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

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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 (Kᵢ = 0.71 ± 0.25 nM, Bₘₐₓ = 69.9 ± 16.1 fmol/µg protein) and 1,25(OH)₂D₃ (Kᵢ = 0.16 ± 0.07 nM, Bₘₐₓ = 38.1 ± 5.5 fmol/µg protein; both n = 3 separate binding assays, P < 0.001 for Kᵢ and Bₘₐₓ). 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 1α-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 1α- 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. 2006). 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(OH)₂D₃ (Gacad & Adams 1998). Functional analyses showed that overexpression of hsc70 in human kidney cells enhanced mitochondrial metabolism of 25-OHD₃ via the enzyme 25-hydroxyvitamin D₃-1α-hydroxylase which catalyzes synthesis of 1,25(OH)₂D₃ (Wu et al. 2002). However, hsc70 also stimulated VDR-mediated transactivation in the presence of 1,25(OH)₂D₃ (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(OH)₂D₃ 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(OH)₂D₃ 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(OH)₂D₃, we have assessed the effects of the hsc70 co-chaperone Becl2-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(OH)₂D₃ was greater than that observed for 25-OHD₃. 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 ([³H]25-OHD₃, specific activity, 187 Ci/mmol) and ([³H]1,25(OH)₂D₃, 179 Ci/mmol) were purchased from Amersham Biosciences. Unlabeled 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and 25-hydroxyvitamin D (25-OHD₃) 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 [³H]25-OHD₃ or [³H]1,25(OH)₂D₃ 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-OHD₃ or 1,25(OH)₂D₃, 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 EDT 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 fmols [³H]25-OHD₃ or [³H]1,25(OH)₂D₃ bound to μg recombinant BAG-1, and plotted against total concentration of [³H]25-OHD₃ or [³H]1,25(OH)₂D₃ in the binding assay (saturation analyses) or bound metabolite/free metabolite (Scatchard plots). The latter plots were used to define binding kinetics: maximal binding (Bmax, intercept with x axis); dissociation constant (Kd, slope of regression line). Three separate binding assays for [³H]25-OHD₃ or [³H]1,25(OH)₂D₃ were carried out and used to present mean Kd and Bmax values ± 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 Na₂HPO₄ and 150 mM NaCl₂ (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-OHΔD 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**

1α- 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 25OHD₃ and [³H]25-OHD₃; 180 G/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 Zobax 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-OHΔD, 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-OHΔD, 24,25(OH)₂D₃ or 1,25(OH)₂D₃. Data were reported as mean fmoles metabolite synthesized/h per mg cellular protein ± 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 Kₘ (Kᵢ) for binding of 25-OHΔD₃ 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-OHΔD₃ (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
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-OHD₃ or 1,25(OH)₂D₃ (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 transfec tant 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-OHD₃ and 1,25(OH)₂D₃. 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 1 BAG-1 specifically binds vitamin D metabolites. (A) and (B) Dose-dependent binding of [³H] 25-OHD₃ and [³H]1,25(OH)₂D₃ to recombinant BAG-1. Data show bound metabolite (fmoles/µg recombinant BAG-1 protein) versus concentration of metabolite added (nM). Total bound [³H]25-OHD₃ or [³H]1,25(OH)₂D₃ 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 [³H]25-OHD₃ and [³H]1,25(OH)₂D₃ binding to recombinant BAG-1. Data show bound/free metabolite versus specifically bound metabolite (fmoles/µg protein). Kₘ values were derived from the slope of each plot and maximal binding (Bₘₐₓ) values were derived from the intercept with the x axis.

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).
responses to the former were due to endogenous conversion to (V), 25-OHD3 (25D) or 1,25(OH)2D3 (1,25D) to confirm that promoter–reporter assays in the absence or presence of vehicle mutant BAG-1Sm (Sm), or empty CMV vector (C) 3C. The 1 constructs for human BAG-1L (L), BAG-1M (M), BAG-1S (S) 24 h in HKC-8 kidney cells transiently transfected with expression (0.1% ethanol), 1,25(OH)2D3 (5 nM) or 25-OHD3 (200 nM) for CYP24 gene promoter vehicle were treated with either vehicle the met abolite 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 1α- 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 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).

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 1α-hydroxylase inhibitor ketoconazole (1 μM) 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 1,25(OH)2D3. Data shown are the mean luciferase reporter units ± 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 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 1α-hydroxylase inhibitor ketoconazole (1 μM) 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 1,25(OH)2D3. Data shown are the mean luciferase reporter units ± 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.
that transfection of empty vector, or expression constructs for BAG-1S or mutant BAG-1Sm had no effect on cytosolic binding of 25-OH-D$_3$. However, transfection of wild type BAG-1S resulted in a twofold increase in 1,25(OH)$_2$D$_3$ binding when compared with cells receiving empty vector only. This effect was completely abrogated when the mutant form of BAG-1S was used.

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 (Bimston 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-D$_3$ and 1,25(OH)$_2$D$_3$ 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-D$_3$ and 1,25(OH)$_2$D$_3$ 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.
There are some important differences between the two proteins. First, the binding kinetics for 25-OHD$_3$ and 1,25(OH)$_2$D$_3$ are clearly different, with BAG-1 showing greater affinity for active 1,25(OH)$_2$D$_3$, whilst hsc70 exhibits greater affinity for the inactive precursor 25-OHD$_3$ (Gacad & Adams 1998). As a consequence, 1,25(OH)$_2$D$_3$ may favor BAG-1 as a binding site, whilst 25-OHD$_3$ 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)$_2$D$_3$ 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)$_2$D$_3$). If this is the case, then we can further speculate that the putative change in binding of 1,25(OH)$_2$D$_3$ will facilitate transfer of the steroid to hsc70-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)$_2$D$_3$ is similar to that observed with hsc70 in the absence of ATP ($K_d<$0.5 nM), but it is significantly greater than ATPase-occupied hsc70 ($K_d>1.0$ nM). Thus, ATPase-mediated association between BAG-1 and hsc70 would favor binding of 1,25(OH)$_2$D$_3$ 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)$_2$D$_3$-mediated transactivation and mitochondrial CYP27b1-mediated metabolism of 25-OHD$_3$ to 1,25(OH)$_2$D$_3$ (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-OHD$_3$. Moreover, when over-expressed in HKC-8 cells BAG-1 enhanced intracellular binding of 1,25(OH)$_2$D$_3$ whilst having no significant effect on 25-OHD$_3$ binding. These data provide further evidence that BAG-1 has greater specificity for 1,25(OH)$_2$D$_3$ when compared with 25-OHD$_3$ 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)$_2$D$_3$. Specifically, mutation of amino acids 169 and 172 of BAG-1S inhibited binding of 1,25(OH)$_2$D$_3$. 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)$_2$D$_3$ 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)$_2$D$_3$ and 25-OHD$_3$, 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-OHD$_3$). The doses of 25-OHD$_3$ (200 nM) and 1,25(OH)$_2$D$_3$ (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)$_2$D$_3$ would be considered supra-physiological. It was therefore interesting to note that 25-OHD$_3$ was as effective as 1,25(OH)$_2$D$_3$ in stimulating CYP24 promoter activity, and that BAG-1 isoforms were able to potentiate responses to both 1,25(OH)$_2$D$_3$ and 25-OHD$_3$. 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. 1998, Kullmann et al. 1999, Guzey M, Takayama S & Reed JC 2000 Vitamin D receptor and its ligand in human monocyte-derived dendritic cells. Molecular and Cellular Endocrinology 195 31–38).

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