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Multidomain sumoylation of the ecdysone receptor (EcR) from *Drosophila melanogaster*

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ABSTRACT

The 20-hydroxyecdysone receptor (EcR) is a transcription factor belonging to the nuclear receptor superfamily. Together with the ultraspiracle nuclear receptor (Usp) it coordinates critical biological processes in insects such as development and reproduction. EcR and its ligands are used in commercially available ecdysone-inducible expression systems and are considered to be artificial gene switches with potential therapeutic applications. However, the regulation of EcR action is still unclear, especially in mammals and as far as posttranslational modifications are concerned. Up until now, there has been no study on EcR sumoylation. Using bioinformatic predictors, a Ubc9 fusion-directed sumoylation system and mutagenesis experiments, we present EcR as a new target of SUMO1 and SUMO3 modification. Our research revealed that EcR undergoes isoform-specific multisumoylation. The pattern of modification remains unchanged in the presence of the ligand and the dimerization partner. The SUMO acceptor sites are located in the DNA-binding domain and the ligand-binding domain that both exhibit structural plasticity. We also demonstrated the existence of a sumoylation site in the F region and EcRA-A/B region, both revealing characteristics of intrinsically disordered regions. The consequences of modification and the resulting impact on conformation and function may be especially crucial for the disordered sequences in these two areas. The isoform-specificity of sumoylation may explain the differences in the transcriptional activity of EcR isoforms.

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1. Introduction

During the life of *Drosophila melanogaster* particular developmental events such as molting, puparium formation, and metamorphosis are regulated by the ecdysteroid 20-hydroxyecdysone (20E). 20E binds directly to a transcription factor, the ecdysone receptor (EcR, NR1H1), which is a member of the nuclear receptor (NR) superfamily [1]. Three EcR isoforms (EcRA, EcRB1 and unique for *Drosophila* EcRB2) are produced from the *EcR* gene by the use of two promoters and alternative splicing [2]. Like other nuclear receptors, EcR exhibits a characteristic modular structure comprised of six regions marked with letters A to F. Some of these regions constitute an autonomous functional domain: the DNA-binding domain (DBD) or the ligand-binding domain (LBD), which are common for all EcR isoforms, or the A/B region, which is distinct for each isoform (see Fig. 1A for more details). The EcR isoforms exhibit various temporal and spatial expression patterns. This may be an explanation for the diversity of cellular responses to ecdysteroids [2–8]. In general, there are high levels of the expressed EcRB1 isoform in larval cells. In contrast, the EcRA isoform is correlated with tissues that eventually make up the adult structures [2]. Moreover, the A/B region of EcRA shows weaker transactivation activity in comparison with the strongest activator, EcRB1, but the molecular cause of these differences is unknown [5–7].

Each of the EcR isoforms is capable of forming a heterocomplex with Ultraspiracle protein (Usp, NR2B4) [9], another member of the NR superfamily and an orthologue of the vertebrate retinoid X receptor (RXR, NR2B3) [10]. Although EcR binds 20E alone [11], ligand binding is greatly enhanced by dimerization with Usp [9]. Therefore, the transcriptionally active EcR/Usp heterodimer is generally thought to be the only functional form of the 20E receptor [4]. Because of this characteristic EcRs are related rather to the vertebrate family of receptors heterodimerizing with RXR than to steroid hormone receptors (NR3).

Abbreviations: 20E, 20-hydroxyecdysone; DBD, DNA-binding domain; EcR, ecdysone receptor; HEK293, human embryonic kidney 293 cells; ID, intrinsically disordered; LBD, ligand-binding domain; MS, mass spectrometry; NR, nuclear receptor; RXR, retinoid X receptor; SUMO, small ubiquitin-like modifier; UFDS, Ubc9 fusion-directed sumoylation; Usp, ultraspiracle.

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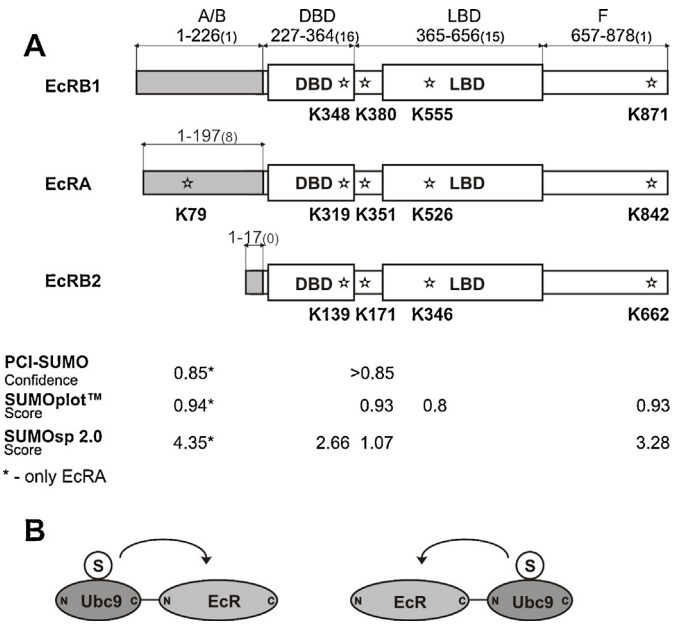

Fig. 1. Sumoylation of EcR from *Drosophila melanogaster*.
(A) Location of SUMO modification sites that were predicted by bioinformatic tools: PCI-SUMO, SUMOplot™, SUMOsp 2.0 with the highest confidence level or scores. Because all three isoforms of EcR from *Drosophila melanogaster* (EcRB1, EcRA and EcRB2) have a common F region with the DNA-binding domain (DBD) and the ligand-binding domain (LBD), sumoylation sites predicted in these regions are equivalent. The EcRA isoform possesses an additional potential sumoylation site in the A/B region which is absent in the EcRB1 and EcRB2 isoforms. The numbers above the arrows refer to amino acid residues in the respective EcR isoform that were chosen by dividing the protein sequence into fragments analyzed in the UFDS system. The numbers in brackets refer to the amount of all the K residues in each fragment. (B) The principle of UFDS of EcR. The fusion of Ubc9 to the N-terminus (on the left) or C-terminus (on the right) of the protein (here EcR) induces the ligase independent sumoylation of the protein in the Ubc9 fusion-directed sumoylation (UFDS) method [31]. S refers to SUMO.

Interestingly, several laboratories have demonstrated that EcR can also function in mammalian cell lines which are deficient in ecdysteroid-like ligands, and for this reason EcR is considered to be a good source of elements for ecdysteroid inducible gene switches [12–15]. In such systems proteins would be produced after induction by EcR ligands bound to its LBD. Then, thanks to fused activation domain, transcription process would be initiated. These switches are believed to possess potential usage in mammals for ecdysone-regulated expression of therapeutic gene, especially since ecdysteroids do not bind to vertebrate steroid receptors [12]. Unfortunately, EcR action is still poorly understood. In particular, nothing is known about its regulation by posttranslational modifications in mammalian cells, and therefore, the application of EcR-based molecular switches in gene therapy is currently limited. For many vertebrate NRs, the dramatic impact of covalent modifications such as phosphorylation, acetylation, ubiquitylation and sumoylation have been described in recent years [16 and references therein]. Sumoylation is a protein conjugation resembling ubiquitylation, based on the reversible attachment of the small ubiquitin-like modifier protein (SUMO), that plays a crucial role in the regulation of many cellular proteins including NRs [17–19 and references therein]. SUMO proteins are expressed in every eukaryotic organism. Some organisms have a single SUMO gene, others, such as plants and vertebrates, possess several SUMO genes [18]. The process of sumoylation begins with SUMO maturation, where SUMO-specific isopeptidases cleave the SUMO precursor at its C terminus [20]. Then, the mature SUMO is activated in an ATP-dependent manner by the E1 activating enzyme, the AOS1-UBA2 complex [21–24]. Next, SUMO is transferred to the E2 conjugating enzyme Ubc9 [25,26] and finally, Ubc9 transfers SUMO to the acceptor protein directly or supported by SUMO E3 ligases [27–29]. The process culminates in the formation of an isopeptide bond between the C-terminal glycine residue of SUMO and the ε-amino group of the K residue of the target protein [18]. The conjugation may be reversed owing to the action of SUMO-specific proteases [20]. The molecular consequences of sumoylation depend on the function of the target protein and may affect intercellular localization, stability and activity. SUMO conjugation can interfere with the interaction between other molecules, such as a substrate or protein partner. SUMO can also create a new binding surface by inducing conformational changes or recruit other binding partners [19].

Despite the fact that all the key elements involved in the sumoylation pathway have been studied and many targets have been identified in organisms such as *Drosophila* [30 and references therein], to our knowledge, there is no experimental data about the sumoylation of EcR in *D. melanogaster*, or more importantly, in mammalian cells, which would provide a clue to the role of EcR action in the potential gene therapy mentioned above. Sumoylation of EcR could potentially interfere its protein–protein interaction surfaces and alter its activity [18]. In this paper we present our research on EcR modification by SUMO obtained with the Ubc9 fusion-directed sumoylation system (UFDS) [31]. This method is ligase-independent and does not require any additional stimulation to strongly enhance the sumoylation process in the cell. UFDS was developed to increase the amount of the sumoylated fraction of the protein of interest in the cell, and thus to facilitate the investigation of protein modification. The method was also used to discover new sumoylation targets [32]. All experiments were performed in the mammalian cell line HEK293, as a model for the cellular environment of commercially available ecdysone-inducible expression systems or the future therapeutic artificial expression factors previously mentioned. The experimental results obtained, together with *in silico* predictions, both indicate that EcR from *D. melanogaster* may undergo isoform specific sumoylation within all domains. These results are quite surprising, since the majority of NRs have only a distinct region or regions involved in sumoylation, as for example in Usp sumoylation sites present in the A/B region and the LBD [33]. The sumoylation pattern of EcR is not affected by the presence of the ligand and Usp, the natural dimerization partner of EcR. Our findings provide valuable insight into the EcR regulation molecular mechanism and set the stage for a new field of research, particularly with regard to the consequences of the posttranslational modifications.

2. Materials and methods

2.1. Sumoylation site prediction

Analysis of the EcR amino acid sequence from *D. melanogaster* (accession number: NP_724460) was performed using three bioinformatic tools: SUMOplotTM, SUMOsp 2.0 and PCI-SUMO. The SUMOplotTM Analysis Program (http://www.abgent.com/doc/sumoplot2) is a predictor evaluating the probability for the SUMO consensus sequence to be involved in SUMO attachment. The SUMOplot™ score system matches directly amino acid residues to the consensus sequence and substitute them with amino acid residues with similar hydrophobicity. PCI-SUMO [34] (http://bioinf.sce.carleton.ca/SUMO/start.php) uses parallel cascade identification with no assumptions regarding either the subcellular localization or the presence of a sequence motif. SUMOsp 2.0 [35] (http://sumosp.biocuckoo.org/prediction.php) is a web server using a training data set from scientific literature. All

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