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ATP hydrolysis is essential for Bag-1M-mediated inhibition of the DNA binding by the glucocorticoid receptor

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ABSTRACT

The 70-kDa heat shock protein (Hsp70) is involved in providing the appropriate conformation of various nuclear hormone receptors, including the glucocorticoid receptor (GR). The Bcl-2 associated athanogene 1M (Bag-1M) is known to downregulate the DNA binding by the GR. Also, Bag-1M interacts with the ATPase domain of Hsp70 to modulate the release of the substrate from Hsp70. In this study, we demonstrate that ATP hydrolysis enhances Bag-1M-mediated inhibition of the DNA binding by the GR. However, the inhibitory effect of Bag-1M was abolished when the intracellular ATP was depleted. In addition, a Bag-1M mutant lacking the interaction with Hsp70 did not influence the GR to bind DNA, suggesting the interaction of Bag-1M with Hsp70 in needed for its negative effect. These results indicate that ATP hydrolysis is essential for Bag-1M-mediated inhibition of the DNA binding by the GR and Hsp70 is a mediator for this process.

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Introduction

Glucocorticoid receptor (GR) exists, in the absence of hormone, in the cytoplasm of the target cells in an inactive form. Maintenance of the inactive state is brought about by a large group of nuclear receptor associated proteins: the chaperones and cochaperones [1]. Chaperones are a group of evolutionarily conserved proteins that bind to and orchestrate the folding of other proteins, in association with cochaperones that dictate the affinity of the chaperone for the substrate [2]. Upon binding ligand, the receptor dissociates from the multiprotein chaperone complex and translocates into the nucleus where it binds specific DNA sequence to regulate the transcription of the target genes [1,3].

The most important chaperones for nuclear receptors are the heat shock proteins [4], for example, the 70-kDa heat shock protein (Hsp70) family that are involved in a variety of functions in eukaryotic cells, including folding of newly synthesized or unfolded polypeptides, supporting protein translocation, delivery of proteins for degradation and regulation of the transcription factors [5–10]. Hsp70 structurally consists of the NH_2-terminal ATPase domain and the COOH-terminal substrate-binding domain, both of which cooperate closely. Nucleotide binding to the ATPase domain of this chaperone regulates the substrate-binding properties [11,12] and Hsp70 binds adenosine triphosphate (ATP) with high affinity and slowly hydrolyzes it to adenosine diphosphate (ADP) [13]. The function of Hsp70 during such diverse cellular processes is strongly influenced by its nucleotide state and cochaperones [14–16]. A well-known example is the interaction of Hsp70 with cochaperone Bag-1 [17–19].

Bag-1 was first identified by virtue of its interaction with the anti-apoptotic protein Bcl-2 and was shown to promote cell survival [20]. 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 [21,22]. Bag-1 utilizes the Bag domain to directly interact with the Hsp70 ATPase domain rather than the substrate-binding domain and promotes the ADP/ATP exchange reaction and/or enhances the ATPase activity of this chaperone, suggesting that Bag-1 is a nucleotide exchange factor for Hsp70 [23–25]. Apart from interaction with Hsp70, Bag-1M also directly interacts with a number of client proteins, including the protein kinase Raf-1 and growth factor receptors [26,27].

The other function of Bag-1 of likely importance for cancer is regulation of nuclear receptors. Bag-1 isoforms exert diverse effects on the action of steroid hormone receptors. For example, Bag-1L enhances the transcriptional activity of both the androgen receptor and estrogen receptor and promote the survival of prostate and breast cancer cells [28,29]. Evidence also showed

Abbreviations: Bag-1, Bcl-2 associated athanogene 1; Bag-1Mmut, Bcl-2 associated athanogene 1M R237A mutant; GR, glucocorticoid receptor; Hsp, heat shock protein; hMTIIa, human metallothionein IIa; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; IB, immunoblot; GRE, glucocorticoid response element.

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that Bag-1 represses peroxisome proliferator activated receptor (PPAR) γ-induced apoptosis in breast cancer [30]. Recent study demonstrated that Bag-1M inhibits the DNA binding by the GR [31]. It has been shown that Bag-1M could be transported from cytoplasm into the nucleus by the liganded GR and this process needs the involvement of Hsp70 [32]. A trimeric complex can be formed with Hsp70 as a mediator between Bag-1M and the GR [33].

In this work, we elucidate that Hsp70 is a mediator of Bag-1M-mediated repression of the DNA binding by the GR. ATP hydrolysis is needed by Bag-1M to inhibit the GR to bind the synthesized DNA probe and the *in vivo* genomic response element as shown by the electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). In line with this, depletion of intracellular ATP abrogated Bag-1M-mediated downregulation of the DNA binding by the GR.

Materials and methods

Cell culture. COS-7 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% CO_2. All culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

Plasmid constructs. The plasmids expressing the wild-type human GR, Bag-1M, the hMTIIa reporter plasmid H1S CAT have previously been reported [31]. pGex-2T-Bag-1M [31] was used to prepare the purified Bag-1M protein. pSG5Bag-1MR237A and pGex-2T-Bag-1MR237A were constructed based on a point mutation of Bag-1M (Bag-1MR237A) [24] generated with QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA). pEGFP-C2 was commercial available from Clontech, Palo Alto, CA.

Transfection assay. Transient transfection was carried out with Fugene 6 (Roche Diagnostics, Basel, Switzerland) reagent according to the manufacturer's instructions. For EMSA, COS-7 cells were transiently transfected with 0.2 μg of empty vector or expression vector encoding GR. For ChIP assay, COS-7 cells were transiently transfected with 0.15 μg of hMTIIa reporter plasmid, 0.2 μg of expression vectors encoding GR, Bag-1M or Bag-1MR237A. 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%.

EMSA and IB. COS-7 cells were transfected with either an empty vector or the GR expressing vector. 48 h after transfection, the cells were treated with dexamethasone (10^{-7} M) for 30 min and whole cell extracts were prepared. EMSA was performed by adding 4 μl of the whole cell extract from the transfected COS-7 cells to 10 μl 2× reaction buffer (20 mM Hepes, pH 7.9, 2 mM ethylenediaminetetraacetic acid, 2 mM DTT, and 5% Ficoll 100) and 0.5 μl of polydIdC. The mixture was supplemented with or without 500 ng of bacterially purified Bag-1M or Bag-1MR237A and adjusted to 19 μl and kept on ice for 10 min. Then, 1 μl of the radioactively labeled DNA probe together with 1 mM ATP, or 1 mM ADP or 1 mM γsATP (pH 6.5) were added to the mixture and further incubated for 15 min at room temperature. Anti-GR (PA1–512, Affinity Bioreagents INC, CA) and anti-Hsp70 (K-20, Santa Cruz, CA) antibodies were used for IB.

Preparation of purified protein. GST-Bag-1M and GST-Bag-1MR237A proteins were prepared using the same method for preparing the GST-Bag-1M protein as previously described [31]. The GST-fusion proteins containing a thrombin recognition site were treated by thrombin solution to cleave the GST tag. Once digestion is completed, GST can be further removed by extensive dialysis against 1× phosphate buffered saline (PBS) followed by purification on Glutathione Sepharose 4B. The flow-through fraction contains the Bag-1M and Bag-1MR237A proteins.

ATP depletion. Depletion of cellular ATP was performed by rinsing the cells twice with PBS followed by incubation at 37 °C with PBS supplemented with 10 mM 2-deoxyglucose (Sigma, MO) for 4 h. Non-ATP-depleted cells, used as control, were incubated in PBS supplemented with 10 mM glucose. The cells were lysed with passive lysis buffer (Promega, WI) and intracellular ATP concentrations were determined using a luciferase–luciferin assay (Sigma, MO).

ChIP. 48 h after transfection, the cells were treated with dexamethasone (10^{-7} M) or ethanol for 30 min. ChIP was performed as previously described [31]. 10% of the precleared supernatant from the lysis of the transfected COS-7 cells was taken out as input. The PCRs 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 CTG CA (hMTIIa for), GCG GGA GGA CAC AGT GTA CC (hMTIIa rev). Antibodies used were: anti-GR (P-20), anti-Bag-1 (FL-274) anti-Hsp70 (W-27) (all from Santa Cruz, CA).

Results

BAG-1M-mediated downregulation of DNA binding by the GR is dependent on ATP hydrolysis

As Bag-1-dependent dissociation of Hsp70/GR complex requires the presence of ATP [24], the effect of this nucleotide on Bag-1M-mediated downregulation of DNA binding by the GR was therefore examined. EMSA was firstly carried out with extracts of COS-7 cells, which are devoid of GR and Bag-1M [31], transfected with the GR and the bacterially purified Bag-1M in the presence or absence of ATP. DNA binding by the ligand-bound GR was repressed in the presence of Bag-1M (Fig. 1). Addition of ATP to the reaction mixture showed a drastic inhibition of DNA binding by the GR in the presence of Bag-1M. However, addition of γsATP did not show a much stronger effect on the downregulation of GR binding to DNA by Bag-1M, neither was the effect of Bag-1M on DNA binding by the GR significantly affected by ADP (Fig. 1). Immunoblot demonstrated that addition of Bag-1M and the nucleotides did not alter the levels of GR and Hsp70. Therefore, Bag-1M-mediated inhibition of DNA binding by GR is significantly influenced by ATP hydrolysis.

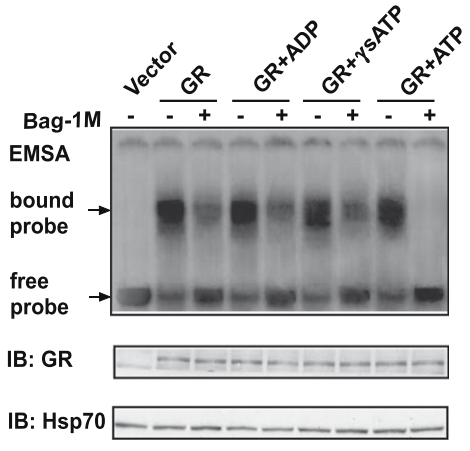

Fig. 1. Bag-1M inhibits DNA binding by the GR under condition of ATP hydrolysis. COS-7 cells were transiently transfected with empty vector or expression vector for the GR. 48 h after transfection, the cells were treated with 10^{-7} M dexamethasone for 30 min. Whole cell extracts were prepared and incubated with bacterially purified Bag-1M. The reaction mixture was incubated on ice for 10 min and was further incubated with radioactively labeled DNA probe covering the GRE together with either ADP, or γsATP, or ATP, and the bound fragments were separated by gel eletrophoresis. Aliquots of the reaction mixture were used for immunoblot with anti-GR and anti-Hsp-70 antibodies.

ATP depletion abrogates BAG-1M-mediated inhibition of DNA binding by the GR

To explore the role of ATP hydrolysis in the action of Bag-1M, the effect of this protein on DNA binding by the GR was determined under conditions of diminished cellular ATP levels. For the depletion of ATP, the GR transfected COS-7 cells were treated with 2-deoxyglucose, a non-hydrolyzable analogue of glucose that is used extensively to produce rapid and severe ATP depletion in cells [34]. Intracellular ATP concentration measured by a modified luciferase assay showed an 80% reduction of the ATP concentration in the COS-7 cells following the addition of 2-deoxyglucose for 4 h (Fig. 2A). The whole cell extracts were then prepared for EMSA.

Ligand-bound GR from the ATP depleted cells interacted with DNA but the addition of Bag-1M had virtually no effect on the DNA binding by the GR (Fig. 2B). Addition of γsATP or ADP also had no significant effect on the ability of Bag-1M to repress DNA binding by the GR. However, addition of ATP showed a strong inhibition of receptor binding to DNA in the presence of Bag-1M, indicating ATP hydrolysis is required by Bag-1M to repress the DNA binding by the GR.

To further investigate whether the ATP-dependent effect of Bag-1M to repress the GR to bind DNA demonstrated in EMSA *in vitro* also occurs *in vivo*, ChIP assay was carried out using transfected COS-7 cells. The cells were cotransfected with GR, Bag-1M together with a glucocorticoid responsive human metallothionein IIa (hMTIIa) reporter gene. The ATP depletion of these transfected cells was performed and the cells were further treated with or without dexamethasone, a synthesized glucocorticoid, for 30 min. PCR was applied to amplify a fragment covering the glucocorticoid response element (GRE) of the reporter gene.

In the absence of hormone, no factor was found to bind the GRE of the transfected hMTIIa reporter gene as demonstrated by no PCR products (Fig. 2C). In the cells treated with dexamethasone, the GR, Hsp70 and Bag-1M were all appeared on the GRE. However, in the ATP depleted cells, the binding to the GRE by the receptor and Hsp70 was enhanced, but the binding by Bag-1M was decreased, confirming that ATP hydrolysis is required by Bag-1M to inhibit

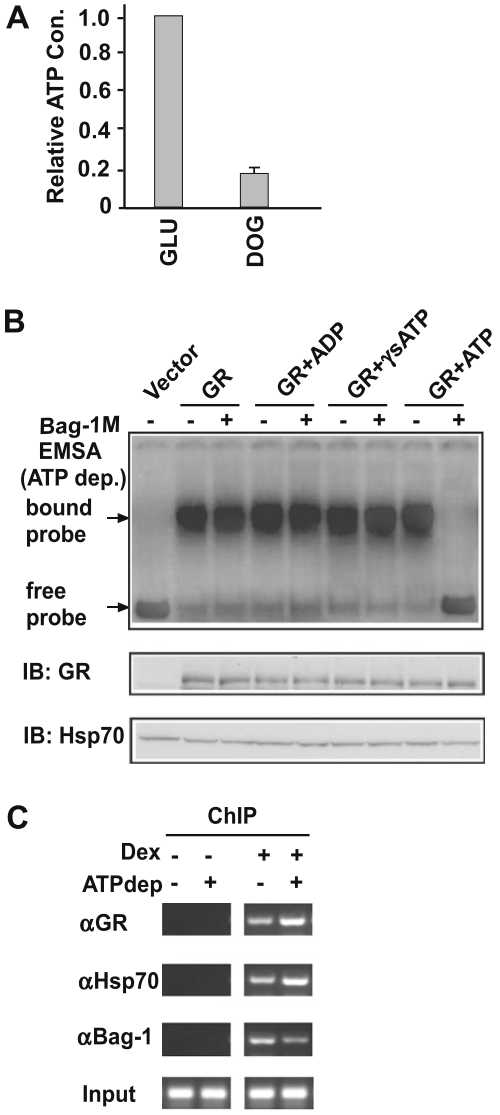

Fig. 2. ATP depletion abolishes BAG-1M-mediated inhibition on the DNA binding activity by the GR. (A) COS-7 cells were transiently transfected with an empty vector or a vector expressing GR. 48 h after transfection, the cells were incubated with either PBS supplemented with 10 mM glucose (GLU) or PBS supplemented with 10 mM 2-deoxyglucose (DOG) for 4 h. The cells were lysed and the intracellular ATP was measured using the luciferin–luciferase assay. Shown is the relative cellular ATP concentration and the bar charts represent the mean and the standard deviation of three independent experiments. (B) Whole cell extracts from the ATP depleted cells (dep) were prepared for EMSA and immunoblot as described in Fig. 1. (C) COS-7 cells were transiently cotransfected were expressing vectors encoding GR and Bag-1M, and a vector harboring hMTIIa gene. 44 h after transfection, the cells were depleted for ATP and treated with either 10^{-7} M dexamethasone or ethanol as described previously. The cells were analyzed for factor occupancy at the hMTIIa gene promoter by the ChIP assay.

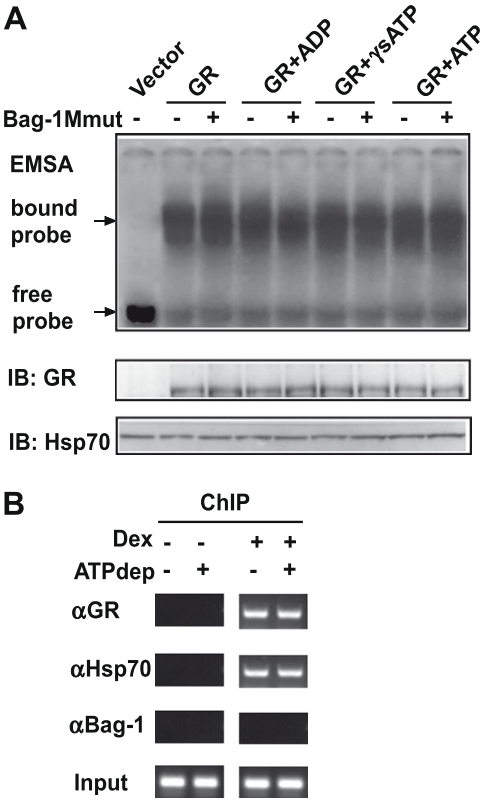

Fig. 3. Interaction with Hsp70 is essential for BAG-1M to repress the DNA binding by GR. (A) COS-7 cells were transiently transfected with empty vector or expression vector for the GR. 48 h after transfection, the cells were treated with 10^{-7} M dexamethasone for 30 min. Whole cell extracts were prepared and incubated with bacterially purified Bag-1MR237A (Bag-1Mm) for EMSA and immunoblot as described in Fig. 1. (B) COS-7 cells were transiently cotransfected were expressing vectors encoding GR and Bag-1MR237A, and a vector harboring hMTIIa gene. 48 h after transfection, the cells were treated with either 10^{-7} M dexamethasone or ethanol for 30 min. Factor occupancy was determined by ChIP.

the GR to bind the DNA. Taken together, these data indicate that Bag-1M could be brought to the GRE, by the ATP-bound Hsp70, where it represses the DNA binding by the GR through ATP hydrolysis.

Hsp70 as a mediator for Bag-1M to downregulate the DNA binding by the GR

Previous studies on modulation of glucocorticoid action by Bag-1M showed that Bag-1M only binds the GR after prior interaction of the receptor with the molecular chaperone Hsp70 [32], and it is known that Bag-1M uses its carboxyl terminal BAG domain to bind the second lobe in the ATPase domain of Hsp70 [25]. To check whether involvement of Hsp70 is essential for Bag-1M-meidated repression on the DNA binding activity by the GR, a purified Bag-1M mutant (Bag-1Mmut, Bag-1MR237A) protein, which does not interact with Hsp70 [24], was added to the reaction mixture of EMSA. As shown in Fig. 3A, no effect of the Bag-1MR237A was observed on DNA binding by the GR, even in the presence of ATP.

The interaction of Bag-1M with Hsp70 in Bag-1M-mediated downregulation of the DNA binding by the GR was also investigated *in vivo*. ChIP experiment was performed by using the transfected COS-7 cells, with the exception of transfecting the Bag-1MR237A rather than the wild-type Bag-1M, as described previously. As shown by the PCR product (Fig. 3B), there was no difference of the DNA binding by the GR in normal and ATP depleted COS-7 cells. Notably, Bag-1MR237A was not bound to the GRE, indicating that interaction with Hsp70 is essential for Bag-1M to its function. Thus, Bag-1M needs interaction with Hsp70 and ATP hydrolysis to exert its negative regulatory action on DNA binding by the GR. It is most likely that ATP hydrolysis and binding of Bag-1M to the GR produces an altered Bag-1M-GR conformation that is less efficient in DNA binding.

Discussion

We have previously reported that Bag-1M is a component of the DNA-GR complex that also harbors Hsp70 [31]. In this present work, we have analysed the effect of ATP hydrolysis on action of Bag-1M to inhibit the DNA binding by the GR. Hsp70 has been identified as an adaptor protein for the interaction of Bag-1M with the GR [32]. In surface plasmon resonance (SPR) measurements, Bag-1M was demonstrated not to interact with the GR unless Hsp70 was bound to the receptor. Furthermore, in confocal immunofluorescence assay a nuclear transport of Hsp70 by the liganded GR was observed [32]. In addition, it has been found as well that Hsp70 interacts the unliganded GR, indicating that Hsp70 bound both the non-activated as well as the ligand-activated receptor [32]. In ChIP assay, we further confirmed those findings in that Hsp70 is indeed in the same GR containing complex bound to the promoter of the glucocorticoid target genes, suggesting a nuclear role of Hsp70 [31].

The fact that interaction of Bag-1M with Hsp70 is essential for the activity of this cochaperone is evident from the finding that Bag-1MR237A, a BAG domain interaction site mutant incapable of interacting with Hsp70, abrogated inhibition of the DNA binding by the GR. Although chaperone Hsp70 is certainly the major direct binding partner of BAG-1M which employs the C-terminal BAG domain, the existence of some other proteins involved in nuclear events and interacting with the same domain cannot be excluded.

As Bag-1M binds to Hsp70–GR complex [32] and can release the bound GR in an ATP-dependent process [13,24], we postulate that this may form the basis of its action. The GR released from the complex with the molecular chaperones, is presumably in an unfavorable conformation for DNA binding and this may be the reason for the reduced DNA binding by the receptor. ATPase activity plays an important role in the action of Hsp70 and ATP hydrolysis is reported to be important for the release of bound substrates from Hsp70 [12]. It is therefore likely that during ATP hydrolysis, GR–Hsp70–Bag-1M complex is released from other bound accessory proteins and may be one of the steps leading to the release of the GR from the heterocomplex with the molecular chaperones. In any case, the downregulation of DNA binding by Bag-1M seen during ATP hydrolysis is most likely due to a conformational change in the receptor molecule and not Bag-1M-mediated degradation of the receptor (data not shown). The whole regulatory action of Bag-1M takes place on DNA since the decrease of bound GR is also associated with a decrease of Hsp70, and perhaps other factors that associate with the GR on DNA.

Another important finding of this study is that molecular chaperones and cochaperones are present on glucocorticoid response elements and play an important role in controlling binding of the receptor to DNA. This finding is believed important in view of many reports on the contribution of molecular chaperones to ligand binding of the nuclear receptors [35], which are so far thought to be their main function in nuclear action. This result agrees with the finding that Bag-1L and Hsp70 are present on the response element of an androgen target gene [36]. Furthermore, it has been reported that the cochaperone p23 is present on glucocorticoid response element and release the bound GR to allow its recycling [37]. This process may also require ATP hydrolysis as p23 couples the ATPase activity of Hsp90 to polypeptide dissociation and thus function as a substrate release factor of Hsp90 [38]. Against this background, it is not so easy to determine which GR molecules are released by p23 and which ones by Bag-1M on a glucocorticoid target gene, since both processes require ATP hydrolysis. However, the two cochaperones, Bag-1M and p23 seem to be functionally opposed to each other. While Bag-1M functions to attenuate GR signaling, p23 functions to restore the original status of the receptor for DNA binding by allowing for recycling of the receptor. This positive and negative regulation of GR action may be important control processes in glucocorticoid physiology.

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