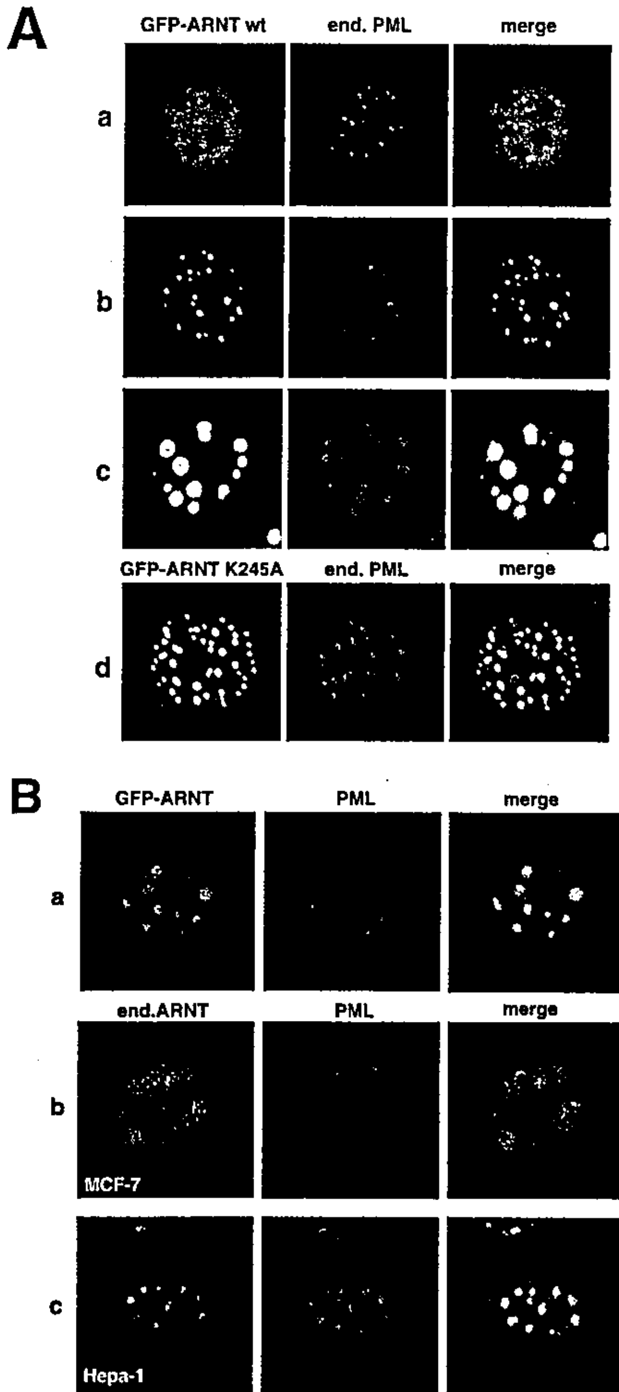

FIG. 4. **Localization of part of ARNT in PML nuclear bodies.** *A*, GFP-ARNT partly colocalizes with endogenous PML. GFP-ARNT WT (*panels a–c*) or GFP-ARNT K245A (*panels d*) (*green*) was transiently expressed in MCF-7 cells, and endogenous (*end.*) PML (*red*) was counterstained by anti-PML antibodies. *B*, overexpressed PML induces translocation of a portion of ARNT into NBs. GFP-ARNT (*green*) and PML (*red*) were coexpressed in MCF-7 cells (*panels a*). PML alone was expressed in MCF-7 cells (*panels b*) or Hepa-1 cells (*panels c*). Endogenous ARNT (*green*) and PML (*red*) were detected by anti-ARNT and anti-PML antibodies, respectively.

generated two plasmids that express wild-type and mutant ARNT as a fusion with green fluorescent protein, termed GFP-ARNT WT and GFP-ARNT K245A, respectively, and transfected them into MCF-7 cells for confocal laser scanning microscopic observation (Fig. 4*A*). Wild-type ARNT showed a few patterns of distribution in the nucleus: a diffuse localization (*panels a*) and discrete nuclear dots (10–50 in each single nucleus) (*panels b* and *c*). However, most of the transfected cells showed the diffuse pattern (*panels a*), whereas the focus formations (*panels b* and *c*) were seen in ~10–30% of the cells. The K245A mutant similarly localized in the nucleus compared with wild-type ARNT (*panels d*) (data not shown). These observations seem to be consistent with a recent report (53).

Because several SUMO-1-modified proteins associate with nuclear domain structures known as PML-NBs (29–34), we investigated whether GFP-ARNT colocalizes with endogenous PML-NBs using immunostaining with anti-PML antibodies (Fig. 4*A*). The foci produced by GFP-fused wild- and mutant ARNT partly colocalized with PML-NBs in MCF-7 cells (*panels b–d*), suggesting the possible relationship of PML with ARNT. Although ARNT was diffusely present in the nucleus (*panels a*), PML is known to localize not only in NBs, but also throughout the nucleus. Next, to investigate whether PML mediates the translocation of ARNT into NBs, PML was expressed in MCF-7 and Hepa-1 cells (Fig. 4*B*). Increased PML induced relatively large sized NBs in transfected cells, leading to the translocation of both GFP-fused and endogenous ARNT into the NBs (*panels a–c*). In the absence of PML overexpression, endogenous ARNT showed a diffuse localization in the nuclei, similar to the frequent case of GFP-ARNT (Fig. 4*A*, *panels a*) (data not shown). Thus, a portion of ARNT colocalized with PML in NBs, but the sumoylation of ARNT appeared not to affect the intracellular distribution in the nucleus. The coexistence of ARNT with PML in NBs suggests the possibility that PML modulates the transcriptional activities of ARNT.

Sumoylation of ARNT Alters Its Ability to Interact with PML—To examine the effect of PML on the transcriptional ability of ARNT, we expressed Gal4-ARNT WT or Gal4-ARNT K245A in combination with PML and SUMO-1 in MCF-7 cells. Luciferase analysis was performed in a similar way as shown in Fig. 3 (Fig. 5*A*). PML increased the transcriptional activities of both wild-type and sumoylation-resistant ARNT. Interestingly, the coexistence of SUMO-1 significantly repressed the effect of PML on transcription by wild-type ARNT, but much less by ARNT K245A. Thus, PML positively regulates ARNT-dependent transcription, and the sumoylation of ARNT at Lys^{245} inhibits the transcriptional role of PML.

The SUMO-1 modification of substrates can affect protein-protein interactions (20, 22). It is of interest that the SUMO-1 acceptor site of ARNT at Lys^{245} resides in the PAS domain, which is required for dimerization with bHLH/PAS partner proteins (3, 42). We therefore examined whether the sumoylation of ARNT affects its ability to dimerize with AHR *in vivo* (Fig. 5*B*). For immunoprecipitation, Myc-tagged AHR was expressed in MCF-7 cells in combination with FLAG-ARNT (WT or K245A), HA-SUMO-1, and HA-UBC9, and the cells were lysed at 48 h after transfection. The Myc precipitates were subjected to Western blot analysis with anti-FLAG antibodies (*right panel*). Unconjugated forms of both wild-type ARNT and ARNT K245A were similarly immunoprecipitated with AHR, and sumoylated ARNT also complexed with AHR in proportion to the original amount in the input lysate. Nonspecific bands reacting to anti-FLAG antibodies were detected between the unconjugated and conjugated forms of ARNT, indicating the presence of equal amounts of cellular proteins in each lane.

To further investigate whether the sumoylation of ARNT affects the interaction of ARNT with PML, we employed a GST pull-down assay (Fig. 5*C*). Bacterially expressed GST-PML (amino acids 5–452) was incubated with whole cell lysates from MCF-7 cells expressing FLAG-ARNT, HA-SUMO-1, and HA-UBC9. The precipitates on glutathione-agarose beads were analyzed by immunoblotting with anti-FLAG antibodies. Interestingly, unconjugated ARNT (WT or K245A) bound PML, but

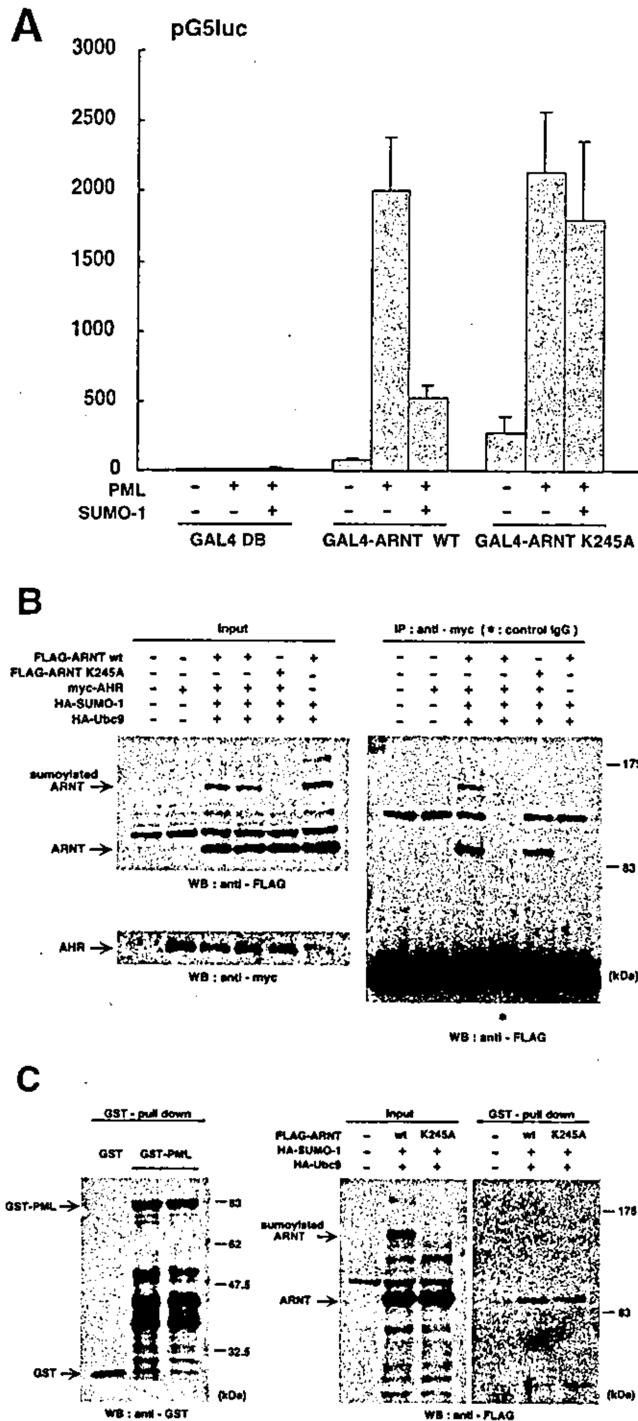

FIG. 5. **Sumoylation of ARNT affects the interaction with PML.** *A*, PML modulates the transcriptional ability of ARNT. MCF-7 cells were transfected with pG5luc (0.5 μg) and Gal4-ARNT WT, Gal4-ARNT K245A, or Gal4 DBD (1 μg) in combination with pCGN-HA-SUMO-1 (1 μg) and/or pcDNA3-Myc-PML (1 μg). The relative luciferase activities with the Gal4 DBD (*DB*) alone were normalized to 10. The values represent the means ± S.D. from at least three independent experiments. *B*, the dimerization of unmodified and sumoylated ARNT with AHR. FLAG-ARNT (WT or K245A), Myc-AHR, HA-SUMO-1, and HA-UBC9 were expressed in MCF-7 cells. Whole cell lysates were probed with anti-FLAG and anti-Myc antibodies (*left panels*), and the Myc immunoprecipitates (*IP*) were examined with anti-FLAG antibodies (*right panel*). *C*, loss of binding of sumoylated ARNT to PML. GST or GST-PML (amino acids 5–452) was incubated with whole cell lysates from MCF-7 cells expressing FLAG-ARNT (WT or K245A), HA-SUMO-1, and HA-UBC9. GST pull-down analysis was performed, and the complexes were probed with anti-GST (*left panel*) and anti-FLAG (*right panels*) antibodies. The whole cell lysates (*input*) were also tested with anti-FLAG antibodies. *WB*, Western blot.

sumoylated ARNT did not associate with PML. Collectively, these data suggest that PML enhances transcription by ARNT and that the sumoylation of ARNT abolishes the interaction with PML to suppress the enhancement of ARNT-mediated transcription by PML.

DISCUSSION

In this study, we have reported that ARNT is a substrate for SUMO-1 modification at the major target Lys^{245} within the PAS domain. UBC9 and PIAS1 stimulated the sumoylation of ARNT when they coexpressed with ARNT and SUMO-1. Because free SUMO-1 is known to be sparsely present in mammalian cells, the increase in SUMO-1 and its conjugating activities facilitated our initial detection of the sumoylated form of ARNT. PIAS1 is a member of the E3-like ligase family, involved in the sumoylation of some target proteins (27, 35). Our data imply that PIAS1 functions as a SUMO-1 ligase of ARNT. Both the ubiquitin and SUMO-1 conjugation systems show very similar enzymatic cascades, but these pathways seem to be different in some aspects discussed below. First, in contrast to ubiquitin ligases, PIAS proteins are not always required for the sumoylation of substrates, and they appear to function as factors that stimulate the SUMO-1 conjugation reaction via UBC9 (54, 55). Overexpressed UBC9 was sufficient for inducing the sumoylation of ARNT in the absence of PIAS1. Second, there is a difference in substrate specificity between ubiquitin and SUMO-1 ligases. Ubiquitin ligases strictly recognize their substrates for ubiquitination, whereas PIAS proteins generously determine the targets. PIAS1 has been reported to mediate the sumoylation of p53 and c-Jun (27, 35) and to interact with the androgen receptor, which is one of the SUMO-1 substrates (56). From our results, there may be two other possible target sites for sumoylation in addition to Lys^{245}, which are unlikely to be located within a sequence matching the consensus motif. SUMO-1 conjugation at these sites may depend on the sumoylation at Lys^{245}. Although many SUMO-modified proteins possess more than one sumoylated site, such coordinated attachment of SUMO-1 molecules to the different target sites has been demonstrated in only a few substrates such as c-Myb. As shown in this study, a relatively small proportion of ARNT was detected in the conjugated form, even when sumoylation was stimulated by UBC9 and PIAS1. This may be explained by the idea that the sumoylation of ARNT is transient and that there is a dynamic equilibrium between SUMO-1-conjugated and -unconjugated forms. Another possibility is that the presence of certain limiting factors affects this sumoylation. We detected a small amount of a SUMO-1-modified form of endogenous ARNT in MCF-7 cells by immunoprecipitation. Sumoylation of some substrates has been shown to be enhanced in the presence of ligand and upon cell treatment (for example, the androgen receptor and heat shock transcription factor-1). The sumoylation of endogenous ARNT might be precisely controlled by certain regulatory mechanisms *in vivo*.

The sumoylation of transcription factors and other related proteins produces diverse influences on their transcriptional activities (11, 23, 24, 26, 28, 29, 57, 58). In our study, the use of the luciferase reporter constructs together with the expression of ARNT (WT or K245A) indicated that the lack of the major sumoylation of ARNT had little effect on AHR/ARNT- and HIF1α/ARNT-mediated transcription. On the other hand, transactivation by Gal4-ARNT was evidently stimulated by the sumoylation-resistant K245A mutation compared with wild-type ARNT. One possible explanation is that the difference in transcriptional activities between wild-type ARNT and ARNT K245A was masked by the involvement of the partner, AHR or HIF1α. In fact, it has been reported that AHR plays a more prominent role than ARNT in AHR/ARNT-mediated transcrip-

tion (51, 52). ARNT is required for transcription by the AHR/ARNT heterodimer, but basically plays a supportive role in this pathway.

Alternatively, SUMO-1 conjugation to ARNT at target site(s) except for Lys^{245}, even if it is infrequent and unstable, may have a sufficient effect on AHR/ARNT- and HIF1α/ARNT-mediated transcription. Furthermore, SUMO-1 and UBC9 synergistically enhanced both AHR/ARNT- and HIF1α/ARNT-mediated transcription, independent of the Lys^{245} sumoylation of ARNT. SUMO-1 also enhanced the transcriptional activities of both Gal4-ARNT WT and Gal4-ARNT K245A in a dose-dependent manner. In other words, the activation of the SUMO-1 conjugation system induces the sumoylation of many substrates as well as ARNT. In agreement with our data, molecules in the SUMO conjugation system, including SUMO-1, UBC9, and PIAS, have been shown to modulate the transcriptional activities of p53, p73α, the androgen receptor, and lymphoid enhancer factor-1, even when they were mutants lacking their major SUMO-1 acceptor site (27, 31, 57, 58). The sumoylation of certain key factor(s) such as PML might be crucial for the general mechanism of transcriptional control. Moreover, it was reported that UBC9 modulates transcription by ETS-1 and TEL independent of its enzymatic ability to conjugate them with SUMO-1 (59, 60) and that UBC9 mediates the nuclear localization of Vsx-1 without its SUMO-1-conjugating activity (61). Thus, UBC9 may possess some other functions in addition to the role of a SUMO-1-conjugating enzyme.

The Lys^{245} target of ARNT resides in the PAS domain, which has been implicated in dimerization with bHLH/PAS members and with interacting molecules such as the transcription factor Sp1 (3, 41, 42). As shown in Fig. 5, sumoylation did not affect the ability of ARNT to dimerize with AHR. This is consistent with the result that the sumoylation of ARNT had little effect on transcription by AHR/ARNT. Recent reports have shown that the SUMO-1 modification of PML, heat shock factor-1 and -2, and lymphoid enhancer factor-1 localizes to nuclear domain structures known as PML-NBs (29–34). In addition, sumoylation leads to the localization of homeodomain-interacting protein kinase-2 and TEL to another type of nuclear subregion (62, 63). These lines of evidence suggest that sumoylation functionally regulates substrate proteins through changing their intracellular distribution. Our observations show that part of ARNT colocalizes with PML or PML-NBs. Furthermore, sumoylation significantly reduced the interaction with PML, resulting in a weakening of the ability of PML to enhance transcription by ARNT. Although the overall distribution of the ARNT K245A mutant was similar to that of wild-type ARNT, we did not exclude the possibility that sumoylated ARNT dislocates from PML-NBs because a small part of ARNT interacting with PML is modified by SUMO-1 in the cells. Thus, the SUMO-1 conjugation of ARNT changes the affinity for PML, but not for AHR. There is a possibility that the association of ARNT with some other proteins may be affected by this modification. In conclusion, our study has shed light on the significant role of the sumoylation of ARNT in modulating its transcriptional roles through affecting the interaction with cooperative proteins.

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