Bag-1M inhibits the transactivation of the glucocorticoid receptor via recruitment of corepressors

Wei Hong\textsuperscript{a,*}, Aria Baniahmad\textsuperscript{b,*}, Juan Li\textsuperscript{a}, Chenglin Chang\textsuperscript{a}, Weizhen Gao\textsuperscript{a}, Yunde Liu\textsuperscript{a}

\textsuperscript{a}Department of Laboratory Medicine, Tianjin Medical University, 300203 Tianjin, China
\textsuperscript{b}Institute for Human Genetics and Anthropology, Jena University Hospital, 07740 Jena, Germany

\textbf{A B S T R A C T}

The Bcl-2 associated athanogene 1M (Bag-1M) is known to repress the transactivation of the glucocorticoid receptor (GR). We report here that Bag-1M inhibits the action of GR via recruitment of corepressors, including nuclear receptor corepressor (NcoR) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), and histone deacetylase (HDAC)\textsubscript{3} to the genomic response element of a glucocorticoid-regulated human metallothionein IIa (hMTIIa) gene. A mutant GR lacking the interaction with BAG-1M fails to recruit the corepressors NcoR and SMRT. RNAi-mediated knock down of corepressors and the use of HDAC inhibitor relieved Bag-1M-induced repression on the transactivation of the GR. In addition, Bag-1M is not involved in the degradation of the receptor. These findings indicate a novel mechanism by which Bag-1M acts as a corepressor and downregulates the activity of the GR.

\textbf{Structural summary:}

MINT-7216164: HDAC\textsubscript{3} (uniprotkb:O15379) physically interacts (MI:0914) with Bag1 (uniprotkb:Q99933) by anti-bait co-immunoprecipitation (MI:0006)
MINT-7216183: NCOR (uniprotkb:O75376) physically interacts (MI:0914) with Bag1 (uniprotkb:Q99933) by anti-bait co-immunoprecipitation (MI:0006)
MINT-7216175: SMRT (uniprotkb:Q9Y618) physically interacts (MI:0914) with Bag1 (uniprotkb:Q99933) by anti-bait co-immunoprecipitation (MI:0006)

\textcopyright 2009 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

The Bcl-2 associated athanogene 1 (Bag-1) proteins are cochaperones that are involved in the regulation of nuclear receptor action [1–5]. In human, the Bag-1 gene encodes four isoforms of the Bag-1 proteins (Bag-1L, Bag-1M, Bag-1S and p29), all of which are expressed through alternative translation initiation sites from the same mRNA [6]. Although it was initially identified as a Bcl-2 binding protein to suppress apoptosis [7], Bag-1 has been known to interact with and regulate the activity of other proteins. For example, Bag-1 interacts with and inhibits the function of the tumor suppressor p73 [8]. Furthermore, the medium isoform, Bag-1M is increased in the hippocampus of Alzheimer’s disease patients and binds to Tau protein and amyloid precursor protein (APP), and overexpressed Bag-1M induces increased level of Tau and APP that are related with the pathology and treatment of Alzheimer disease [9,10].

The different Bag-1 proteins exert varying effects on the transactivation function of nuclear receptors. The largest isoform, Bag-1L, enhances the transactivation of androgen receptor (AR) [11] but inhibits the action of glucocorticoid receptor (GR) [12], whereas, Bag-1M, downregulates the transactivation of GR [13]. Recent study revealed that Bag-1M localized to the glucocorticoid response element (GRE) in a hormone sensitive manner and repressed the DNA binding by the GR [14]. This provides another evidence that molecular chaperones and cochaperones can modulate the nuclear receptor action at the genomic response element apart from the previous finding that molecular chaperone p23 was present at the response element in the presence of hormone and disrupted receptor-mediated transcriptional activation [15].
Moreover, the Bag-1 co-chaperone also affects the protein level of the GR through its ubiquitin-like domain that is involved in the degradation of GR in a hormone dependent manner in the presence of another co-chaperone chromatin C-terminus of heat shock protein (Hsp) 70 interacting protein (CHIP) [16].

Glucocorticoid regulates the expression of target genes involved in many pathophysiological processes. A human metallothionein IIa (hMTIIa) gene has been identified and used as a model to investigate the action of GR [17]. Although heavy metal cations are the most potent inducers of MT in mammals, other agents also initiate increases in MT expression [18]. For example, glucocorticoid transactivates the expression of hMTIIa gene through the GR that binds to the GRE located upstream of the gene [17].

Here, we report that Bag-1M is not involved in the degradation of GR through the ubiquitin–proteosome pathway as a potential mechanism for down-regulation of GR activity. Rather, our data suggest that Bag-1M recruits corepressors and histone deacetylase (HDAC), in a hormone dependent manner, to the GR genomic response element of the hMTIIa gene. In line with this, knock-down of corepressors and inhibition of HDAC activity reversed Bag-1M-mediated negative effect.

2. Materials and methods

2.1. Cell culture

COS-7 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum at 37 °C and in an atmosphere of 5% CO2. All culture media contained 100 units/ml penicillin and 100 μg/ml streptomycin.

2.2. Plasmid constructs

The plasmids expressing the wild-type human GR, the mutant GR, Bag-1M, the indicator plasmid pGL3MMTV, control plasmid pRenilla-luc and the hMTIIa reporter plasmid H1S CAT have previously been reported [14]. pEGFP-C2 was commercial available from Clontech, Palo Alto, CA.

2.3. Antibodies

For ChIP assay, GR (P-20), Bag-1 (FL-274), nuclear receptor corepressor (NcoR) (H-303), silencing mediator for retinoid acid and thyroid hormone receptor (SMRT) (C-19) and HDAC3 (H-99) antibodies and rabbit IgG and goat IgG (all from Santa Cruz Biotechnolog, Santa Cruz, CA) were used. The following antibodies were used for immunoprecipitation: Bag-1 (FL-274), NcoR (H-303), SMRT (C-19), HDAC3 (H-99), all from Santa Cruz. For immunoblot (IB), Bag-1 (C-16, Santa Cruz), Actin (1-19, Santa Cruz), SMRT (N-20, Santa Cruz) and NcoR (06-892, Upstate) antibodies were employed.

2.4. Transfection assay and luciferase reporter gene assay

COS-7 cells were transiently transfected with plasmid constructs using FuGene 6 reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. The efficiency of transfection was determined as the percentage of green fluorescent cells following cotransfection of pEGFP-C2, a mammalian expression plasmid encoding for enhanced green fluorescent protein. It remained stable throughout the reported experiments at a level of 75–80%. The reporter gene assay has previously been described [14]. In this assay, three independent experiments were performed and the data were analyzed, by a software for the student’s t-test, to calculate the probability value (P-value) to check, whether or not, a significant difference exists between two groups of data obtained under conditions of different treatments.

2.5. Immunoprecipitation and IB

The transiently transfected and dexamethasone treated COS-7 cells were further treated with 1 mM dimethyl 3,3’-dithiobispropionimidate (Pierce Biotechnology, Rockford, IL) for 30 min to cross-link the protein. Cells were lysed in buffer (0.5% NP-40, 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol) and preincubated with protein A sepharose (Amersham Bioscience, Shanghai, China). Bag-1, NcoR, SMRT or HDAC3 antibodies were used for immunoprecipitation and IB was followed with Bag-1 antibody.

2.6. ChIP and re-ChIP

ChIP and re-ChIP were performed as previously described [19]. For ChIP in transfected COS-7 cells, 10% of the precleared supernatant was taken out as input. The PCR reactions of input and immunoprecipitated material were run with 28 cycles. The primers used for the PCR amplification are (in 5′–3′ direction): CCG GTT ACT GTG ATG CTC GA (hMTIIa for); GCG GGA GGA CAC AGT GTA CC (hMTIIa rev). For re-ChIP, 20% of the elute after the first precipitation but before the second precipitation was obtained as input. The PCR reactions of input and immunoprecipitated material were run with 32 cycles. For ChIP in HeLa cells, 20% of the precleared supernatant was used as input and the PCR reactions were run with 28 cycles.

2.7. RNA interference

NcoR small interfering RNA (siRNA) (M-003518-01), SMRT siRNA (M-020145-02) and the luciferase GL3 siRNA used as control, were all purchased from Dharmacon. Transfection of COS-7 and HeLa cells with the siRNA was performed using an Oligofectamine reagent (Invitrogen, Shanghai, China) at a final concentration of 150 nM. The cells were finally collected for measurement of luciferase activity, real-time PCR and immunoblot.

2.8. Real-time PCR

The primers (in 5′–3′ direction): TCG GAT ACG TCA TCA GCA CC (for) and TCC CTC CTG TCG TGT ACT CGA (rev) were used for the detection of hMTIIa gene expression. Human β-actin gene expression was detected as a control by the primers: TCA CCC ACA CTG TGC CCA T (for) and CTC TTT CTC GAA GTC CAG GG (rev). The ABI PRISM 7000 sequence detection system and the SYBR Green PCR Master Mix (Applied Biosystem, Shanghai, China) and 40 cycles were used for the amplification. Three independent real-time PCRs were run and the data were analyzed, by a software for the Student’s t-test, to calculate the P-value to check, whether or not, a significant difference exists between two groups of data obtained under conditions of different treatments.

3. Results and discussion

3.1. Bag-1M recruits corepressors and HDAC

Since we have previously shown that Bag-1M and GR are present at the GRE of a glucocorticoid-regulated human metallothionein IIa (hMTIIa) gene [14], it is worthwhile to investigate how Bag-1M alters the action of the GR complex at the GRE. ChIP was therefore carried out to identify the possible factors that are involved in the GR complex. COS-7 cells that are devoid of GR and Bag-1M [12] were transfected with an hMTIIa reporter gene. In addition, Bag-1M, the wild-type GR or a mutant GR (glucocorticoid
receptor K496L/I497G mutant (GRmt), which is unable to interact with Bag-1M but mediates hormone response [21], were cotransfected with the reporter plasmid. No protein was found to bind the GRE of the hMTIIa gene, in the absence of hormone but in the presence of Bag-1M, as shown by no detectable amplified DNA fragment (Fig. 1A). When the cells were only transfected with either the wild-type GR or the mutant GR, the receptors were present at the GRE after hormone treatment (Fig. 1A). In the cells cotransfected with vectors coding for Bag-1M and GR, both factors bind to the GRE in the presence of hormone (Fig. 1A). Intriguingly, the presence of Bag-1M and the wide type GR at the GRE lead to the recruitment of the endogenously expressed corepressors, including NcoR and SMRT, as well as histone deacetylase3 (HDAC3). However, in the cells cotransfected with Bag-1M and GRmt, only GRmt was present at the GRE, indicating Bag-1M is recruited to the response element by interacting with specific amino acid sequences of the GR (Fig. 1A), and suggests that NcoR, SMRT and HDAC3 require BAG-1M for recruitment to GR.

To demonstrate that the GR, Bag-1M, corepressors and HDAC3 are all present at the same promoter, a re-ChIP analysis was performed for which the anti-Bag-1 antibody was employed for the first immunoprecipitation and resulted in a PCR product in the presence of hormone (Fig. 1B). The second immunoprecipitation of the Bag-1 first immunoprecipitate using the GR, NcoR, SMRT and HDAC3 antibodies revealed a PCR product of the same genomic locus (Fig. 1B). Control experiment showed that the Bag-1 antibody was completely inactivated before the use of the second set of antibodies (Fig. 1B) and no unspecific immunoprecipitation was occurred by IgG (Fig. 1B). These data further confirm that Bag-1M recruits corepressors and HDAC to the GRE. We assume that the effect of Bag-1M may be due to its ability to interact with the corepressors and HDAC. Accordingly, we carried out the immunoprecipitation experiments and could show that NcoR and Bag-1 antibodies could immunoprecipitated Bag-1M (Fig. 1C). Accordingly, SMRT and Bag-1 specific antibodies could also immunoprecipitate Bag-1M (Fig. 1D). The use of HDAC3 and Bag-1 antibodies immunoprecipitated Bag-1M as well (Fig. 1E), but Bag-1M could not be immunoprecipitated by the control IgG as shown in the IBs (Fig. 1C-E). The data suggest that BAG-1M is complexed with SMRT, NcoR and HDAC3 on chromatin.

While Bag-1M appears to be recruited to GRE through interaction with distinct amino acid sequences of the GR, the exact sequence requirements for interacting with GR need to be further identified. Several corepressor complexes associated with nuclear receptors have been elucidated, among which the NcoR–SMRT complex harboring HDACs is the most notable one [20]. Bag-1M interacts with NcoR and SMRT which in turn to bind the HDAC and this may explain how these proteins are recruited to the GRE. Our previous finding revealed that Bag-1M inhibits the DNA binding by the GR [14]. Thus, there might be two mechanisms utilized by Bag-1M to downregulate the transactivation of GR. One is to reduce the DNA binding by the GR and the other is to recruit corepressors to the response element. Reduced DNA binding by the GR was mostly seen in vitro indicating that on chromatin level, the reduced GR binding plays a subordinated role in attenuating the action of GR.

3.2. Corepressors and HDAC are essential for Bag-1M-mediated inhibition of the transactivation of the GR

To further confirm whether corepressors and HDAC are involved in the repression of the GR activity by Bag-1M, siRNA was employed to knock down corepressors NcoR and SMRT. In the cells cotransfected with GR and Bag-1M, Bag-1M represses the action of GR in a hormone-dependent manner (Fig. 2A, compare the value of lane 2 with that of lane 4, statistical analysis showed P < 0.05), and Bag-1M-mediated repression on the transactivation of the GR was not altered when control siRNA was introduced into the cells (Fig. 2A, compare lane 4 with lane 8, P > 0.05). In contrast, when siRNA of NcoR or SMRT was transfected to the cells, respectively, Bag-1M-induced inhibition on the transactivation of the GR was partially released and cotransfection of the NcoR and SMRT siRNAs had a synergic effect, suggesting that NcoR and SMRT are involved in Bag-1M-mediated negative regulation on the action of GR (Fig. 2A, compare lanes 12, 16 and 20 with lane 8, the respective P < 0.05). None of the transfected siRNAs affected the ligand-dependent transactivation of the GR (Fig. 2A, compare lanes 6, 10, 14 and 18 with lane 2, the respective P > 0.05). In addition, no significant differences could be found among the basal levels of transactivation

Fig. 1. Corepressors and histone deacetylase are recruited by Bag-1M to the GR target gene. (A) Corepressors are bound to the GRE of hMTIIa in the presence of Bag-1M and the wild-type GR but not the mutant GR (GRmt, K496L/I497G). COS-7 cells were transiently cotransfected with a vector harbouring an hMTIIa indicator gene, expression vectors encoding either the wild-type GR or GRmt, together with or without an expression vector for Bag-1M. Forty-eight hours after transfection, the cells were treated with dexamethasone (10^{-7} M) or ethanol for 1 h and were analysed for factor occupancy at the hMTIIa gene promoter by the ChIP assay. The ChIP assay was carried out on the hMTIIa gene promoter in transfected COS-7 cells with the anti-Bag-1 antibody for the first immunoprecipitation. These immunoprecipitates were subjected to a second immunoprecipitation with antibodies against GR, Bag-1, NcoR, SMRT and HDAC3, respectively. IgG and inactivated antibody were used as controls. Lower panel shows the inputs. (C–E) An aliquot from the ChIP assay was applied for immunoprecipitation with anti-NcoR (C), anti-SMRT (D), anti-HDAC3 (E), and anti-Bag-1 antibodies. Immunoblot was performed with anti-Bag-1M antibody and IgG as a control.
(Fig. 2A, when any two of the basal level values were compared, the respective $P > 0.05$). IBs showed that the protein level of NcoR and SMRT was effectively knocked down, respectively, by siRNA compared with those from the non-transfected or control siRNA transfected cells (Fig. 2B and C).

Since HDAC3 was also found to be present at the GRE of the glucocorticoid-regulated gene, we used trichostatin A (TSA), an HDAC inhibitor, to inhibit the activity of HDAC to investigate the expression of the reporter gene. As previously shown, Bag-1M inhibits the transactivation of the GR, but its negative effect was largely reversed by the use of TSA, which did not affect the transactivation mediated by the GR (Fig. 2D, compare lane 4 with lane 8, $P < 0.05$; compare lane 2 with lane 6, $P > 0.05$). However, there were no significant differences among the basal level transactivations (Fig. 2D, when any two of the basal level values were compared, the respective $P > 0.05$). This confirmed that Bag-1M recruits corepressors that subsequently interact with HDAC to suppress the expression of the glucocorticoid-induced reporter gene.

To further confirm, whether or not, the recruitment of corepressors and HDAC3 is due to the overexpressed Bag-1M in the transfection experiments, ChIP assays were carried out in which the binding of the GR to genomic GRE of the hMTIIa gene in HeLa cells was analyzed. In the absence of dexamethasone, we did not detect factors binding to the GRE (Fig. 3A). In contrast, GR, Bag-1M, NcoR, SMRT and HDAC3 were all present at the response element in a ligand-dependent manner (Fig. 3B). To characterize the effect of corepressors on GR action, we made use of HeLa cells in which the endogenous level of NcoR and SMRT has been knocked down by transfection of siRNA to investigate the expression of the hMTIIa gene. As shown in Fig. 3C, dexamethasone treatment induced the expression of the hMTIIa gene and transfection of siRNAs did not affect the basal level expression of the gene (Fig. 3C, compare lanes 2–5 with lane 1, the respective $P > 0.05$). Also, transfection of control siRNA had no effect on the expression of the hMTIIa gene (Fig. 3C, compare lane 7 with lane 6, $P > 0.05$). In contrast, transfection of either NcoR or SMRT siRNA, or a cotransfection of NcoR and SMRT siRNAs, led to increased expression of the hMTIIa gene (Fig. 3C, compare lanes 8–10 with lane 7, the respective $P < 0.05$), indicating these corepressors are involved in the downregulation of the activity of the GR by the endogenous Bag-1M. The endogenous level of NcoR and SMRT was effectively knocked down by siRNA, as shown by the IBs, compared with those from the non-transfected or control siRNA transfected cells (Fig. 3D and E).
of the genomically encoded GR-regulated gene, confirming the importance of corepressor recruitment for the inhibitory activity of Bag-1M. NcoR and SMRT are potent corepressors that are present in protein complexes containing HDACs and their corepressor activity is mediated through HDAC-dependent mechanism. Accordingly, the HDAC inhibitor TSA largely relieved the repression of GR by Bag-1M as demonstrated from the transfected indicator gene as well as the endogenous glucocorticoid responsive gene, suggesting that the negative activity of Bag-1M is, at least in part, HDAC-dependent.

### 3.3. Bag-1M is not involved in the degradation of GR

Since Bag-1M contains a ubiquitin-like domain, we investigated whether Bag-1M-mediated downregulation of the action of the GR is due to its involvement in the ubiquitin–proteosome pathway for receptor degradation. Therefore, COS-7 cells were cotransfected with vectors coding for GR and Bag-1M. In addition, the transfected cells were treated with a proteosome inhibitor MG132 and then were lysed for IB to determine the receptor level. The data showed that when MG132 was used to inhibit the proteosome activity, the receptor level was enhanced implying that under basal condition the GR was degraded through the proteosome (Fig. 4). While in the presence of Bag-1M, the receptor levels were not altered in the absence or presence of MG132 (Fig. 4, compare lanes 7 and 8 with 3 and 4; lanes 9 and 10 with 5 and 6), suggesting that Bag-1M did not target GR for degradation. Our data is different from a previous finding that Bag-1M is involved in the GR degradation in the presence of another coactivator CHIP that ubiquitinates the receptor for subsequent degradation [16].

However, our finding is consistent with the observation that several nuclear receptors including GR are ubiquitinated and degraded in the course of their nuclear activities [21–26]. In addition to receptors, studies have revealed that coactivators, including steroid receptor coactivator 1 (SRC1), SRC2, SRC3, CBP and E6-associated protein (E6-AP), could also be ubiquitinated and degraded, through the proteosome, in order to disassemble and reassemble coactivator complexes, thereby promoting enhanced transcription [27]. It is conceivable that the ubiquitination and degradation of the p160 coactivators could possibly contribute to a preinitiation complex, allowing for transcription elongation to proceed. In this manuscript, we have demonstrated that Bag-1M was not involved in ubiquitin–proteosome-mediated degradation of the GR, but the role of Bag-1M in the ubiquitination of the p160 coactivators still needs to be elucidated.

It has been recognized that molecular chaperones and cochaperones exert an important role in the modulation of the action of nuclear receptors in addition to the formation of a complex to keep...
the receptors in an appropriate conformation for ligand binding [28,29]. Recently, heat shock protein 90 (Hsp90) has been reported to enhance the DNA binding by the liganded vitamin D receptor and is essential for optimal vitamin D responsiveness [30]. GCUNC45, an Hsp90 cochaperone, effectively blocks the progression of progesterone receptor chaperoning in the presence of Hsp90beta [31]. Hsp27 was known to dysregulate the interaction of estrogen receptor with chromatin and acts as a suppressor of estrogen-induced transcription [32,33]. Hsp27 was also shown to be phosphorylated in an androgen-dependent manner and enhanced the AR stability, shuttling and transactivation, thereby promoting the survival of prostate cancer cells [34]. In addition, a histone chaperone termed nucleophosmin (NPM/B23) interacts with AR to promote AR binding to the androgen response element and subsequently modulate the transactivation of the AR [35]. Indeed, our data provide additional evidence to support the notion that chaperones modulate the activity of nuclear receptors and this mode of modulation can be fulfilled in different manners.

In conclusion, our data suggest that BAG-1M downregulates GR-mediated transactivation by recruiting the corepressors NcoR, SMRT and HDAC3 to GR and indicates BAG-1M is a novel corepressor for GR to modulate its agonist-induced transactivation.

Acknowledgements

This work was supported by a grant from Tianjin Medical University (2007 KY 11, W.H.) and a grant for the “11-5” New Century Outstanding Scientist from Tianjin Medical University (W.H.).

References


[15] Chadli, A., Felts, S.J. and Toft, D.O. (2008) GCUNC45, an Hsp90 beta subunit, is upregulated in Hsp90beta [31]. Hsp27 was known to dysregulate the interaction of estrogen receptor with chromatin and acts as a suppressor of estrogen-induced transcription [32,33]. Hsp27 was also shown to be phosphorylated in an androgen-dependent manner and enhanced the AR stability, shuttling and transactivation, thereby promoting the survival of prostate cancer cells [34]. In addition, a histone chaperone termed nucleophosmin (NPM/B23) interacts with AR to promote AR binding to the androgen response element and subsequently modulate the transactivation of the AR [35]. Indeed, our data provide additional evidence to support the notion that chaperones modulate the activity of nuclear receptors and this mode of modulation can be fulfilled in different manners.

In conclusion, our data suggest that BAG-1M downregulates GR-mediated transactivation by recruiting the corepressors NcoR, SMRT and HDAC3 to GR and indicates BAG-1M is a novel corepressor for GR to modulate its agonist-induced transactivation.

Acknowledgements

This work was supported by a grant from Tianjin Medical University (2007 KY 11, W.H.) and a grant for the “11-5” New Century Outstanding Scientist from Tianjin Medical University (W.H.).