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Ecdysone receptor-dependent gene regulation mediates histone poly(ADP-ribosyl)ation

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Abstract

While the ecdysone dependency of puff formation in giant polytene chromosomes from fly salivary glands has been well documented, the molecular mechanisms underlying this process remain unknown. However, it does appear to involve chromatin remodeling and modification mediated by ecdysone receptor (EcR). As *Drosophila* poly(ADP-ribose) polymerase (dPARP) has recently been reported to be involved in ecdysone-induced puff formation, we decided to test the possible role of dPARP in ligand-induced dEcR transactivation in an insect system. dPARP co-activated the ligand-induced transactivation function of EcR in the insect cell line S2, and appeared to physically interact with EcR in a ligand-dependent manner. ChIP analysis of an EcR target gene promoter revealed ligand-dependent recruitment of dPARP with poly(ADP-ribosyl)ation of histones in the EcR binding site and, surprisingly, also in a distal region of the promoter. Our results indicated that EcR-mediated gene regulation may be coupled with chromatin modification through poly(ADP-ribosyl)ation.

Keywords: Ecdysone receptor; Poly(ADP-ribose) polymerase; Poly(ADP-ribosyl)ation; Puff; Co-activator

In vertebrates, nuclear receptors (NRs) serve as critical regulators in many tissues, during both developmental and adult stages, through the transcriptional control of target genes. NRs form a gene superfamily across species and function as ligand-inducible DNA sequence-specific regulators. Most NRs are transcriptionally activated by their cognate ligands, while some NRs, called orphan receptors, are thought to have no endogenous ligands but instead promote constitutive transcription. Like other sequence-specific regulators, NRs require a number of nuclear complexes to exert transcriptional control [1–3]. One class of nuclear complexes contains components that can covalently modify histone tails through acetylation, phosphorylation, methylation, or other protein modifications [4]. One of the best characterized histone-modifying complexes is the p300/CBP and p160 histone acetyltransferase (HAT) complex [5–8]. Although not all the components of this complex have been biochemically identified, it is known that HAT complexes include p300/CBP and three p160 family members. This HAT complex, along with the GCN5/TRRAP HAT complex, are thought to be common co-activators of NR function [9], at least in mammals. Another significant class of nuclear complex is the ATP-dependent chromatin remodeling complex [10], as nucleosome arrays in NR target gene promoters are generally inhibitory to specific DNA binding and consequent activation of gene expression by NRs. Indeed, several chromatin remodeling complexes have recently been shown to both directly and indirectly assist in NR transcriptional control [11,12]. Although it is believed that the co-operative functions of these two nuclear complex classes allow the NR-mediated control target of

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gene transcription, the extent of interaction between nuclear complexes remains largely unknown at the molecular level.

Drosophila puff, local decondensed chromatin structure, in the giant polytene chromosome of the salivary glands is presumed as a best model to address the issue above in terms of a fly NR ecdysone receptor (EcR) function, since its endogenous ligand, ecdysone, is well described to induce puff [13–15], presumably through EcR in larvae approaching pupation [16–19]. While this ecdysone-induced puff formation clearly requires chromatin remodeling and modification, most of the factors and complexes involved in this process have not yet been characterized, despite the similarities in NR and co-regulator functions between insects and vertebrates. Recently, *Drosophila* poly(ADP-ribose) polymerase (dPARP) was reported as a candidate factor, with the poly(ADP-ribosyl)ation of chromatin proteins by dPARP appearing to be crucial for puff formation [20–22]. However, the functional relationship between dPARP and EcR has not yet been established [23,24].

Therefore, in the present study we decided to examine the possible role of dPARP in EcR function in insect cells. We report that dPARP co-activates the ligand-induced transactivation function of EcR through functional association with EcR bound to an EcR target gene promoter, presumably through histone poly(ADP-ribosyl)ation [20].

Materials and methods

Plasmids. Full-length cDNA of EcR-B1, USP, dPARP, and *Renilla* luciferase (RL) (Promega) were inserted into pAct5 to give protein expression under the control of the *Drosophila* actin 5C promoter. Four copies of hsp27 EcRE were inserted into the promoter of the luciferase pGL3-basic vector (Promega) to generate EcRE-TATA-Luc [25,26].

Transfection and luciferase assay. Drosophila embryonic Schneider (S2) cells were cultured in Schneider's *Drosophila* medium, supplemented with 10% charcoal-stripped fetal calf serum. S2 cells were transfected with the indicated plasmids (0.5 μg EcRE-TATA-Luc, 0.05 μg EcR-B1, USP, and 0.025 μg or 0.125 μg dPARP) using SuperFect Transfection Reagent (Qiagen) in 12-well petri dishes. Total amounts of cDNA were adjusted by supplementing with empty vector up to 1.0 μg. Cells were treated with the synthetic EcR ligand Muristerone A (2.5×10^{-7} M) for 16 h. Luciferase activity was determined using the Luciferase Assay System (Promega) [27]. As a reference plasmid to normalize transfection efficiency, 0.2 ng pAct5-RL plasmid was cotransfected in all experiments. Results were calculated as means ± SD from at least three independent experiments.

RNAi. For RNAi, gene-specific primer pairs were designed to amplify dPARP that also incorporated the binding sites for 5′ T7 RNA polymerase (5′-TAATACGACTCACTATAGGGTACTGCTGAGT ATGCAAGAAC-3′ and 5′-TAATACGACTCACTATAGGGTACT ATCGCGAAACCTGAAG-3′) or for the transcriptional coactivator *Taiman* (5′-TAATACGACTCACTATAGGGTACTATGTCAATT GCTGCAG-3′ and 5′-TAATACGACTCACTATAGGGTACTCAG ATTGACCTTTGAATC-3′). PCR products were purified and quantified by OD 260 and used to generate dsRNA using the T7 MEGAscript kit (Ambion). RNA was resuspended in water and added directly to Effectene Transfection Reagent (Qiagen) mixture [28]. S2 cells were transfected with dsRNA (8 μg for each gene targeted) in 100 mm dishes followed by incubation for ~48 h to allow protein turnover and degradation of targeted mRNA.

Immunoprecipitation. Whole cell extracts were used for immunoprecipitation with anti-FLAG M2 resin (Sigma) followed by Western blotting with anti-FLAG (Immunology Consultants Laboratory) or anti-PARP (Neo Markers) antibody. For immunoprecipitation of overexpressed proteins, cells were transfected as indicated with FLAG-tagged EcR-B1 (5 μg) and dPARP (3 μg) in the presence or absence of Muristerone A (2.5×10^{-7} M) [29].

Chromatin immunoprecipitation. Soluble chromatin from S2 cells was prepared using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology) and immunoprecipitated with antibody against EcR (DDA2.7 from DSHB), poly(ADP-ribosyl)ated proteins (anti-PAR from Trevigen) or acetyl-histone H3 (anti-AcH3 from Upstate Biotechnology). Specific primer pairs were designed to amplify the promoter (5′-AAAGAACGGCAAACATGAGG-3′ and 5′-CTTC TGGCTCTTTCTAGCACAG-3′) and upstream (5′-GTTTTTCTGG TTGGTTATGGG-3′ and 5′-CCTTGACTGAAGTTCCTTAC-3′) regions of the *hsp27* gene and a microsatellite loci (ac004640: 5′-CCGTA AGCCCATAAGCGTAA-3′ and 5′-GGCTACGGCTAGAGTTCG TG-3′) [30] from extracted DNA. PCR conditions were optimized to allow semiquantitative measurement on 2% agarose/Tris–acetate–EDTA gels [9]. Cells were usually treated with 2.5×10^{-7} M Muristerone A for 2 h.

Results

Drosophila PARP acts as an ecdysone receptor co-activator

While PARP has been reported to act as a co-repressor for nuclear receptors in human cells [31], it remained unknown whether PARP functions as a co-regulator with respect to nuclear receptor transactivation function in *Drosophila*. Therefore, we first assessed whether *Drosophila* PARP modulated the ligand-induced transactivation function of EcR. Co-regulatory activity on EcR was tested in a transient expression assay that used a luciferase reporter gene containing a consensus EcRE in the promoter transfected into S2 cells. dPARP potently co-activated the ligand-induced transactivation function of EcR in the presence of the EcR synthetic ligand Muristerone A (Fig. 1A, lanes 5 and 6), but did not alter basal transcription levels in the absence of Muristerone A (Fig. 1A, lanes 2 and 3). PARP-induced transactivation was significantly attenuated in the S2 cells transfected with dsRNA for dPARP RNAi (Fig. 1A, lanes 4 and 7). *Drosophila Taiman*, a homologue of human AIB1, one of the three p160 HAT members, has been demonstrated genetically to be a major EcR co-activator, and indeed *Taiman* RNAi attenuated the ligand-induced transactivation function of EcR (Fig. 1A lanes 4 and 8). We then tested whether *Taiman* co-regulated ligand-induced EcR transactivation along with dPARP. As *Taiman* RNAi did not reduce the dPARP co-activator activity (Fig. 1B, lanes 5

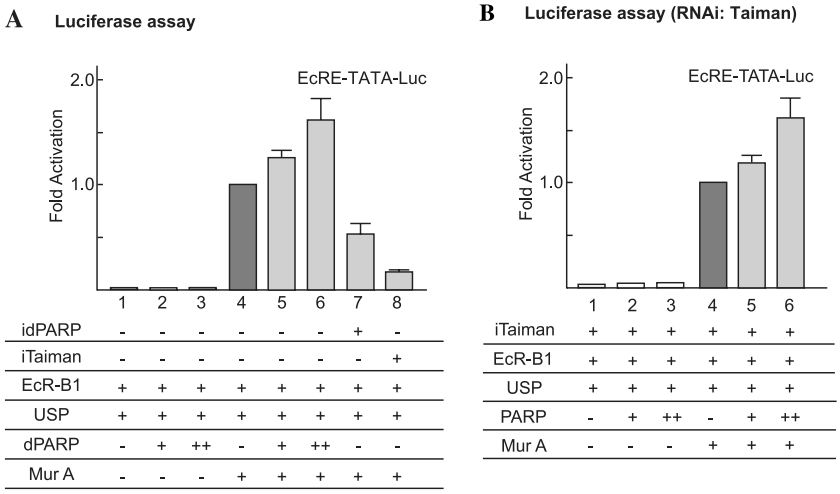

Fig. 1. Co-activation of liganded EcR function by dPARP. (A) Enhanced ligand-induced transactivation function of EcR in S2 cells expressing dPARP. Cells were transfected with the indicated plasmids (0.5 μg EcRE-TATA-Luc, 0.05 μg EcR-B1, USP, and 0.025 or 0.125 μg dPARP). For RNAi, cells were transfected with dsRNA (0.15 μg for each gene targeted) in 12-well petri dishes followed by incubation for ~48 h. Cells were then incubated for 16 h in the presence or absence of synthetic EcR ligand Muristerone A (Mur A) (2.5×10^{-7} M) and assayed for luciferase activity. Results are given as means ± SD of at least three independent experiments. (B) RNAi against *Taiman* had no effect on ligand-dependent EcR transactivation function.

and 6), it appears likely that the co-activation of dPARP on EcR-mediated gene activation was independent of *Taiman*.

dPARP associates with ecdysone receptor in a ligand-dependent manner

Based on our findings that dPARP appeared to play a role in ligand-induced transactivation, we examined the physical interaction between dPARP and EcR using a co-immunoprecipitation assay in *Drosophila* S2 cells. dPARP co-immunoprecipitated with EcR, but only in the presence of EcR ligand (Fig. 2A). Reflecting the luciferase assay findings that dPARP co-activated EcR function independent of *Taiman* function, ligand-dependent interaction between EcR and dPARP was not affected by *Taiman* RNAi (Fig. 2B).

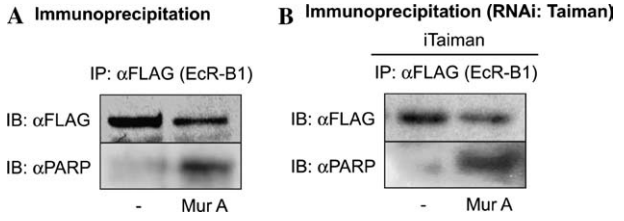

Fig. 2. Ligand-dependent interaction of dPARP with EcR. (A) Muristerone A-dependent interaction of PARP with EcR in S2 cells. For immunoprecipitation (IP) with mouse anti-FLAG antibody, cells were cotransfected as indicated with FLAG-tagged EcR-B1 (5 μg) and PARP (3 μg) and cultured in the presence or absence of Mur A (2.5×10^{-7} M) for 2 h. (B) RNAi against *Taiman* had no effect on ligand-dependent interaction of dPARP with EcR. For RNAi, S2 cells were transfected with dsRNA (8 μg for each gene targeted) in 100 mm dishes followed by incubation for ~48 h.

PARP is recruited to endogenous EcR-target genes

To test whether dPARP is indeed recruited to EcR bound to ecdysone response elements (EcREs) in target gene promoters in vivo, we performed a ChIP assay using an endogenous *Drosophila hsp27* gene promoter, known to be an EcR target gene. In agreement with previous reports, 2 h after the addition of ligand, EcR

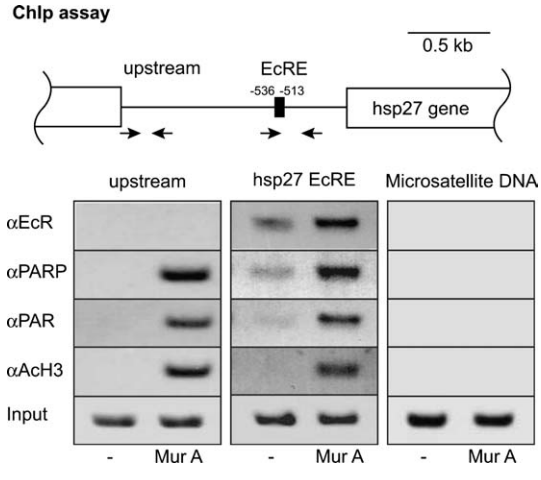

Fig. 3. Ligand-dependent recruitment of dPARP to EcR and accumulation of poly(ADP-ribose) polymers on the EcR target gene promoter. Muristerone A-dependent recruitment of EcR/PARP complexes bound on the *hsp27* gene promoter in S2 cells. For ChIP analysis, soluble chromatin prepared from S2 cells treated with or without Mur A (2.5×10^{-7} M) for 2 h was immunoprecipitated with the indicated antibodies. An anti-PAR antibody immunoprecipitates poly(ADP-ribosyl)ated proteins. Final DNA extracts were amplified using specific primer pairs to detect the *hsp27* gene promoter, the upstream region of the *hsp27* gene promoter, or a microsatellite loci.

was recruited to the EcRE of the *Drosophila hsp27* gene, but not to an upstream region (about 1 kb upstream relative to EcRE) or to a microsatellite loci, as a negative control (Fig. 3). We also found that dPARP was recruited to the EcRE in the *hsp27* promoter in a ligand-dependent manner. Surprisingly, ligand-induced association of dPARP was also detected in the distal upstream region that did not associate with EcR. Thus, we concluded that PARP was recruited to the EcR in a ligand-dependent manner and then appeared to be sequentially recruited to the promoter region by an as yet unknown mechanism.

Discussion

Similar to mammals, the *Drosophila* homologue of p160/AIB1, *Taiman* (TAI), is known to be a coactivator of EcR [32]. TAI potentiates the ligand-induced transactivation function of EcR and interacts with EcR only in the presence of ligand. Our results showed that dPARP also appeared to act as an EcR co-activator based on its ability to potentiate ecdysone-inducible transcription (Figs. 1A and B) and ecdysone-dependent binding to EcR (Fig. 2A). Furthermore, the co-activation function of dPARP and association between dPARP and EcR did not require TAI (Figs. 1C and 2B). Taken together, our results indicated that dPARP may form part of a complex separate from the HAT co-activator p160/TAI complex. As dPARP was recruited to the *hsp27* promoter through ligand-induced association with EcR, dPARP may serve as an EcR co-activator to allow the clustered gene expression of a number of heat shock proteins and other ecdysone-inducible genes during puff formation [33].

Our experiments showed that dPARP physically bound to EcR in a ligand-dependent manner and resulted in the induction of chromatin poly(ADP-ribosyl)ation of the EcR target promoter. However, to our surprise, this EcR ligand-induced poly(ADP-ribosyl)ation was also detected at a promoter region distal from the EcR binding site. Together with the findings of ligand-dependent association between dPARP and EcR, it appears that once dPARP is recruited to EcR bound to specific DNA elements in EcR target gene promoters, sequential poly(ADP-ribosyl)ation may take place from EcR binding sites to more distal promoter regions. As puff formation is likely to involve numerous histone tail modifications [32,34,35], it is likely that poly(ADP-ribosyl)ation plays a significant role in EcR-mediated histone modification [22], although it is unclear at this stage whether poly(ADP-ribosyl)ation triggers or follows the other histone modifications. This issue will be addressed by evaluating the timing of recruitment of histone modifying complexes to EcR target gene promoters in future experiments.

In conclusion, during transient activation of gene expression in response to environmental stimuli, such as fat-soluble ligands that activate NR function [36], we propose that PARP is recruited by sequence-specific regulators to decondense chromatin into an active state for transcriptional control through dissociation of nucleosomes by poly(ADP-ribosyl)ation of histones [20,37,38]. Such decondensation of nucleosomal arrays is likely to enable basal transcription machinery accessible to transcribe target genes.

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