Co-chaperone potentiation of vitamin D receptor-mediated transactivation: a role for Bcl2-associated athanogene-1 as an intracellular-binding protein for 1,25-dihydroxyvitamin D₃

R F Chun, M Gacad, L Nguyen, M Hewison and J S Adams

Division of Endocrinology, Diabetes and Metabolism, Burns and Allen Research Institute, Cedars-Sinai Medical Center, Room D-3088, 8700 Beverly Boulevard, Los Angeles, California 90048, USA

(Requests for offprints should be addressed to M Hewison; Email: martin.hewison@cshs.org)

Abstract

The constitutively expressed member of the heat shock protein-70 family (hsc70) is a chaperone with multiple functions in cellular homeostasis. Previously, we demonstrated the ability of hsc70 to bind 25-hydroxyvitamin D₃ (25-OHD₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). Hsc70 also recruits and interacts with the co-chaperone Bcl2-associated athanogene (BAG)-1 via the ATP-binding domain that resides on hsc70. Competitive ligand-binding assays showed that, like hsc70, recombinant BAG-1 is able to bind 25-OHD₃ ($K_d = 0.71 \pm 0.25$ nM, $B_{max} = 69.9 \pm 16.1$ fmoles/ μ g protein) and 1,25(OH)₂D₃ ($K_d = 0.16 \pm 0.07$ nM, $B_{max} = 38.1 \pm 3.5$ fmoles/ μ g protein; both $n=3$ separate binding assays, $P < 0.001$ for K_d and B_{max}). To investigate the functional significance of this, we transiently overexpressed the S, M, and L variants of BAG-1 into human kidney HKC-8 cells stably transfected with a 1,25(OH)₂D₃-responsive 24-hydroxylase (CYP24) promoter-reporter construct. As HKC-8 cells also express the enzyme 1 α -hydroxylase, both 25-OHD₃ (200 nM) and 1,25(OH)₂D₃ (5 nM) were able to induce CYP24 promoter activity. This was further enhanced following overexpression of all the three BAG-1 isoforms. By contrast, BAG-1 isoforms had no effect on metabolism of 25-OHD₃ by HKC-8 cells (either via 1 α - or 24-hydroxylase activities). Further studies showed that a mutant form of BAG-1S exhibited decreased binding of 1,25(OH)₂D₃ and this resulted in a concomitant loss of potentiation of CYP24 promoter transactivation. Similar effects were not observed for 25-OHD₃. These data highlight a novel role for BAG-1 as an intracellular-binding protein for 1,25(OH)₂D₃ and further suggest that BAG-1 is able to potentiate vitamin D receptor-mediated transactivation by acting as a nuclear chaperone for 1,25(OH)₂D₃.

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Introduction

Steroid hormones mediate their effects by binding to intracellular receptors which then act as transcriptional regulators by forming a regulatory nuclear complex that includes the liganded receptor, its dimer partner and other accessory proteins (Novac & Heinzel 2004). Our understanding of the molecular mechanisms involved in steroid hormone signaling has expanded greatly over the last 20 years with the result that nuclear receptors are now major targets for drug discovery (Gronemeyer *et al.* 2004). Despite this, there are many facets of steroid hormone receptor biology that have yet to be elucidated. Chief amongst these is the pathway by which the lipophilic steroid hormone moves from the cell exterior to its cognate intracellular receptor. Whether the unliganded receptor is located within the cytoplasm (as is the case for type 1 receptors such as the glucocorticoid and androgen receptors) or the nucleus (as is the case for type 2 receptors such as the retinoid and vitamin D receptors; VDRs), it seems unlikely that the process of binding a specific ligand will

depend simply on localized concentration gradients. Whereas the mechanisms that define the intracellular localization and movement of receptor proteins themselves are well documented (Hager *et al.* 2000, Kawata *et al.* 2001, Pemberton & Paschal 2005, Kumar *et al.* 2006), much less is known concerning the trafficking of their ligands.

In a series of recent studies, we have shown that estradiol (E₂) and the vitamin D metabolites, 25-hydroxyvitamin D₃ (25-OHD₃), and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) bind specifically to intracellular chaperone proteins of the heat-shock protein (hsp) family (Gacad *et al.* 1997, Gacad & Adams 1998). For 25-OHD₃ and 1,25(OH)₂D₃, these chaperones were initially cloned from New World primates and referred to as intracellular vitamin D-binding proteins (IDBP) 1 and 2 (Wu *et al.* 2000); subsequent sequence analysis of human cDNAs showed that the homologs of these proteins were hsp70 (IDBP2) and its constitutive counterpart hsc70 (IDBP1; Adams *et al.* 2004). Both hsc70 and hsp70 bound pro-hormone 25-OHD₃ with a greater affinity than the active hormonal form of

vitamin D, $1,25(\text{OH})_2\text{D}_3$ (Gacad & Adams 1998). Functional analyses showed that overexpression of hsc70 in human kidney cells enhanced mitochondrial metabolism of 25-OHD_3 via the enzyme 25-hydroxyvitamin D_3 -1 α -hydroxylase which catalyzes synthesis of $1,25(\text{OH})_2\text{D}_3$ (Wu *et al.* 2002). However, hsc70 also stimulated VDR-mediated transactivation in the presence of $1,25(\text{OH})_2\text{D}_3$ (Wu *et al.* 2000), indicating that it can act as a facilitator of both nuclear and mitochondrial functions of vitamin D metabolites. That this occurs despite the fact that the chaperone exhibits relatively low affinity binding for $1,25(\text{OH})_2\text{D}_3$ when compared with the VDR (Gacad & Adams 1998) suggests that additional factors are involved in modulating the intracellular trafficking of this hormone. The conventional protein chaperone functions of hsc70 are known to be dependent on its endogenous ATPase activity which enhances protein-binding avidity by hydrolyzing ATP to ADP (Erbse *et al.* 2004). ATP also modulates the binding of $1,25(\text{OH})_2\text{D}_3$ to hsc70, but in this case hormone binding was enhanced rather than suppressed by ATP prior to its hydrolysis to ADP (Chun *et al.* 2005). To identify alternative mechanisms involved in hsc70-mediated intracellular trafficking of $1,25(\text{OH})_2\text{D}_3$, we have assessed the effects of the hsc70 co-chaperone Bcl2-associated athanogene (BAG)-1 on vitamin D binding, metabolism, and signal transduction in human kidney cells. In common with hsc70, overexpression of BAG-1 isoforms potentiated VDR-mediated transactivation but, in contrast to hsc70, the co-chaperones had no effect on vitamin D metabolism. Data also showed that, like hsc70, BAG-1 was able to bind 25-hydroxylated vitamin D metabolites, but, in this case, the binding affinity for $1,25(\text{OH})_2\text{D}_3$ was greater than that observed for 25-OHD_3 . We therefore propose a novel role for BAG-1 as an additional intracellular-binding protein and nuclear chaperone for vitamin D metabolites.

Materials and methods

Reagents

Radiolabeled ($[^3\text{H}]25\text{-OHD}_3$, specific activity, 187 Ci/mmol) and ($[^3\text{H}]1,25(\text{OH})_2\text{D}_3$, 179 Ci/mmol) were purchased from Amersham Biosciences. Unlabeled $1,25\text{-dihydroxyvitamin D}_3$ ($1,25(\text{OH})_2\text{D}_3$) and $25\text{-hydroxyvitamin D}$ (25-OHD_3) were purchased from Biomol, Plymouth Meeting, PA, USA. Recombinant human BAG-1 protein corresponding to amino acids 241–345 of BAG-1L (112–216 of BAG-1S) was purchased from AbNova Corporation, Taipei, Taiwan, ROC. Rabbit polyclonal antibody directed against human BAG-1 was kindly provided by Dr Graham Packham, University of Southampton, UK. Polyclonal VDR antibody and

monoclonal hsc70 antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Cell line

HKC-8 human proximal tubule cells (a kind gift from Dr Lorraine Racusen, Johns Hopkins, Baltimore, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco) supplemented with 5% (v/v) fetal calf serum (FCS) as described previously (Bland *et al.* 1999).

Analysis of binding of vitamin D metabolites to recombinant BAG-1 protein

Binding of $[^3\text{H}]25\text{-OHD}_3$ or $[^3\text{H}]1,25(\text{OH})_2\text{D}_3$ to purified recombinant human BAG-1 was measured in competitive ligand-binding assays as described previously (Gacad & Adams 1998). Briefly, increasing concentrations of radiolabeled vitamin D metabolites (0.05–5.0 nM), in the presence or absence of excess (100 nM) unlabeled 25-OHD_3 or $1,25(\text{OH})_2\text{D}_3$, were dispensed into borosilicate glass tubes and evaporated to complete dryness under nitrogen. Each tube received 0.4 μg BAG-1 protein, solubilized in 0.5 M NaCl ETD buffer (1.0 mM EDTA, 10 mM Tris-HCl (pH 7.4), 5.0 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). After overnight incubation at 4 °C, bound and unbound sterols were separated by adding dextran-coated charcoal buffer into each tube which was then incubated on ice for 1 h. Following centrifugation of tubes for 30 min at 2500 *g* at 4 °C, supernatant containing protein-bound sterol was decanted into scintillation vials and counted for radioactivity. Data were reported as fmoles $[^3\text{H}]25\text{-OHD}_3$ or $[^3\text{H}]1,25(\text{OH})_2\text{D}_3$ bound to μg recombinant BAG-1, and plotted against total concentration of $[^3\text{H}]25\text{-OHD}_3$ or $[^3\text{H}]1,25(\text{OH})_2\text{D}_3$ in the binding assay (saturation analyses) or bound metabolite/free metabolite (Scatchard plots). The latter plots were used to define binding kinetics: maximal binding (B_{max} , intercept with *y* axis); dissociation constant (K_d , slope of regression line). Three separate binding assays for $[^3\text{H}]25\text{-OHD}_3$ or $[^3\text{H}]1,25(\text{OH})_2\text{D}_3$ were carried out and used to present mean K_d and B_{max} values $\pm$ s.d.

Analysis of binding of vitamin D metabolites to cellular BAG-1 proteins following transfection of BAG-1 cDNA expression constructs

Confluent cultures of HKC cells transfected with expression constructs containing cDNA for BAG-1S, or mutant BAG-1Sm (Q169A/K172A; a kind gift from Dr Graham Packham, University of Southampton, UK), or transfected with empty vector control plasmid were

harvested, pelleted, and washed twice in PBS (20 mM Na_2HPO_4 and 150 mM NaCl (pH 7.2). Cell pellets were resuspended in ETD buffer (1 mM, 10 mM Tris-HCl (pH 7.4), and 5 mM dithiothreitol) containing 1 mM PMSF and homogenized by Polytron on ice in five 15 s bursts. Nuclei, with associated nuclear steroid receptor proteins, were pelleted at 4000 *g* for 30 min at 4 °C. Supernatant was subjected to further centrifugation at 100 000 *g* for 1 h at 4 °C and the supernatant from this procedure was then used in binding assays with a single saturating dose of [^3H]25-OHD₃ or [^3H]1,25(OH)₂D₃ (both 5 nM) in the presence or absence of 100 nM unlabeled 25OHD₃ or 1,25(OH)₂D₃.

Western blot analysis

Aliquots (10 µg) of total protein, solubilized in RIPA buffer, pH 8.0 (50 mM Tris-HCl, 0.5% sodium deoxycholate, 0.1% SDS, 1.0% nonidet P-40) with protease inhibitors were loaded onto Precise 10% Tris-HEPES PAGE mini-gels (Pierce Biochemicals, Rockford, IL, USA) and electrophoresed for 1 h at 100 v. Western blotting was performed by standard methods. Briefly, resolved proteins were transblotted onto Hybond-P PVDF membrane (Amersham Biosciences) for 1 h at 100 v, blocked overnight at 4 °C and then incubated with primary antibody for 1.5 h at 23 °C on a rocker platform. Specific protein was detected using the Western-Light Detection Kit (Tropix, Bedford, MA, USA).

Steroid transactivation assays

For transactivation studies, HKC-8 cells were stably transformed with a CYP24 gene promoter vitamin D response element (VDRE) luciferase reporter plasmid (Arbour *et al.* 1998) and G418-resistant colonies exhibiting 1,25(OH)₂D₃-induced luciferase activity were selected. Cells were cultured in 24-well plates to approximately 80% confluence and each well was transfected with 0.2 µg of BAG-1 expression plasmid or empty CMV vector (pcDNA 3.1+, Invitrogen, Carlsbad, CA), together with 0.002 µg renilla luciferase control plasmid (pRL-TK; Promega) and 50 µl Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). Post-transfection, DNA-liposome complexes were removed and vehicle, 25-OHD₃ or 1,25(OH)₂D₃ in Opti-MEM were added to the cells. After 24 h media were removed, cells washed with PBS and then lysed with 250 µl buffer from the Dual-Luciferase Reporter Assay system (Promega). Aliquots (30 µl) of lysate were then assayed for firefly luciferase (45 s) and renilla luciferase (15 s) and quantified using an EG&G Berthold Auto Lumat LB953 (Berthold Technologies, Oak Ridge, TN, USA).

Analysis of 25-OHD₃ metabolism

1 α - and 24-hydroxylase activities in HKC-8 cells were assessed by quantifying the conversion of radiolabeled 25OHD₃ to 1,25(OH)₂D₃ or 24,25(OH)₂D₃ in serum-free cultures of these cells. For each assay, 50 nM [^3H]25-OHD₃ (a 1 in 10 mixture of unlabeled 25-OHD₃ and [^3H]25-OHD₃; 180 Ci/mmol, Amersham Biosciences) was added to cells in 200 µl of serum-free medium and then incubated for 5 h at 37 °C, with the reaction being terminated by freezing at -20 °C. Protein from these samples was initially precipitated with added acetonitrile (1:1). Vitamin D metabolites were then extracted from the reaction mixtures by elution on C18-OH columns according to manufacturer's instructions (Diasorin, Stillwater, MN, USA). The resulting eluent was resuspended in 25 µl elution solvent hexane:methanol:isopropanol (90:5:5), vortexed for 15 s, and individual metabolites separated by HPLC using a Beckman Gold system with an Agilent Technologies Zorbax Sil normal phase column (Agilent Technologies, Palo Alto, CA, USA) eluted at a rate of 1.5 ml/min for 20 min. Elution profiles for standard vitamin D metabolites (25-OHD₃, 24,25(OH)₂D₃, 1,25(OH)₂D₃) were determined by u.v. absorbance at 264 nm. Elution of metabolites of [^3H]25-OHD₃ was assessed using a β -Ram Model 4 in-flow detector (IN/US, Tampa, FL, USA) in conjunction with Ultima-Flo M scintillation fluid (Perkin-Elmer, Boston, MA, USA) at a 2:1 ratio with a 5 s dwell time to designate the increments for data collection. Lauralite 3 software (LabLogic, Sheffield, UK) was used to quantitate peaks of radioactivity corresponding to 25-OHD₃, 24,25(OH)₂D₃ or 1,25(OH)₂D₃. Data were reported as mean fmoles metabolite synthesized/h per mg cellular protein $\pm$ s.d. following *n* = 3 separate experiments.

Results

To determine whether BAG-1 was able to associate with vitamin D metabolites in a similar fashion to its chaperone partner hsc70, binding analyses were carried out using recombinant BAG-1 protein as the recipient and tritiated 25-OHD₃ or 1,25(OH)₂D₃ as ligand. Dose response analyses (Fig. 1A and B) showed that BAG-1 was able to specifically bind both metabolites with saturable kinetics. Linearization of these data by Scatchard plot analysis (Fig. 1C and D) indicated that the K_{d} s (K_{d}) for binding of 25-OHD₃ and 1,25(OH)₂D₃ were 0.71 ± 0.25 and 0.16 ± 0.07 nM respectively (*n* = 3 separate binding assays, *P* < 0.001). Maximal binding (B_{max}) was higher for 25-OHD₃ (69.9 ± 16.1 fmoles/µg protein) than for 1,25(OH)₂D₃ (38.1 ± 3.5 fmoles/µg protein; *n* = 3 separate binding assays, *P* < 0.001). Further evidence for the specificity of vitamin D

Figure 1 BAG-1 specifically binds vitamin D metabolites. (A) and (B) Dose-dependent binding of $[^3\text{H}]25\text{-OHD}_3$ and $[^3\text{H}]1,25(\text{OH})_2\text{D}_3$ to recombinant BAG-1. Data show bound metabolite (fmol/ μg recombinant BAG-1 protein) versus concentration of metabolite added (nM). Total bound $[^3\text{H}]25\text{-OHD}_3$ or $[^3\text{H}]1,25(\text{OH})_2\text{D}_3$ data (T) are shown as closed circles, non-specifically bound (NSB) as open circles and specifically bound (S) as closed triangles. (C) and (D) Scatchard plot (linearized data) analysis of $[^3\text{H}]25\text{-OHD}_3$ and $[^3\text{H}]1,25(\text{OH})_2\text{D}_3$ binding to recombinant BAG-1. Data show bound/free metabolite versus specifically bound metabolite (fmol/ μg protein). K_d values were derived from the slope of each plot and maximal binding (B_{max}) values were derived from the intercept with the x axis.

metabolite binding to BAG-1 was obtained by carrying out parallel-binding analyses using heat-denatured BAG-1 protein. In this case, no specific binding was observed for either 25-OHD_3 or $1,25(\text{OH})_2\text{D}_3$ (data not shown).

To assess the potential functional impact of interaction between vitamin D metabolites and BAG-1, further studies were carried out using stable transfectant variants of HKC-8 cells that constitutively expressed a luciferase promoter-reporter construct containing a 24-hydroxylase gene (CYP24) promoter VDRE. The transfectant HKC-8 cells also expressed endogenous VDR and retained the parental cell line's capacity for 1α - and 24-hydroxylase activities (Bland *et al.* 1999), thereby enabling quantification of responses to both 25-OHD_3 and $1,25(\text{OH})_2\text{D}_3$. In the first series of studies, expression plasmids containing cDNAs for BAG-1L (L), BAG-1M (M), BAG-1S (S) or a mutant form of BAG-1S (Sm; Fig. 2) were transfected into the luciferase-expressing HKC-8 cells to investigate their effect on VDR-VDRE-mediated transactivation (Fig. 3). In the resulting cells, protein for each co-chaperone was expressed at similar levels (Fig. 3A). Parental HKC-8

Figure 2 Schematic of BAG-1 isoforms used in the study. BAG-1L, BAG-1M, and BAG-1S all contain an ubiquitin-like domain (ULD) and a BAG domain. BAG-1Sm contains an altered protein sequence within helix 2 of the BAG domain at amino acids 169 (glutamine (Q) to alanine (A)) and 172 (lysine (K) to alanine (A)). Amino acids 169 and 172 of BAG-1S corresponded to amino acids 298 and 301 of BAG-1L and are present in the recombinant protein used in initial binding studies (241–345 of BAG-1L).

Figure 3 Effect of transient overexpression of BAG-1L, BAG-1M, BAG-1S, or mutant BAG-1Sm on VDRE-mediated transactivation and 25-OHD₃ metabolism in HKC-8 cells. (A) relative levels of expression of BAG-1 in HKC-8 cells transfected with empty vector (C), BAG-1S (S), mutant BAG-1Sm (Sm), BAG-1L (L), or BAG-1M (M) as determined by western blot analysis of transfectant cells. Low-level endogenous expression of BAG-1 was not detectable for the exposure times used to derive the western blots (see empty vector transfectants). (B) Stable transfectant variants of HKC-8 cells expressing a luciferase reporter construct containing a 1,25(OH)₂D₃-inducible vitamin D response element from the CYP24 gene promoter vehicle were treated with either vehicle (0.1% ethanol), 1,25(OH)₂D₃ (5 nM) or 25-OHD₃ (200 nM) for 24 h in HKC-8 kidney cells transiently transfected with expression constructs for human BAG-1L (L), BAG-1M (M), BAG-1S (S) mutant BAG-1Sm (Sm), or empty CMV vector (C) 3C. The 1 α -hydroxylase inhibitor ketoconazole (1 μ M) was added to promoter-reporter assays in the absence or presence of vehicle (V), 25-OHD₃ (25D) or 1,25(OH)₂D₃ (1,25D) to confirm that responses to the former were due to endogenous conversion to

cells expressed very low endogenous levels of BAG-1S, M, and L, which were not detected using the autoradiograph exposure times employed in Fig. 3A. The transfectant variants of HKC-8 were then assessed for transcriptional responses to treatment with either 1,25(OH)₂D₃ or 25-OHD₃ (both for 24 h) using concentrations representing the relative physiological levels of these metabolites (5 and 200 nM respectively). Induction of VDRE-mediated promoter activity in the HKC-8-luciferase transfectants was achieved by both 1,25(OH)₂D₃ and 25-OHD₃ (Fig. 3B), the latter reflecting the ability of HKC-8 cells to endogenously convert 25-OHD₃ to 1,25(OH)₂D₃. Inhibition of CYP27b1 activity by treatment with ketoconazole suppressed 25-OHD₃-mediated luciferase activity but was without effect on 1,25(OH)₂D₃-mediated transcription (Fig. 3C). Following overexpression of each of the BAG-1 isoforms, the induction of CYP24 promoter activity by 25-OHD₃ was further increased approximately twofold when compared with cells treated with the metabolite and plasmid-only cDNA ($P < 0.001$). Similar responses were also observed in cells treated with 1,25(OH)₂D₃, although, in this case, the effects of BAG-1M and BAG-1S (both $P < 0.001$) were more pronounced than the effects of BAG-1L ($P < 0.05$). In contrast to the effect of BAG-1S, transfection of a mutant form of BAG-1S that incorporated a glutamine (Q) to alanine (A) change at amino acid 169 and a lysine (K) to alanine (A) change at amino acid 172, had no stimulatory effect on 25-OHD₃ or 1,25(OH)₂D₃-induced transactivation (Fig. 3B).

As 25-OHD₃ requires conversion to 1,25(OH)₂D₃ to facilitate VDR-mediated transcription, the effects of BAG-1 on transcriptional responses to the inactive precursor metabolite could have been due to either: 1) BAG-1-enhanced transcriptional responses following conversion of 25-OHD₃ to 1,25(OH)₂D₃ or 2) BAG-1-enhanced synthesis of 1,25(OH)₂D₃ leading in turn to potentiated transcription. To address the latter, we assessed the effect of BAG-1S, M, or L on 25-OHD₃ metabolism in untreated HKC-8 cells which exhibit basal 1 α - and 24-hydroxylase activities. Data in Fig. 4 revealed that transfection of the BAG-1 isoforms had no effect on synthesis of either 24,25(OH)₂D₃ or 1,25(OH)₂D₃.

To determine whether or not the data in Figs 3 and 4 were associated with altered cytosolic binding of 25-OHD₃ or 1,25(OH)₂D₃, similar to that reported in Fig. 1, further binding assays were carried out using BAG-1-transfected HKC-8 cells. Data in Fig. 5 showed

1,25(OH)₂D₃. Data shown are the mean luciferase reporter units $\pm$ s.d. normalized for transfection efficiency represented by control renilla luciferase expression ($n = 4$ separate assays). Statistically different from equivalent CMV empty vector control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistically different from vehicle-treated CMV empty vector control: # $P < 0.05$; ## $P < 0.01$.

Figure 4 Effect of transient expression of BAG-1S, BAG-1M, or BAG-1L on vitamin D metabolism. HKC-8 cells were treated with either vehicle (0.1% ethanol), 1,25(OH)₂D₃ (5 nM), or 25-OH-D₃ (100 nM) for 24 h in the presence or absence of transiently transfected expression constructs for human BAG-1S (S), BAG-1M (M), BAG-1L (L), or empty CMV vector (C). Cells were then analyzed for 1 α - (CYP27b1) and 24-hydroxylase (CYP24) activities using [³H]25-OH-D₃ as substrate (50 nM) with products ([³H]1,25(OH)₂D₃ or [³H]24,25(OH)₂D₃) separated and quantified by HPLC. Data were reported as fmol vitamin D metabolite produced/h per mg cellular protein $\pm$ s.d. ($n=4$).

that transfection of empty vector, or expression constructs for BAG-1S or mutant BAG-1Sm had no effect on cytosolic binding of 25-OH-D₃. However, transfection of wild type BAG-1S resulted in a twofold increase in 1,25(OH)₂D₃ binding when compared with cells receiving empty vector only. This effect was completely abrogated when the mutant form of BAG-1S was used.

Figure 5 Over-expression of BAG-1 in HKC-8 cells alters cytosolic binding of 1,25(OH)₂D₃. HKC-8 cells were transiently transfected with empty vector (C), or expression constructs for BAG-1S (S) or mutant BAG-1Sm (Sm) and then assessed for cytosolic binding of either 25-OH-D₃ or 1,25(OH)₂D₃. Data are shown as DPM-specific binding of [³H]25-OH-D₃ or [³H]1,25(OH)₂D₃ at a single saturating dose (5 nM for each). *** = statistically different from vector-only transfectant cells, $P<0.001$.

Discussion

As befits its name, BAG-1 was originally cloned as a protein that binds to and promotes the anti-apoptotic properties of mouse Bcl-2 via an ATP- and raf-1 kinase-dependent mechanism (Takayama *et al.* 1995, Wang *et al.* 1996, Wang & Reed 1998). Significantly, the homolog of BAG-1, RAP46, was originally identified as a glucocorticoid receptor (GR)-binding peptide (Zeiner & Gehring 1995). Since then RAP46/BAG-1 has been shown to associate with several other members of the steroid receptor superfamily, with functional responses varying from potent enhancement of transactivation to complete inhibition (Froesch *et al.* 1998, Kullmann *et al.* 1998, Brimmell *et al.* 1999, Guzey *et al.* 2000, Cato & Mink 2001, Witcher *et al.* 2001, Schmidt *et al.* 2003, Shatkina *et al.* 2003). The molecular basis for these effects has been only partially defined and is complicated by the fact that the term BAG-1 in fact refers to the three isoforms of this protein: RAP46 is also referred to as BAG-1M, but longer (BAG-1L) and shorter (BAG-1S) forms are also expressed (Packham *et al.* 1997, Froesch *et al.* 1998). BAG-1 isoforms are also known to bind to many other signaling factors, most prominently the heat-shock proteins hsp70 and hsc70, where they act to attenuate chaperone function with respect to the facilitation of protein folding (Takayama *et al.* 1997). In this case, the effects of BAG-1 have been shown to be due to binding of its C-terminal region to the hsp70/hsc70 ATPase site of the heat-shock proteins resulting in the inhibition of ATP hydrolysis and the concomitant uncoupling of chaperone-mediated protein folding (Bimston *et al.* 1998). In previous studies, we have shown that hsp70 and hsc70 can also act as intracellular steroid chaperones by binding the vitamin D metabolites 25-OH-D₃ and 1,25(OH)₂D₃ and potentiating their mitochondrial metabolism and nuclear signaling (Wu *et al.* 2000, 2002, Chun *et al.* 2005). This led us to propose a model by which binding and protein-to-protein transfer of vitamin D metabolites is linked to hsc70 ATP hydrolysis and protein substrate binding (Wu *et al.* 2000). In view of the fact that BAG-1 inhibits hsc70 function by interfering with the normal chaperone cycle (Bimston *et al.* 1998), we carried out specific experiments to investigate its effects on the metabolism and function of vitamin D metabolites.

The data presented in this study show that BAG-1 is able to bind 25-OH-D₃ and 1,25(OH)₂D₃ with relatively high affinity suggesting a potential new function for this co-chaperone. Specifically, we propose that, in addition to its established role as a facilitator of steroid hormone receptor action, BAG-1 may also function as an intracellular chaperone for the actual ligands of these receptors. Although this postulate is similar to that which has already been described for hsc70 (Wu *et al.*

2000, 2002, Adams *et al.* 2004, Chun *et al.* 2005), there are some important differences between the two proteins. First the binding kinetics for 25-OHD₃ and 1,25(OH)₂D₃ are clearly different, with BAG-1 showing greater affinity for active 1,25(OH)₂D₃, whilst hsc70 exhibits greater affinity for the inactive precursor 25-OHD₃ (Gacad & Adams 1998). As a consequence, 1,25(OH)₂D₃ may favor BAG-1 as a binding site, whilst 25-OHD₃ would favor hsc70. Co-immunoprecipitation and *in vitro* heterodimerization studies indicate that there is a close association between BAG-1 and hsc70 (Takayama *et al.* 1997). It is therefore possible that vitamin D metabolites will be able to transfer from one protein to another. This would be dependent in part on the relative-binding affinities of individual chaperones but may also involve more active and directed mechanisms. We have shown previously that the binding of 1,25(OH)₂D₃ to hsc70 is enhanced by occupancy of the chaperone's ATPase domain but then decreases following hydrolysis of ATP to ADP via hsc70 ATPase (Chun *et al.* 2005). By contrast, protein substrate binding to hsc70 is less avid when ATP is docked within the ATPase site of hsc70 but is then enhanced following ATP hydrolysis (Luders *et al.* 2000, Young *et al.* 2003). Based on these observations, it is possible that the high affinity, ATPase-dependent, binding of protein substrate (e.g. BAG-1) to hsc70 will conversely result in decreased hsc70 affinity for sterol (i.e., 1,25(OH)₂D₃). If this is the case, then we can further speculate that the putative change in binding of 1,25(OH)₂D₃ will facilitate transfer of the sterol to hsc70s bound protein substrate (Chun *et al.* 2005). At present, this remains conjecture but it is interesting to note that the affinity of BAG-1 for 1,25(OH)₂D₃ is similar to that observed with hsc70 in the absence of ATP ($K_d < 0.3$ nM), but it is significantly greater than ATPase-occupied hsc70 ($K_d > 1.0$ nM). Thus, ATPase-mediated association between BAG-1 and hsc70 would favor binding of 1,25(OH)₂D₃ to BAG-1 rather than hsc70, providing a potential mechanism for the preferential transfer of the active vitamin D metabolite from one intracellular chaperone to another.

A second crucial difference between BAG-1 and hsc70 with respect to vitamin D is that, when over-expressed, hsc70 was able to potentiate both 1,25(OH)₂D₃-mediated transactivation and mitochondrial CYP27b1-mediated metabolism of 25-OHD₃ to 1,25(OH)₂D₃ (Wu *et al.*, 2000, 2002). By contrast, BAG-1 isoforms stimulated VDR-mediated transactivation but had no effect on either CYP27b1 or CYP24 metabolism of 25-OHD₃. Moreover, when over-expressed in HKC-8 cells BAG-1 enhanced intracellular binding of 1,25(OH)₂D₃ whilst having no significant effect on 25-OHD₃ binding. These data provide further evidence that BAG-1 has greater specificity for 1,25(OH)₂D₃ when compared with 25-OHD₃ but they

also highlight binding differences between recombinant BAG-1 and BAG-1 expressed by actual cells. The intracellular expression studies using mutant BAG-1S also highlight a potential region of the protein that is involved in binding 1,25(OH)₂D₃. Specifically, mutation of amino acids 169 and 172 of BAG-1S inhibited binding of 1,25(OH)₂D₃. This region of the BAG domain (helix 2) has been shown to be important in mediating interaction with chaperones such as hsc70 (Briknarova *et al.* 2001), further endorsing a potential mechanism by which binding of 1,25(OH)₂D₃ may transfer from one protein to another.

The data presented in this study also address a feature of intracellular sterol chaperones that we have not previously considered, namely the extent to which chaperones are able to influence responses to exogenously added versus endogenously synthesized sterols. Human proximal tubule HKC-8 cells were used in this study because they constitutively exhibit both CYP27b1 and CYP24 activities. Thus, we were able to stimulate promoter-reporter activity using both 1,25(OH)₂D₃ and 25-OHD₃, with the latter being due to endogenous conversion to the former via the enzyme CYP27b1 (BAG-1 does not affect vitamin D metabolism and does not appear to alter intracellular binding of 25-OHD₃). The doses of 25-OHD₃ (200 nM) and 1,25(OH)₂D₃ (5 nM) used in this study were chosen to reflect the different circulating levels of these metabolites in humans, although the concentration of 1,25(OH)₂D₃ would be considered supra-physiological. It was therefore interesting to note that 25-OHD₃ was as effective as 1,25(OH)₂D₃ in stimulating CYP24 promoter activity, and that BAG-1 isoforms were able to potentiate responses to both 1,25(OH)₂D₃ and 25-OHD₃. These data suggest that intracellular chaperones such as BAG-1 are as effective, if not more effective, in handling locally generated versus exogenously added ligand.

Previous publications have reported divergent vitamin D responses to overexpression of BAG-1 isoforms despite the fact that in each of these studies BAG-1 has been shown to physically interact with VDR (Guzey *et al.* 2000, Witcher *et al.* 2001). It is therefore possible that in different cells the functional remit of BAG-1 will be subtly different, with effects varying between conformational changes in VDR, modulation of co-activator/co-repressor function and, as outlined in this study, effects on ligand binding. As indicated above, this is likely to be influenced by the relative level of BAG-1 expression when compared with other chaperones and VDR but may also be dependent on the spectrum of BAG-1 isoforms in a cell. The potentiation of VDR-mediated responses by BAG-1 reported by Reed and colleagues was most pronounced following expression of the full-length isoform, BAG-1L, in COS monkey kidney or 293 human kidney cells (Guzey *et al.* 2000). By contrast, the same group showed

that the shorter isoform of BAG-1 (BAG-1S) was able to antagonize retinoid function via direct interaction with the retinoid acid receptor (RAR; Liu *et al.* 1998). Based on these observations, they concluded that the potentiation of VDR responsiveness by BAG-1L involved several regions including the hsc70 ATPase-interacting BAG domain which is common to all the isoforms. It is therefore tempting to speculate that at least part of the function of this region of BAG-1 is to act as both a recipient of hsc70 and its ligand cargo, namely 1,25(OH)₂D₃.

There is increasing evidence that localized autocrine or paracrine mechanisms play a key role in mediating many of the novel non-classical responses to vitamin D with respect to its proposed immunomodulatory (Hewison *et al.*, 2003, 2004, Liu *et al.* 2006) and anti-cancer (Townsend *et al.* 2005a,b) actions. It therefore seems likely that the BAG-1-mediated intracellular co-chaperone effects described in this study will be applicable to both the classical renal and non-classical extra-renal vitamin D system. As such, further analysis of the impact of BAG-1 on vitamin D signaling in other cells, may help to shed light on the reported variability in sensitivity to 1,25(OH)₂D₃ that is associated with some diseases (Ebert *et al.* 2006). It is also unclear whether binding of vitamin D metabolites or other sterols will affect the more conventional actions of BAG-1 such as its ability to bind antiapoptotic Bcl-2 and promote cell survival (Takayama *et al.* 1995, Wang *et al.* 1996, Wang & Reed 1998). Finally, BAG-1 is known to interact with the signal transduction pathways for various other steroid hormone receptors, including androgen and glucocorticoid receptors (Froesch *et al.* 1998, Kullmann *et al.* 1998, Brimmell *et al.* 1999, Guzey *et al.* 2000, Cato & Mink 2001, Witcher *et al.* 2001, Schmidt *et al.* 2003, Shatkina *et al.* 2003), raising the possibility that the co-chaperone is able to bind other sterol ligands in a similar fashion to that described here for 1,25(OH)₂D₃. If this is the case, then it is intriguing to contemplate BAG-1 as a new and potentially important target for the disruption or therapeutic manipulation of steroid hormone action.

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References

- Adams JS, Chen H, Chun R, Gacad MA, Encinas C, Ren S, Nguyen L, Wu S, Hewison M & Barsony J 2004 Response element binding proteins and intracellular vitamin D binding proteins: novel regulators of vitamin D trafficking, action and metabolism. *Journal of Steroid Biochemistry and Molecular Biology* **89–90** 461–465.
- Arbour NC, Ross TK, Zierold C, Prah J & DeLuca HF 1998 A highly sensitive method for large-scale measurements of 1,25-dihydroxy-vitamin D. *Analytical Biochemistry* **255** 148–154.
- Bimston D, Song J, Winchester D, Takayama S, Reed JC & Morimoto RI 1998 BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release. *EMBO Journal* **17** 6871–6878.
- Bland R, Walker EA, Hughes SV, Stewart PM & Hewison M 1999 Constitutive expression of 25-hydroxyvitamin D3-1alpha-hydroxylase in a transformed human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. *Endocrinology* **140** 2027–2034.
- Briknarova K, Takayama S, Brive L, Havert ML, Knee DA, Velasco J, Homma S, Cabezas E, Stuart J, Hoyt DW *et al.* 2001 Structural analysis of BAG1 co-chaperone and its interactions with Hsc70 heat shock protein. *Nature Structural Biology* **8** 349–352.
- Brimmell M, Burns JS, Munson P, McDonald L, O'Hare MJ, Lakhani SR & Packham G 1999 High level expression of differentially localized BAG-1 isoforms in some oestrogen receptor-positive human breast cancers. *British Journal of Cancer* **81** 1042–1051.
- Cato AC & Mink S 2001 BAG-1 family of co-chaperones in the modulation of nuclear receptor action. *Journal of Steroid Biochemistry and Molecular Biology* **78** 379–388.
- Chun R, Gacad MA, Hewison M & Adams JS 2005 Adenosine 5'-triphosphate-dependent vitamin D sterol binding to heat shock protein-70 chaperones. *Endocrinology* **146** 5540–5544.
- Ebert R, Schutze N, Adamski J & Jakob F 2006 Vitamin D signaling is modulated on multiple levels in health and disease. *Molecular and Cellular Endocrinology* **248** 149–159.
- Erbse A, Mayer MP & Bukau B 2004 Mechanism of substrate recognition by Hsp70 chaperones. *Biochemical Society Transactions* **32** 617–621.
- Froesch BA, Takayama S & Reed JC 1998 BAG-1L protein enhances androgen receptor function. *Journal of Biological Chemistry* **273** 11660–11666.
- Gacad MA & Adams JS 1998 Proteins in the heat shock-70 family specifically bind 25-hydroxyvitamin D3 and 17beta-estradiol. *Journal of Clinical Endocrinology and Metabolism* **83** 1264–1267.
- Gacad MA, Chen H, Arbelle JE, LeBon T & Adams JS 1997 Functional characterization and purification of an intracellular vitamin D-binding protein in vitamin D-resistant new world primate cells, amino acid sequence homology with proteins in the hsp-70 family. *Journal of Biological Chemistry* **272** 8433–8440.
- Gronemeyer H, Gustafsson JA & Laudet V 2004 Principles for modulation of the nuclear receptor superfamily. *Nature Reviews. Drug Discovery* **3** 950–964.
- Guzey M, Takayama S & Reed JC 2000 BAG1L enhances trans-activation function of the vitamin D receptor. *Journal of Biological Chemistry* **275** 40749–40756.
- Hager GL, Lim CS, Elbi C & Baumann CT 2000 Trafficking of nuclear receptors in living cells. *Journal of Steroid Biochemistry and Molecular Biology* **74** 249–254.
- Hewison M, Freeman L, Hughes SV, Evans KN, Bland R, Eliopoulos AG, Kilby MD, Moss PA & Chakraverty R 2003 Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *Journal of Immunology* **170** 5382–5390.
- Hewison M, Zehnder D, Chakraverty R & Adams JS 2004 Vitamin D and barrier function: a novel role for extra-renal 1 alpha-hydroxylase. *Molecular and Cellular Endocrinology* **215** 31–38.
- Kawata M, Matsuda K, Nishi M, Ogawa H & Ochiai I 2001 Intracellular dynamics of steroid hormone receptor. *Neuroscience Research* **40** 197–203.
- Kullmann M, Schneikert J, Moll J, Heck S, Zeiner M, Gehring U & Cato AC 1998 RAP46 is a negative regulator of glucocorticoid receptor action and hormone-induced apoptosis. *Journal of Biological Chemistry* **273** 14620–14625.

- Kumar S, Saradhi M, Chaturvedi NK & Tyagi RK 2006 Intracellular localization and nucleocytoplasmic trafficking of steroid receptors: an overview. *Molecular and Cellular Endocrinology* **246** 147–156.
- Liu R, Takayama S, Zheng Y, Froesch B, Chen GQ, Zhang X, Reed JC & Zhang XK 1998 Interaction of BAG-1 with retinoic acid receptor and its inhibition of retinoic acid-induced apoptosis in cancer cells. *Journal of Biological Chemistry* **273** 16985–16992.
- Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schaubert J, Wu K, Meinken C *et al.* 2006 Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **311** 1770–1773.
- Luders J, Demand J, Papp O & Hohfeld J 2000 Distinct isoforms of the cofactor BAG-1 differentially affect Hsc70 chaperone function. *Journal of Biological Chemistry* **275** 14817–14823.
- Novac N & Heinzel T 2004 Nuclear receptors: overview and classification. *Current Drug Targets. Inflammation and Allergy* **3** 335–346.
- Packham G, Brimmell M & Cleveland JL 1997 Mammalian cells express two differently localized Bag-1 isoforms generated by alternative translation initiation. *Biochemical Journal* **328** 807–813.
- Pemberton LF & Paschal BM 2005 Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic* **6** 187–198.
- Schmidt U, Wochnik GM, Rosenhagen MC, Young JC, Hartl FU, Holsboer F & Rein T 2003 Essential role of the unusual DNA-binding motif of BAG-1 for inhibition of the glucocorticoid receptor. *Journal of Biological Chemistry* **278** 4926–4931.
- Shatkina L, Mink S, Rogatsch H, Klocker H, Langer G, Nestl A & Cato AC 2003 The cochaperone Bag-1L enhances androgen receptor action via interaction with the NH2-terminal region of the receptor. *Molecular and Cellular Biology* **23** 7189–7197.
- Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA & Reed JC 1995 Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell* **80** 279–284.
- Takayama S, Bimston DN, Matsuzawa S, Freeman BC, Aime-Sempe C, Xie Z, Morimoto RI & Reed JC 1997 BAG-1 modulates the chaperone activity of Hsp70/Hsc70. *EMBO Journal* **16** 4887–4896.
- Townsend K, Evans KN, Campbell MJ, Colston KW, Adams JS & Hewison M 2005a Biological actions of extra-renal 25-hydroxy-vitamin D-1 α -hydroxylase and implications for chemoprevention and treatment. *Journal of Steroid Biochemistry and Molecular Biology* **97** 103–109.
- Townsend K, Banwell CM, Guy M, Colston KW, Mansi JL, Stewart PM, Campbell MJ & Hewison M 2005b Autocrine metabolism of vitamin D in normal and malignant breast tissue. *Clinical Cancer Research* **11** 3579–3586.
- Wang HG & Reed JC 1998 Bcl-2, Raf-1 and mitochondrial regulation of apoptosis. *Biofactors* **8** 13–16.
- Wang HG, Takayama S, Rapp UR & Reed JC 1996 Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *PNAS* **93** 7063–7068.
- Witcher M, Yang X, Pater A & Tang SC 2001 BAG-1 p50 isoform interacts with the vitamin D receptor and its cellular over-expression inhibits the vitamin D pathway. *Experimental Cell Research* **265** 167–173.
- Wu S, Ren S, Chen H, Chun RF, Gacad MA & Adams JS 2000 Intracellular vitamin D binding proteins: novel facilitators of vitamin D-directed transactivation. *Molecular Endocrinology* **14** 1387–1397.
- Wu S, Chun R, Gacad MA, Ren S, Chen H & Adams JS 2002 Regulation of 1,25-dihydroxyvitamin d synthesis by intracellular vitamin D binding protein-1. *Endocrinology* **143** 4135.
- Young JC, Barral JM & Ulrich Hartl F 2003 More than folding: localized functions of cytosolic chaperones. *Trends in Biochemical Sciences* **28** 541–547.
- Zeiner M & Gehring U 1995 A protein that interacts with members of the nuclear hormone receptor family: identification and cDNA cloning. *PNAS* **92** 11465–11469.

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