The vitamin D receptor (VDR) is a member of the steroid/retinoid receptor superfamily of nuclear receptors that has potential tumor-suppressive functions. We show here that VDR interacts with and is regulated by BAG1L, a nuclear protein that binds heat shock 70-kDa (Hsp70) family molecular chaperones. Endogenous BAG1L can be co-immunoprecipitated with VDR from prostate cancer cells (ALVA31; LNCaP) in a ligand-dependent manner. BAG1L, but not shorter non-nuclear isoforms of this protein (BAG1; BAG1M/Rap46), markedly enhanced, in a ligand-dependent manner, the ability of VDR to trans-activate reporter gene plasmids containing a vitamin D response element in transient transfection assays. Mutant BAG1L lacking the C-terminal Hsp70-binding domain suppressed (in a concentration-dependent fashion) VDR-mediated trans-activation of vitamin D response element-containing reporter gene plasmids, without altering levels of VDR or endogenous BAG1L protein, suggesting that it operates as a trans-dominant inhibitor of BAG1L. Gene transfer-mediated elevations in BAG1L protein levels in a prostate cancer cell line (PC3), which is moderately responsive to VDR ligands, increased the ability of natural (1α,25(OH)2 vitamin D3) and synthetic (1α,25-dihydroxy-19-nor-22(E)-vitamin D3) VDR ligands to induce expression of the VDR target gene, p21Waf1, and suppress DNA synthesis. Thus, BAG1L is a direct regulator of VDR, which enhances its trans-activation function and improves tumor cell responses to growth-suppressive VDR ligands.

1α,25(OH)2 vitamin-D3, a steroidal hormone which controls calcium homeostasis and bone formation, increases the expression of VDR. The effects of 1α,25(OH)2 vitamin D3 are mediated via interaction with a specific nuclear receptor (VDR) (1). The VDR is a ligand-dependent transcriptional regulator, belonging to the nuclear receptor (NR) superfamily (reviewed in Ref. 3). VDR primarily interacts with specific DNA sequences composed of a hexanucleotide of direct repeat, binding as either a homodimer or as heterodimer with retinoid X receptors (RXRs) (4).

Known target genes of VDR regulation include the cell cycle inhibitors p21Waft and p27Kip1 (5), perhaps accounting in part for the anti-proliferative effects of VDR ligands on some types of cells. Growth suppressive effects of VDR ligands on epithelial cancer cells in vitro have prompted interest in the possibility of applying natural or synthetic VDR ligands for the treatment of cancer (reviewed in Ref. 6). Prostate cancer is among the types of tumor cells with documented sensitivity to VDR ligands. 1α,25(OH)2 vitamin D3 and its less calcemic synthetic analogues have been shown to inhibit in vivo growth of established human prostate carcinoma cell lines and primary cultures of normal and prostate cancer cells (7, 8). Depending on the particular cell line tested, VDR ligands can induce cell cycle arrest, differentiation, apoptosis, or combinations of these events (9). Functional VDR is necessary for the growth-inhibitory effect of VDR. However, prostate cancer cell lines vary in their sensitivity to 1α,25(OH)2 vitamin D3 and its synthetic analogues in ways that cannot be explained by differences in VDR protein levels or rates of ligand metabolism, suggesting the existence of mechanisms for modulating VDR function at a post-ligand binding step. This variability in bioresponses to synthetic vitamin D3 analogues has also been observed in vivo in clinical trials involving men with advanced prostate cancer (10).

The human BAG1 gene encodes several proteins, including BAG1, BAG1M (Rap46), and BAG1L, which differ in the length of their N-terminal domains but which all share a conserved C-terminal domain that binds the ATPase domain of heat shock 70-kDa (Hsp70) family molecular chaperones (11). Some of the BAG1 protein isoforms have been shown to interact with and regulate the activity of certain members of the steroid hormone/retinoid superfamily of NRs (12–14). For example, BAG1 binds and suppresses retinoic acid receptors (RARs) (13), BAG1M (Rap46) interacts with and inhibits glucocorticoid receptors (15), and BAG1L associates with and enhances the trans-activation function of androgen receptors (AR) (14). In this report, we examined the relation of BAG1 proteins to VDR. Our findings indicate that the longest of the BAG1 protein isoforms, BAG1L, interacts with VDR in a ligand-inducible manner, enhancing VDR function and improving prostate cancer cellular responses to the growth suppressive effects of vitamin D3 analogues. Thus, levels of BAG1L may be one of the determinants of vitamin D3 responses in normal and malignant tissues.

MATERIALS AND METHODS

VDR Ligands—1α,25(OH)2 vitamin D3 and 1α,25-dihydroxy-19-nor-22(E)-vitamin D3 were generously provided by Dr. H. F. DeLuca (Uni-
versity of Wisconsin, Madison, WI) (16). These VDR ligands were prepared as 10⁻³ m stock solutions in ethanol and stored at -20 °C. Stock solution concentrations were confirmed by spectrophotometry (Spectra Max 190, Molecular Devices), using an extinction coefficient of 220–290 nm for 1a,25(OH)2 vitamin D3. The spectrophotometric confirmation for 1a,25-dihydroxyvitamin D3 (22,23-E)-vitamin D3 was slightly modified as described previously (16).

**Cell Culture**—The human prostate cancer cell lines PC-3 and LNCaP, the transformed human embryonal kidney line 293, and monkey kidney COS-7 cell lines were obtained from the American Type Culture Collection (Rockville, MD). The ALVA 31 human prostate cancer cell line was generously provided by Dr. G. Miller (17). Cells were maintained in a humidified atmosphere with 5% CO2 in RPMI 1640 (PC3, LNCaP, ALVA31) or Dulbeco’s modified Eagle’s medium (293 and COS-7) supplemented with 10% fetal calf serum, 1 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). For most experiments, cells were cultured in medium in which the serum had been preabsorbed with activated charcoal to deplete steroid hormones (14).

**Transfections**—COS-7 and 293T cells were transiently transfected by a LipofectAMINE method. Cells at ~50% confluence (~2 × 10⁶ cells) were seeded per well in six-well (9.4 cm²) plates (Corning, New York) in 2 ml/well of steroid-depleted medium. The next day, 2.2 μg of DNA was combined with 6 μl of LipofectAMINE in a total volume of 375 μl of Opti-MEM (Life Technologies, Inc.) and incubated for ~0.5 h. Adherent cells were then washed twice with serum-free pre-warmed Opti-MEM, and then DNA/LipofectAMINE mixtures were applied in 750 μl of Opti-MEM. After culturing at 37 °C and 5% CO2 for 3 h, 1.5 ml of 20% calf serum containing medium was added per well. The following day, various concentrations of 1α,25(OH)₂ vitamin D3 or 1α,25-dihydroxy-19-nor-22(E)-vitamin D3 were added and cells were cultured for up to 3 days before preparing lysates for various assays.

For stable transfections, 2.2 μg of either supercoiled or Scal-cut pUC/CAM-VBAG1L, plasmid DNA, encoding BAG1L protein (11) or pUC/CAM parental vector was transfected into PC-3 cells using the LipofectAMINE method, essentially as described above. After 2 days, cells were cultured in medium containing 6.6 mg/ml (active drug) G418 (Life Technologies, Inc.). Medium was replaced twice weekly, until colonies of stably transfected clones arose. Clones were individually recovered and expanded in culture.

**Reporter Gene Assays**—Transient transfection reporter gene assays were employed for monitoring VDR trans-activation function. Briefly, cells were transiently transfected as described above with various amounts (20, 50, 100, and 150 ng) of pcDNA3 plasmids encoding BAG1L (BAG1LΔC), BAG1L, BAG1LΔC, or BAG1L (20–200 ng) (11, 14) (see Fig. 1), together with 250 ng of a plasmid encoding one copy of a VDRE upstream of a thymidine kinase minimal promoter and the luciferase (CAT) reporter (Promega), normalized for total protein content, and relative levels of luciferase were then measured by incorporation of bromodeoxyuridine (BrdUrd), then expressed as fold activation relative to a defined control, usually cells cultured without VDR ligands or cells transfected without BAG1L-encoding plasmids.

**Cell Cycle Analysis**—The percentage of cells undergoing DNA synthesis was measured by incorporation of bromodeoxyuridine (BrdUrd), essentially as described (21, 22). Stably transfected clones of PC-3 cells containing pUC/CAM control (“Neo”) or pUC/CAM-VBAG1L plasmids were seeded at 7.5 × 10⁴ cells/six-well plate in 2 ml of normal medium for 24 h, then changed to medium in which the serum had been preabsorbed with activated charcoal to deplete steroid hormones. In some cases, VDR ligands were added, as indicated, and cultures were continued for up to 48 h. Cells were then collected by trypsinization, and fixed in 70% ethanol (final concentration) and 1% glacial acetic acid for 30 min. Fixed cells were then washed with phosphate-buffered saline (pH 7.4) containing 0.5% (w/v) bovine serum albumin (BSA), then exposed to 2 μl HCl, 0.5% BSA at room temperature for 20 min before re-washing with PBS/BSA and re-suspending in 0.5 ml of 1 M sodium borate (Na₂B₄O₇) (pH 8.7) for 2 min at room temperature, and washing a final time with PBS/BSA. Cells were incubated in PBS/BSA with 20 μl of fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (PharMingen) for 20 min in the dark. Finally, propidium iodide (50 μg/ml) was added and the cells were analyzed using a fluorescein-activated cell sorter (Becton Dickinson FACStar-Plus) using the Cell Quest and Mod-Fit programs.

**Antibodies and Immunoblotting**—Cell lysates were prepared using radioimunoprecipitation assay buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA), normalized for total protein content (25 μg of protein), and subjected to SDS-PAGE using 12% gels, followed by electrophoresis to 0.45-μm nitrocellulose transfer membranes (Bio-Rad). Blots were incubated as described (11, 14), with the following primary antibodies, including 1:1000 (v/v) of a mouse monoclonal specific for human VDR (IVG8C11) (gift of Dr. H. F. DeLuca) (23), 1:1000 (v/v) of a rabbit polyclonal anti-VDR IgG (Santa Cruz Biotechnology, Inc., Santa Barbara, CA), 1:1000 (v/v) control normal rabbit IgG (Santa Cruz Biotechnology, Inc.), 1:1000 (v/v) control mouse IgG, (Dako, Inc.), 1:1000 (v/v) of mouse anti-human p21 monoclonal antibody (IgG) (PharMingen, San Diego, CA). Immunodetection was accomplished essentially as described (11, 14) using horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) and an enhanced chemiluminescence detection method (ECL) (Amersham/Pharmacia Biotech) with exposure to x-ray film (XAR, Eastman Kodak Co.).

**Co-immunoprecipitations**—Untransfected ALVA31 and LN-CaP cells or 293T cells transfected with plasmids encoding BAG1L or BAG1LΔC were cultured with or without 5 × 10⁻⁶ M 1α,25(OH)₂ vitamin D₃, and collected at 70% confluency. Cells were lysed on ice in HKMEN (10 mM HEPES (pH 7.2), 142 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.2% Nonidet P-40) containing protease inhibitors (Roche Molecular Biochemicals), then either passed several times through a 21-gauge needle to disrupt nuclei or NE-PER nuclear extraction reagent (Pierce) was added according to the manufacturer’s protocol. After centrifugation at 10,000 × g for 25 min, the resulting supernatants from equivalent numbers of cells were subjected to immunoprecipitations in HKMEN using the anti-BAG1 monoclonal KS6C8, anti-VDR monoclonal IVGSC11, or anti-VDR polyclonal antiserum, bound to protein G-agarose (Zymed Laboratories Inc., San Francisco, CA). Control immunoprecipitations were performed using mouse IgG₁, (Dako) or non-immune rabbit serum (Santa Cruz). Immune-complexes were washed three times with 1 ml of HKMEN and analyzed by SDS-PAGE/immunoblotting, as above.

**Results**

**BAG1L Enhances Trans-activation Function of VDR**—We initially explored the effects of BAG1 proteins on the trans-activation function of the VDR using transient transfection reporter gene assays in HEK 293T and COS-7 cells. Several isoforms of the BAG1 protein were compared, as depicted in Fig. 1. These isoforms of BAG1 all contain a conserved C-terminal domain “BAG domain” responsible for high affinity interactions with Hsc70/Hsp70 molecular chaperones and they
FIG. 2. BAG1L enhances trans-activation function of VDR. COS-7 cells at ~50% confluence in six-well plates (area 9.4 cm²) in steroid-depleted medium were transfected by a LipofectAMINE method with VDR encoding plasmid (190 ng), VDRE-CAT reporter plasmid (250 ng), pCMV-βgal (400 ng), and various amounts of BAG1-encoding plasmids as indicated, normalizing total DNA to 2.2 μg/well. After 1 day, cells were stimulated with 5 × 10⁻⁹ M 1α,25(OH)₂ vitamin D₃. Cell extracts were prepared and assayed for CAT and β-galactosidase activity, expressing normalized data as a ratio relative to transfected cells which received pcDNA3 control DNA instead of a BAG1-expression plasmid (mean ± S.E.; n = 3). In A, plasmids encoding BAG1L (triangles), BAG1M (squares), or BAG1L (circles) were transfected in amounts of 20, 50, and 100 ng (reported as ng/cm²). In B, plasmids encoding BAG1L (circles) or BAG1LΔC mutant (squares) were transfected using 20, 50, 100, or 150 ng of these plasmid DNAs, as indicated.

FIG. 3. Immunoblot analysis of expression of BAG1 isoforms in transfected cells. COS-7 cell plasmids in 60-mm dishes (growth area: 21 cm²) were transiently transfected using a LipofectAMINE reagent with 220 ng of VDR and 330 ng of either pcDNA3 (neo) or pcDNA3-plasmids encoding BAG1L, BAG1M, BAG1LΔC, BAG1, or BAG1S. After 2 days, whole cell lysates were prepared, normalized for total protein content (25 μg/lane), and subjected to SDS-PAGE/immunoblot assay, using anti-BAG1 (A) or anti-VDR (B) antibodies in conjunction with an ECL-based detection method. In C, 293T cells were transfected with 220 ng of VDR and increasing amounts of pcDNA3-BAG1L DNA (lanes 2–6, 44, 110, 220, 330, and 440 ng, respectively) (reported as ng/cm²) for area of 21 cm²), normalizing total DNA content (~5 μg in 60-mm dishes) with pcDNA3 (Neo) vector. Lysates were prepared after 2 days and analyzed as above using anti-BAG1 antibody. Molecular size markers are indicated in kilodaltons.

possess an upstream ubiquitin-like domain, but they differ in the length of their N-terminal domains. The various isoforms of BAG1L arise by translation from alternative initiation codons within a common mRNA (11, 25). Among these proteins, only BAG1L contains both nucleoplasmin-like and SV40 large T-like candidate nuclear targeting sequences and is constitutively localized to nuclei (11, 25).

To enforce expression of selected isoforms of BAG1, a cDNA encoding the longest isoform, BAG1L, and various 5’-truncated versions of cDNAs encoding BAG1M, BAG1, and BAG1S were subcloned into an expression plasmid, with additional modifications as described previously (11, 14, 24). These plasmids were then co-transfected in various amounts with a fixed amount of plasmid encoding VDR and a VDRE-CAT reporter gene plasmid. Cells were supplied with physiologically relevant concentrations (5 × 10⁻⁹ M) of 1α,25(OH)₂ vitamin D₃. All data were normalized relative to cells stimulated with 1α,25(OH)₂ vitamin D₃ in the absence of BAG1 expression plasmids.

As shown in Fig. 2, the longest isoform, BAG1L, enhanced the trans-activation function of VDR in a concentration-dependent manner, resulting in a 2–4-fold increase in VDRE-tk-CAT reporter gene activation under these conditions. In contrast, BAG1M, BAG1, and BAG1S had little effect on VDR activity (Fig. 2 and data not shown). Moreover, although full-length BAG1L effectively enhanced VDR activity, a mutant of BAG1L lacking the C-terminal Hsc70/Hsp70-binding domain did not. This observation confirms the specificity of these findings obtained with full-length BAG1L and also suggests that the C-terminal BAG domain of BAG1L is required for potentiating VDR activity.

Immunoblot analysis was performed to verify production of the BAG1L, BAG1LΔC, BAG1M, BAG1, and BAG1S proteins in transfected cells. As shown in Fig. 3, BAG1L, BAG1LΔC, BAG1, and BAG1S were produced at comparable levels. The BAG1M protein was also produced, but due to internal translation initiation from the AUG, which normally gives rise to the shorter BAG1 protein, the steady-state levels of BAG1M achieved were only about half the other isoforms. Nevertheless, BAG1M protein was produced at levels far in excess of endogenous BAG1 and BAG1L, which migrate at ~35 and ~55 kDa and which can be seen as faint bands in the immunoblot analysis (Fig. 3). We conclude, therefore, that the selective enhancement of VDR activity seen with BAG1L but not with other BAG1 isoforms in transient transfection reporter gene assays is unlikely to be due to differences in the levels of production of these proteins. Importantly, expression of the various isoforms of BAG1 had no effect on levels of VDR, thus excluding alterations in the receptor for 1α,25(OH)₂ vitamin D₃ as a trivial explanation for the results obtained in reporter gene assays (Fig. 3B). Immunoblotting also confirmed the concentration dependence of BAG1L protein production in response to transfection of various amounts of expression plasmid DNA (Fig. 3C), further validating the results obtained by reporter gene assays.

1α,25(OH)₂ Vitamin D₃ Induces Association of BAG1L with
BAG1L Enhances VDR Trans-activation

VDR—The functional collaboration of BAG1L with VDR prompted us to explore whether these proteins physically interact, particularly given evidence that BAG1L can associate with certain other members of the NR family (13–15). Co-immunoprecipitation assays were performed using lysates from untransfected ALVA-31 (Fig. 4) and LNCaP (data not shown) prostate cancer cells to explore whether the endogenous BAG1L and VDR proteins can form complexes. These lines were chosen because they contain relatively high intrinsic levels of both BAG1L and VDR, and because they are sensitive to 1α,25(OH)2 vitamin D3-induced growth suppression (10). BAG1L was immunoprecipitated from lysates prepared from unstimulated and 1α,25(OH)2 vitamin D3-treated cells, and the resulting immune complexes were analyzed by SDS-PAGE/immunoblotting using an anti-VDR antibody.

Anti-BAG1 immunoprecipitates prepared from lysates of 1α,25(OH)2 vitamin D3-treated cells contained associated VDR, whereas VDR was not found associated with BAG1L immune complexes derived from unstimulated ALVA31 (Fig. 4) or LNCaP (data not shown) cells. Control immunoprecipitates prepared using mouse IgG1 instead of anti-BAG1 antibody confirmed the specificity of these results. Comparisons of the levels of VDR and BAG1L proteins in ALVA31 and LNCaP lysates before and after treatment with 1α,25(OH)2 vitamin D3 revealed no demonstrable difference, indicating that the association is not merely secondary to ligand-induced changes in the amounts of these proteins (data not shown). We conclude, therefore, that VDR associates with BAG1L in a ligand-dependent manner.

FIG. 4. BAG1L associates with and modulates function of VDR in a ligand-dependent manner. In A, lysates were prepared using HKMEN solution from ALVA31 prostate cells grown in the absence (–) or presence (+) of 5 × 10−8 M 1α,25(OH)2 vitamin D3. Immunoprecipitations were performed using anti-BAG1 monoclonal (IgG1) K86C8 (14) (lanes 2 and 3), rabbit polyclonal IgG VDR antibody (Santa Cruz) (lane 4), rabbit IgG control (lane 5), or mouse IgG1 control (lane 6). Immune complexes were analyzed by SDS-PAGE immunoblotting using a polyclonal rabbit anti-VDR with ECL-based detection. As a control, lysate (25 μg) from 1α,25(OH)2 vitamin D3-treated cells was also directly loaded in the gel (lane 1). The positions of the heavy (IgH) and light (IgL) chains of the primary antibodies are indicated (right), as well as the position of the VDR (left). In B, COS-7 cells in steroid-depleted medium were transfected as described in Fig. 2, using 100 ng of pcDNA3-BAG1L (+) or pcDNA3 control (−) plasmid DNA. After 1 day, either 5 × 10−8 M 1α,25(OH)2 vitamin D3 (+) or control diluent (−) was added to cultures. Lysates were prepared 48 hours later, and relative CAT production from the VDR-CAT reporter gene plasmid was measured, normalizing for β-galactosidase and expressing the data as fold activation relative to cells that received neither the VDR nor BAG1L plasmids and which were not stimulated with VDR ligand.

VDR association with BAG1L was detected BAG1L deficient HKMEN solution from ALVA31 prostate cells grown in the absence (+) or presence (−) of 1α,25(OH)2 vitamin D3. Immunoprecipitations were performed using anti-BAG1 monoclonal (IgG1) K86C8 (14) (lanes 2 and 3), rabbit polyclonal IgG VDR antibody (Santa Cruz) (lane 4), rabbit IgG control (lane 5), or mouse IgG1 control (lane 6). Immune complexes were analyzed by SDS-PAGE immunoblotting using a polyclonal rabbit anti-VDR with ECL-based detection. As a control, lysate (25 μg) from 1α,25(OH)2 vitamin D3-treated cells was also directly loaded in the gel (lane 1). The positions of the heavy (IgH) and light (IgL) chains of the primary antibodies are indicated (right), as well as the position of the VDR (left).

VDR association with BAG1L was detected BAG1L deficient HKMEN solution from ALVA31 prostate cells grown in the absence (+) or presence (−) of 1α,25(OH)2 vitamin D3. Immunoprecipitations were performed using anti-BAG1 monoclonal (IgG1) K86C8 (14) (lanes 2 and 3), rabbit polyclonal IgG VDR antibody (Santa Cruz) (lane 4), rabbit IgG control (lane 5), or mouse IgG1 control (lane 6). Immune complexes were analyzed by SDS-PAGE immunoblotting using a polyclonal rabbit anti-VDR with ECL-based detection. As a control, lysate (25 μg) from 1α,25(OH)2 vitamin D3-treated cells was also directly loaded in the gel (lane 1). The positions of the heavy (IgH) and light (IgL) chains of the primary antibodies are indicated (right), as well as the position of the VDR (left).
Cells were cultured in the presence of either the natural VDR ligand, 1,25(OH)₂D₃, or were transfected with various amounts of plasmid DNA encoding BAG1L, COS-7 or HEK 293T cells were transiently co-transfected with an equal amount of pcDNA3 control plasmid. After 1 day, cells were stimulated with 5 × 10⁻⁹ M 1,25(OH)₂D₃ vitamin D₃ and cell extracts were prepared 2 days later and assayed for CAT and β-galactosidase activity. Data were normalized using β-galactosidase, and results expressed as fold-activation relative to 1,25(OH)₂D₃ vitamin D₃-stimulated cells, which received the VDR expression vector in combination with pcDNA3 control plasmid. B COS-7 cells in 60-mm dishes (area 21 cm²) were transiently transfected with 330 ng of pcDNA3-BAG1L, 220 ng of VDR, and increasing amounts of pcDNA3-BAG1L(ΔC) (lanes 2–5, 44, 110, 220, 330, and 440 ng, respectively) (reported as ng/cm²). After 2 days, whole cell lysates were prepared, normalized for total protein (25 μg/lane), and subjected to SDS-PAGE/immunoblot assay using anti-BAG1 antibody. The positions of the BAG1L and BAG1L(ΔC) proteins are indicated (arrowheads). Molecular size markers are indicated in kilodaltons. C 293T cells in 100-mm dishes were transiently transfected with equivalent amounts of plasmids (~1 μg each) encoding VDR, and either full-length BAG1L or BAG1L(ΔC). Cells were treated 1 day later with 5 × 10⁻⁸ M 1,25(OH)₂D₃ vitamin D₃, then collected at 2 days after transfection, lysed on ice in HKMEN buffer, and immunoprecipitations were performed using either anti-BAG1 monoclonal KS6C8 or mouse IgG control antibody. Immune complexes were directly run in the gel for comparison with immunoprecipitates. The blot was reprobed with anti-BAG1 antibody (lower panel) to verify production of the BAG1L and BAG1L(ΔC) proteins (indicated by arrowheads).

**Fig. 5. BAG1L(ΔC) mutant inhibits VDR function.** A, HEK 293T cells were transiently transfected with 200 ng of VDR, 250 ng of the VDRE-CAT, 400 ng of pCMV-βgal, 250 ng of pcDNA3-BAG1L, and various amounts of pcDNA3-BAG1L(ΔC) (20–800 ng, reported as ng/cm²) or an equal amount of pcDNA3 control plasmid, total DNA normalized to 2.2 μg/well (six-well plates, growth area 9.4 cm²) by addition of pcDNA3 control plasmid. After 1 day, cells were stimulated with 5 × 10⁻⁹ M 1,25(OH)₂D₃ vitamin D₃ and cell extracts were prepared 2 days later and assayed for CAT and β-galactosidase activity. Data were normalized using β-galactosidase, and results expressed as fold-activation relative to 1,25(OH)₂D₃ vitamin D₃-stimulated cells, which received the VDR expression vector in combination with pcDNA3 control plasmid. B COS-7 cells in 60-mm dishes (area 21 cm²) were transiently transfected with 330 ng of pcDNA3-BAG1L, 220 ng of VDR, and increasing amounts of pcDNA3-BAG1L(ΔC) (lanes 2–5, 44, 110, 220, 330, and 440 ng, respectively) (reported as ng/cm²). After 2 days, whole cell lysates were prepared, normalized for total protein (25 μg/lane), and subjected to SDS-PAGE/immunoblot assay using anti-BAG1 antibody. The positions of the BAG1L and BAG1L(ΔC) proteins are indicated (arrowheads). Molecular size markers are indicated in kilodaltons. C 293T cells in 100-mm dishes were transiently transfected with equivalent amounts of plasmids (~1 μg each) encoding VDR, and either full-length BAG1L or BAG1L(ΔC). Cells were treated 1 day later with 5 × 10⁻⁸ M 1,25(OH)₂D₃ vitamin D₃, then collected at 2 days after transfection, lysed on ice in HKMEN buffer, and immunoprecipitations were performed using either anti-BAG1 monoclonal KS6C8 or mouse IgG control antibody. Immune complexes were analyzed by SDS-PAGE immunoblotting, using a polyclonal rabbit anti-VDR antisera with ECL-based detection. Lysates (25 μl) were also run directly in the gel for comparison with immunoprecipitates. The blot was reprobed with anti-BAG1 antibody (lower panel) to verify production of the BAG1L and BAG1L(ΔC) proteins (indicated by arrowheads).

**BAG1L Enhances VDR Trans-activation**

To extend the analysis of BAG1L to an endogenous target of VDR, we explored the effects of VDR ligands on induction of p21Waf1 protein production in PC3 cells. PC3 cells contain moderate levels of VDR (800 fmol/mg protein), but express BAG1L at low levels (10). For these experiments, therefore, PC3 cells were stably transfected with either a control plasmid or BAG1L-encoding plasmid and several clones were characterized. Immunoblot analysis revealed several stably transfected clones with elevated levels of BAG1L protein compared with control-transfected or parental PC3 cells. These clones were then cultured with either natural VDR ligand, 1α,25(OH)₂D₃ vitamin D₃ (5 × 10⁻⁸ M) or vitamin D₃ analogue 1α,25-dihydroxy-19-nor-22(E)-vitamin D₃ (5 × 10⁻⁹ M) (Fig. 6, A and B) or the synthetic vitamin D₃ analogues 1α,25-dihydroxy-19-nor-22(E)-vitamin D₃ (5 × 10⁻⁹ M) (Fig. 6, C and D).

BAG1L induced a dose-dependent increase in VDR-mediated trans-activation of the p21Waf1 promoter in these transient transfection reporter gene assays when VDR ligands were supplied (Fig. 6) but not in the absence of ligands (data not shown). The effect of BAG1L appeared to be more pronounced, in terms of -fold enhancement of reporter gene activation, when the vitamin D₃ analogue 1α,25-dihydroxy-19-nor-22(E)-vitamin D₃ was employed, compared with the natural VDR ligand, 1α,25(OH)₂D₃ vitamin D₃. However, because maximal BAG1L plasmid DNA concentrations (plateau) were not reached in these experiments, quantitative comparisons should be interpreted cautiously.

BAG1L enhances VDR-mediated induction of the endogenous p21Waf1 gene. To extend the analysis of BAG1L to an endogenous target of VDR, we explored the effects of VDR ligands on induction of p21Waf1 protein production in PC3 cells. PC3 cells contain moderate levels of VDR (800 fmol/mg protein), but express BAG1L at low levels (10). For these experiments, therefore, PC3 cells were stably transfected with either a control plasmid or BAG1L-encoding plasmid and several clones were characterized. Immunoblot analysis revealed several stably transfected clones with elevated levels of BAG1L protein compared with control-transfected or parental PC3 cells. These clones were then cultured with either natural VDR ligand, 1α,25(OH)₂D₃ vitamin D₃ (5 × 10⁻⁸ M) or vitamin D₃ analogue 1α,25-dihydroxy-19-nor-22(E)-vitamin D₃ (5 × 10⁻⁸ M) for 1–3 days, and lysates were prepared for immunoblot analysis of p21Waf1 protein levels.

Both natural and synthetic VDR ligands induced greater increases in p21Waf1 protein levels in BAG1L-overexpressing PC3 cells compared with controls. Increases in p21Waf1 were sometimes more rapid in BAG1L-overexpressing compared with control-transfected cells. Re-probing the blots with an antibody to β-actin verified loading of equivalent amounts of total protein. Although representative data are provided for two clones in Fig. 7, similar findings were obtained with others (data not shown).

**BAG1L Sensitizes PC3 Cells to Growth Inhibition by VDR Ligands**—The effects of VDR ligands on p21Waf1 expression in PC3 cells suggested that BAG1L-overexpressing cells might display greater sensitivity to the growth suppressive effects of vitamin D₃ analogues. Accordingly, clones of PC3 control (Neo-)transfected and BAG1L-transfected cells were cultured in steroid-depleted medium with or without 5 × 10⁻⁸ M 1α,25(OH)₂D₃ vitamin D₃ for 2 days, and the percentage of replicating cells was then estimated by labeling with BrdUrd, which is incor-
porated into the DNA of cells in S-phase. Comparisons were made between two Neo control-transfected clones of PC3 and six BAG1L-transfected clones. The percentage inhibition of cell proliferation induced by VDR ligand was significantly greater for the BAG1L-transfected compared with the Neo-control transfected PC3 cell clones, as determined by contrasting BrdUrd incorporation in each clone when cultured without versus with 1α,25(OH)2 vitamin D3 (p < 0.005 by unpaired t test). Similar results were obtained using the synthetic VDR ligand, 1α,25-dihydroxy-19-nor-22(E)-vitamin D3 (19-nor-22(E)) and CAT activity was measured 2 days later, normalizing data relative to β-galactosidase and reporting results as fold-activation relative to cells transfected with pcDNA3 control plasmid instead of pcDNA3-BAG1L. BAG1L did not enhance VDR activity when VDR ligands were omitted from cultures (data not shown).

DISCUSSION

In this report, we provide the first evidence that BAG1L can interact with and regulate the activity of VDR. In contrast, shorter isoforms of BAG1, including BAG1M, BAG1, and BAG1S, lacked the ability to enhance the trans-activation function of VDR. In human cells, at least four BAG1 isoforms can arise from translation-initiation from alternative start codons within a common mRNA, resulting in proteins that all share a common C terminus but that can be distinguished by the length of their N termini: BAG1S, BAG1, BAG1M (previously also termed Rap 46/Hap 46), and BAG1L. Of these, BAG1 and BAG1L are the most abundant in vivo, with only scant amounts of BAG1M or BAG1S generally observed (11). Similarly, in mice, BAG1 and BAG1L are the most prevalent isoforms. Moreover, the bag1 mRNA molecules of mice lack the ATG required for production of BAG1M (11, 25). BAG1 is predominantly, although not exclusively, a cytosolic protein, whereas BAG1L is located entirely in the nucleus of cells (11, 14, 25, 26).

The unique ability of BAG1L to enhance VDR function in cells may be related to the nuclear location of this protein. BAG1L contains candidate nuclear localization sequences, which are not found in other BAG1 isoforms, including nucleo-plasmin-like and SV40 large-T antigen-like basic amino acid
BAG1L Enhances VDR Trans-activation

motifs commonly associated with nuclear import. In contrast, BAG1M contains only a portion of one of these candidate nuclear localization sequences and has been shown to reside in the cytosol unless stimulated to traffic into the nucleus by associating with other proteins, such as glucocorticoid receptors. However, the unique ~50-amino acid N-terminal domain of BAG1L also contains several additional copies of (EEX) repeats, which conceivably may have relevance to the ability of this protein to associate with and enhance the function of VDR. In this regard, it has been suggested that these EEX repeats in the BAG1M protein allow it to associate with DNA (28). Thus, the presence of additional copies of this repeating motif in the longer BAG1 protein conceivably could improve its ability targeting to DNA, indirectly enhancing its functional interactions with nuclear hormone receptors. However, other sequences found in the unique N-terminal region of BAG1L might account for its ability to collaborate with VDR, independently of possible DNA binding activity, such as through interactions with co-activator proteins.

The VDR binds its cognate response elements in target genes as either a homodimer (VDR/VDR) or heterodimer (VDR/RXR), leading to activation or repression of transcription via interaction with tran-scriptional co-factors and the basal transcriptional machinery. Moreover, VDR can also form heterodimers with RAR (29). Previously, we reported that the shorter BAG1 isoform can antagonize RAR activity through what appears to be a direct interaction with RAR, inhibiting binding of RAR/RXR heterodimers to retinoid response elements in vitro and suppressing RAR function in cells (13). In contrast, BAG1 does not interact with RXR and does not interfere with RXR signal-

![Graph](image-url)

**FIG. 8.** BAG1L overexpression increases sensitivity of PC3 cells to growth suppression by 1α,25(OH)2 vitamin D3. A, various clones of control (Neo) or BAG1L-transfected PC3 cells were cultured with or without 5 × 10−8 M 1α,25(OH)2 vitamin D3. BrdUrd was then added to the cultures for 1 h, and cells were recovered, fixed, and stained with anti-BrdUrd antibody, determining the percentage of cells that incorporated BrdUrd into DNA by a flow cytometry method. Data are presented as percentage inhibition based on comparison of untreated and vitamin D3-treated cells. Mean values are indicated by bars. B, immunoblot data are presented for each of the stably transfected PC3 clones, using lysates normalized for total protein content and probing blots with antibodies specific for BAG1 (top) or VDR (bottom). The position of BAG1L is indicated by an arrowhead. The endogenous BAG1 protein is also seen in the gel (not labeled). Molecular size markers are indicated in kilodaltons.

Regarding the ability of this protein to bind Hsp70/Hsc70 molecular chaperones with high affinity (11, 26), thus, if ligand binding to steroid hormone receptors causes conformational changes that permit stable Hsp70/Hsc70 binding, then this could possibly provide a mechanism by which Hsp70/Hsc70 bridges BAG1L to VDR and other members of the NR family. Indeed, deletion of the C-terminal Hsp70/Hsc70-binding domain from BAG1L abrogated its ability to associate with VDR, as determined by co-immunoprecipitation experiments. In this regard, it has been reported that Hsp70 can be found complexed to ligand-activated steroid hormone receptors (i.e. estrogen receptor, glucocorticoid receptor, and prolactin receptor) bound to DNA (32–35), further supporting this idea. However, analysis of a mutant of BAG1L lacking the C-terminal Hsc70/Hsp70-binding domain revealed a trans-dominant inhibitory function for this protein, where it suppressed rather than enhanced VDR trans-activation function. A similar trans-dominant inhibitory effect of BAG1L(ΔC) has also been reported previously for AR (14). If the only mechanism for functional interaction of BAG1L with VDR were through association with VDR/Hsp70 complexes, then we would expect deletion of the Hsp70-binding domain to nullify BAG1L effects on VDR but not to interfere with VDR in a trans-dominant manner. Since physiochemical analysis of recombinant BAG1 protein has revealed it to be a monomer (36), it also seems unlikely that the BAG1L(ΔC) mutant interfered with endogenous BAG1L by forming heterodimer/hetero-oligomers with the wild-type BAG1L protein. Thus, we favor the interpretation that BAG1L requires both the Hsc70-binding domain and other upstream regions of this protein for regulating VDR. The purpose of the upstream regions of BAG1L in this context remains to be clarified, but could include interactions with DNA, chromatin, co-activator proteins, or other types of proteins that influence nuclear hormone receptor function.

The mechanism by which BAG1L enhances the trans-activation function of VDR is currently unknown. Given the ability of this protein to bind Hsp70/Hsc70 molecular chaperones, it seems reasonable to speculate that BAG1L may act in collaboration with Hsp70/Hsc70 to produce conformational changes in VDR or VDR-associated proteins such as co-activators that result in a net enhancement of ligand-dependent transcription. However, alternative models are possible. For example, all BAG1 isoforms contain a conserved ubiquitin-like domain,
which has been suggested based on circumstantial evidence to mediate interactions with the 26 S proteosome (37). Accordingly, BAG1L conceivably could facilitate the turnover of proteins within VDR transcription complexes. Although no effect of BAG1L on the steady-state levels of VDR was observed, we cannot exclude the possibility of effects on the turnover of VDR-associated proteins not examined here.

1α,25(OH)₂ vitamin D₃ and its synthetic analogues can have growth-suppressive effects on certain types of tumor cells, including adenocarcinomas of the prostate (10). However, responses to natural and synthetic VDR ligands vary widely among tumor lines, despite their expression of functional VDR, suggesting that a variety of factors may modulate sensitivity to VDR ligands. The evidence presented here suggests that BAG1L protein levels may represent one determinant of sensitivity to growth inhibition by VDR ligands. Using a prostate cancer cell line, which expresses moderate levels of VDR (800 fmol/mg) but which contains little BAG1L and which displays little responsiveness to VDR ligands, we found that gene transfer-mediated overexpression of BAG1L increased cell cycle arrest induced by VDR ligands. This enhanced sensitivity to growth arrest induced by VDR ligands was associated with increased expression of p21Waf1, a known target gene of VDR (27). However, it remains to be determined whether VDR-mediated growth repression is directly attributable to p21Waf1 gene induction by this steroid hormone receptor, and it should be recognized that VDR is likely to affect expression of a wide variety of target genes in cells. Although it cannot be excluded that the down-regulation of p21Waf1 seen in PC3 cells treated with natural or synthetic VDR ligands is a consequence rather than a cause of the growth suppressive effects of these compounds, our studies of the p21Waf1 promoter in transient transfection reporter gene assays demonstrated that BAG1L is capable of augmenting VDR-mediated transcription of this promoter.

Prostate cancer is the most common lethal form of cancer currently diagnosed in American men, second only to lung cancer as the leading cause of malignancy-associated death among males (38). The primary therapy for men with metastatic disease entails use of anti-androgens and androgen ablation therapy, essentially all patients eventually relapse with hormone-refractory disease (10). Androgen ablation therapy for use in palliative treatment of metastatic prostate cancer. However, variability in responses to 1α,25(OH)₂ vitamin D₃-based therapies limits opportunities for clinical applications. Improved understanding of the role of BAG1L and other factors in the regulation of VDR activity and tumor suppression by VDR ligands may contribute to more rational selection of patients for therapy with 1α,25(OH)₂ vitamin D₃ or its synthetic analogues.

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