Dynamic interactions between L-type voltage-sensitive calcium channel Ca\(_{\alpha}1.2\) subunits and ahnak in osteoblastic cells

Ying Shao, Kirk J. Czymmek, Patricia A. Jones, Victor P. Fomin, Kamil Akanbi, Randall L. Duncan, and Mary C. Farach-Carson

Department of Biological Sciences, University of Delaware, Newark, Delaware

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VOLTAGE-SENSITIVE Ca\(^{2+}\) channels (VSCCs) are key regulators of intracellular Ca\(^{2+}\) homeostasis in osteoblasts that selectively allow Ca\(^{2+}\) to cross the plasma membrane. Such Ca\(^{2+}\) signals play key roles in osteoblast function, including changes in gene expression, signal-secretion coupling, modulation of expression of extracellular matrix proteins that make up osteoid of new bone, and responses to mechanical stimuli that maintain bone density (12, 20). High-voltage-activated L-type VSCCs are composed of the pore-forming \(\alpha_1\)-subunit and the auxiliary \(\alpha_2\delta\) and \(\beta\)-subunits but are devoid of a \(\gamma\)-subunit in osteoblasts (4). The \(\alpha_1\)-subunit determines the fundamental properties of individual VSCCs. This subunit has four homologous domains, I–IV, each with six transmembrane segments linked by cytoplasmic loops and with intracellular NH\(_2\) and COOH termini (10, 20). Among the 10 known \(\alpha_1\)-subunits, the L-type Ca\(_{\alpha}1.2\) (\(\alpha_{1C}\)) subunit is the most abundant and also the primary site for Ca\(^{2+}\) influx into rodent osteoblasts (36, 39). The accessory subunits, especially the \(\beta\)-subunits, regulate the VSCC gating properties and govern channel assembly and membrane trafficking (14, 55). Four distinct \(\beta\)-subunit genes (\(\beta_1\)–\(\beta_4\)) exist, each with multiple splice variants, increasing the diversity of potential gene products (2, 29). The intracellular cytoplasmic \(\beta\)-subunit interacts with the \(\alpha_1\)-subunit at the I-II intracellular loop via a conserved 18-amino acid sequence, the \(\alpha\)-interaction domain, with a corresponding binding site on the \(\beta\)-subunit, the \(\beta\)-interaction domain (16, 44). The \(\beta\)-subunits facilitate VSCC membrane localization by blocking an endoplasmic reticulum (ER) retention signal in the I-II intracellular loop of the \(\alpha_1\)-subunit (7). Coexpression of \(\beta\)-subunits facilitates the appropriate folding of VSCCs through a “chaperone-like” effect and increases the functional expression of Ca\(_{\alpha}1.2\) subunits at the plasma membrane without affecting total channel expression (14, 56). Crystallographic analysis revealed that the \(\beta\)-subunit has structural similarities to the membrane-associated guanylate kinase proteins, which function as scaffolds to cluster ion channels and as receptors and organize intracellular signaling pathways (6, 25, 49). The membrane-associated guanylate kinase-like properties of \(\beta\)-subunits modify \(\alpha_1\)-subunit function (43, 53). \(\beta\)-Subunits also may influence intracellular signal transduction through various protein interactions (18). Although three \(\beta\)-subunits (\(\beta_1\)–\(\beta_3\)) are present in mouse osteoblasts, previous findings in our laboratory showed that the \(\beta_2\)-subunit is the most abundant and is the only \(\beta\)-subunit of which the transcript decreases concomitantly with the reduction of Ca\(_{\alpha}1.2\) subunit mRNA and protein after 1,25-dihydroxyvitamin D3 treatment (K. Akanbi and J. Bergh, unpublished data). The \(\beta_2\)-subunit is also the major subunit in L-type cardiac VSCCs, where it contributes to a higher peak current when complexed with the pore-forming \(\alpha_1\)-subunit (13). Although bone and heart share the common properties of mechanosensitivity and dominance of Ca\(_{\alpha}1.2\), the \(\beta\)-subunit associated with Ca\(_{\alpha}1.2\) in osteoblasts has not been identified.

Human AHNAK\(^1\) is a 700-kDa phosphoprotein identified in neuroblastoma and other tumor cells (51); its gene was mapped to chromosome 11q12 (33). AHNAK is composed of 5,643 amino acids and has a large 4,390-amino acid-long central domain of conserved repetitive motifs flanked by unique NH\(_2\) and COOH termini (51). AHNAK plays a regulatory role in controlling Ca\(^{2+}\) membrane permeability and intracellular Ca\(^{2+}\) homeostasis. Studies using glutathione S-transferase fusion proteins showed that ahnak interacts with the auxiliary \(\beta_2\)-subunits, which anchor the L-type Ca\(_{\alpha}1.2\) VSCCs to the actin cytoskeleton via a region (aa 5456–5643) at the COOH terminus of ahnak (1, 27). After addition of recombinant ahnak protein fragments with the COOH-terminal \(\beta\)-subunit binding

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\(^1\) We use ahnak as the generic term for this protein, inasmuch as the work described here was performed in a mouse model.
antibody was raised in chicken (Sigma Genosys, Woodlands, TX) subunit polyclonal antibody was purchased from Alomone Research.

Intracellular association of ahnak with Ca$^{2+}$ channels thus could play a central role in determining whether a Ca$^{2+}$ response in osteoblastic cells translates into a functional response to alter osteoblast phenotype or behavior.

In the present study, we investigated the associations among L-type VSCC Ca$_{1.2}$ and β-subunits and ahnak protein in growth-phase MC3T3-E1 cells, which simulate hormonally and mechanically sensitive osteoblasts (5, 20, 35). Experiments were designed to 1) identify the nature of the functional VSCC complex associated with ahnak in osteoblasts, 2) determine whether such complexes were assembled in the secretory route, 3) discover whether ahnak association with VSCCs was required for cytoskeletal communication, and 4) investigate the effects of ahnak knockdown on Ca$^{2+}$ influx.

**MATERIALS AND METHODS**

**Cell culture.** Mouse osteoblastic MC3T3-E1 cell line subclone 14 was purchased from American Type Culture Collection (Manassas, VA). When subconfluent, these cells provide a model for the hormone and mechanosensitive preosteoblast that responds to such stimuli to form osteoblasts capable of producing new osteoid (39).

Cells were maintained in α-MEM containing ribonucleosides and deoxyribonucleosides supplemented with 10% (vol/vol) heat-inactivated FBS, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 10 mM HEPES buffer at 37°C with 5% (vol/vol) CO$_2$. Fresh culture medium was supplied every 2 days, and the cells were grown to ~70–80% confluence before each experiment. All culture agents were purchased from Gibco Invitrogen Life Technologies (Carlsbad, CA). For imaging experiments, cells were plated in an eight-well Lab-Tek chambered cover-glass system (Nalge Nunc International, Naperville, IL) at a density of 5 × 10$^5$ cells/cm$^2$ and grown overnight. Cytochalasin D (1 μM; EMD Biosciences, La Jolla, CA) or the DMSO vehicle control was applied to the cells for 30 min in some experiments.

**Primary antibodies.** For indirect antibody-based fluorescence resonance energy transfer (FRET) studies, it was essential to have primary antibodies recognizing target proteins that were raised in different species. An affinity-purified rabbit anti-L-type VSCC Ca$_{1.2}$ subunit polyclonal antibody was purchased from Alomone Research (Jerusalem, Israel). An affinity-purified anti-β$_2$-subunit polyclonal antibody was raised in chicken (Sigma Genosys, Woodlands, TX) against a synthetic peptide sequence of CDKGEVISVKRRSEA corresponding to amino acids 579–593 at the COOH terminus of the β$_2$-subunit (accession no. NP_446303). Other β-subunit antibodies were provided by Dr. Kevin P. Campbell (University of Iowa College of Medicine, Iowa City, IA). A rabbit anti-ahnak polyclonal antibody (KIS) was a kind gift from Dr. Emma Shitivelman (University of California, San Francisco, CA). For FRET studies, an affinity-purified sheep anti-ahnak polyclonal antibody (KIS) was generated for following the published sequence KISMPDVLHLKPKG in the central conserved domain (50) by Bethyl Laboratories (Montgomery, TX). This same peptide was used as the blocking peptide for the demonstration of specificity (see Fig. 4, lane 3).

**Coimmunoprecipitation.** MC3T3-E1 cells were scraped from the plates and solubilized in ice-cold lysis buffer containing 20 mM Na$_2$HPO$_4$ (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.3% (vol/vol) Triton X-100, and 100 μM PMSF with protease inhibitor cocktail III (Calbiochem, San Diego, CA). Cell lysates were centrifuged at 10,000 rpm for 20 min at 4°C, and supernatants were collected. For removal of proteins binding nonspecifically, supernatants were precleared with 50 μl of suspended 50% (vol/vol) protein A-agarose (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h at 4°C with rotation and then centrifuged at 10,000 rpm to remove the resin. The precleared sample was mixed with 5 μg of affinity-purified rabbit anti-Ca$_{1.2}$ subunit polyclonal antibodies and incubated by end-over-end mixing overnight at 4°C. About 50 μl of 50% resin slurry were added to the protein-antibody mixture, which was incubated overnight at 4°C with rotation. The resin was pelleted and washed three times with ice-cold lysis buffer and then resuspended in 40 μl of Laemmli sample buffer. The samples were denatured for 3 min at 95°C and separated from the agarose by microcentrifugation.

The protein sample (20 μl) was used in Western blotting for β-subunit detection with subtype-specific primary antibodies diluted at 1:400 (see below). For negative controls, one sample was immunoprecipitated with protein A-agarose without the primary antibodies, and another sample was incubated with nonimmune rabbit IgG and protein A-agarose, instead of the rabbit anti-Ca$_{1.2}$ subunit polyclonal antibodies.

**Western blotting.** MC3T3-E1 cells were solubilized as previously described, and the protein concentration of the supernatant was determined using bichinonic acid assay (Pierce, Rockford, IL). For β-subunit study, proteins were separated on 10% Tris-glycine SDS-polyacrylamide gel under reducing (β$_1$) or nonreducing (β$_1$, β$_2$, and β$_3$) conditions. For ahnak study, proteins were separated on 3–8% NuPAGE Tris-acetate gel and then transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4°C in 5% (wt/vol) BSA in PBS containing 0.1% (vol/vol) Tween 20 (PBS-T); then primary antibodies (1 μg/ml) were incubated overnight in blocking buffer at 4°C. After three 10-min rinses with PBS-T, the blot was treated with horseradish peroxidase-conjugated donkey anti-sheep antibody (1,200,000 dilution; Jackson ImmunoResearch, West Grove, PA) or horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1,200,000 dilution; Pierce) for 2 h at 4°C. Immunoreactive bands were visualized by the Supersignal West Dura Extended Duration Substrate (Pierce). An equivalent amount of nonimmune IgG was used as a control to ensure the specificity of protein recognition.

**Immunohistochemistry.** Growth-phase MC3T3-E1 cells, which are most sensitive to extracellular signals, were fixed in a 2% (wt/vol) parafomaldehyde-PBS solution for 15 min at room temperature, rinsed in PBS, permeabilized, and blocked for 30 min with PBS containing 0.2% (vol/vol) Triton X-100 and 1% (vol/vol) normal donkey serum. Samples were then incubated with sheep anti-ahnak polyclonal antibody diluted 1:50 in PBS containing 1% (vol/vol) normal donkey serum for 1 h at 37°C. After samples were rinsed three times with PBS, a 1:40 dilution of FITC-conjugated donkey anti-sheep secondary antibody (Jackson ImmunoResearch) was applied at 37°C for 40 min in darkness. Samples then were counterstained with ToPro-3 (1,2,000 dilution; Molecular Probes, Eugene, OR). F-actin filaments were visualized using a 1:100 dilution of Alexa Fluor 488-labeled phallolidin (Molecular Probes). Images were collected using a Zeiss LSM 510 VIS confocal microscope with 488-nm laser line of a 30-mW argon krypton and 633-nm laser line of a 5-mW helium-neon laser (Carl Zeiss, Oberkochen, Germany). Negative controls were performed using nonimmune IgGs diluted at concentrations equivalent to primary antibody concentrations or without primary antibodies. Immunohistochemistry of long bone sections (femur) was performed as described previously (48), with substitution of anti-ahnak antibody for antibody to VSCC.

**Small interfering RNA.** MC3T3-E1 cells were grown to 50% confluency by visual inspection in α-MEM without antibiotics before the small interfering RNA (siRNA) treatment. ON-TARGETplus SMARTpool siRNA directed against murine ahnak protein (accession nos. NM_009643 and NM_175108) was purchased from...
Dharmacon (Lafayette, CO). Transfections were performed with Oligofectamine (Invitrogen) according to the manufacturer’s protocol. Protein assays to access knockdown were performed at 24–96 h after transfection. Functional assays were performed during maximum knockdown.

Sample preparation for FRET analysis. MC3T3-E1 cells were grown and fixed as described above. For FRET analysis of L-type Cav1.2 subunit (donor) and β2-subunit (acceptor), cells were incubated with rabbit anti-Cav1.2 subunit polyclonal antibodies diluted 1:50 in PBS for 1 h at 37°C and then with a 1:100 dilution of cyanine 3 (Cy3)-conjugated monovalent Fab fragments of donkey anti-rabbit IgG for 40 min at 37°C (Jackson ImmunoResearch). After three rinses with PBS, chicken anti-β2-subunit polyclonal antibodies diluted 1:50 were applied for 1 h at 37°C, and then cyanine 5 (Cy5)-conjugated Fab fragments of goat anti-chicken IgG diluted 1:100 were applied for 40 min at 37°C in darkness. For FRET analysis of L-type VSCC β2-subunit (donor) and ahnak (acceptor), the cells were incubated with chicken anti-β2-subunit polyclonal antibodies diluted 1:50 in PBS for 1 h at 37°C and then with a 1:100 dilution of Cy3-conjugated Fab fragments of goat anti-chicken IgG for 40 min at 37°C in darkness. After three rinses with PBS, sheep anti-ahnak polyclonal antibodies and then with a 1:1 mixture of Cy3- and Cy5-conjugated Fab fragments of donkey anti-rabbit secondary antibodies. The acceptor photobleaching approach was utilized to detect whether FRET occurred between the two secondary antibodies, and FRET efficiency was calculated. Positive controls also were conducted with anti-β2-subunit and anti-ahnak primary antibodies. For negative controls, we used 1) secondary, but no primary, antibodies, 2) nonimmune IgGs diluted at equivalent concentrations to replace the primary antibodies, 3) donor (Cy3) or acceptor (Cy5) single-labeled cells, and 4) substitution of rabbit anti-osteopontin (OPN) primary antibody for the anti-Cav1.2 subunit and anti-ahnak primary antibodies. OPN was selected, inasmuch as it is abundant in the secretory pathway for these cells but should never associate with the β2-subunit in the cytoplasm.

Data analysis using acceptor photobleaching method. Energy transfer was detected as an increase of donor fluorescence intensity signal after photobleaching of the acceptor fluorophore (30). This method can directly measure the presence or absence of FRET and does not require extensive corrections used for sensitized emission. Repeat photobleaching provides the additional advantage of continuous monitoring for 2-axis drift, and it allows extrapolation of 100% photobleaching by linear regression (41). An initial Cy3-Cy5 dual-channel image was obtained before photobleaching. A designated area in the Cy5 channel was then selected and subjected to intense laser power (100%) to destroy the acceptor molecule’s fluorescence in short exposure. A dynamic series of fluorescent images were acquired from Cy3 (donor) and Cy5 (acceptor) channels during the repeat bleaching process. Donor and acceptor threshold levels were defined from a 40 × 40 pixel cell-free area in split donor and acceptor channels. Regions of interest (ROIs) were selected from the cell interior, along the plasma membrane, and in the nucleus of the cells in the bleached and unbleached area, where fluorescent intensity of donor and acceptor was determined and averaged over the whole area after background subtraction using LSM FRET Macro software (version 1.5, Carl Zeiss). Because 100% photobleaching of the acceptor fluorescence was not reached in our experiments, the theoretical value of donor fluorescence intensity after 100% photobleaching was extrapolated from a line fit of a plot of percent increase in donor fluorescence vs. percent decrease in acceptor fluorescence, as described elsewhere (41). FRET efficiency (E) was calculated as follows: E (%) = 100 × (donor fluorescence intensity after photobleaching – donor fluorescence intensity before photobleaching) / donor fluorescence intensity after photobleaching (31). Inside a particular selected ROI, areas with no FRET or no substantial FRET were included in the calculations. Positive values obtained from the software indicated FRET, whereas slightly negative or zero values indicated no FRET, between the donor and acceptor pair. Multiple ROIs were selected and analyzed from different sample collections to obtain the average FRET efficiency (Eavg).

Data analysis using sensitized emission method. FRET was detected as an increase of acceptor fluorescence intensity signal in the FRET channel after donor excitation (17). Three image sets were recorded from the single-labeled donor, the single-labeled acceptor, and the double-labeled FRET samples. ROIs were selected from different areas of the cells, where corrected FRET image (FRET) values were evaluated by Zeiss LSM FRET Macro software. On the basis of the experimental conditions and appropriate controls, areas with >10% increase in FRET was considered positive for FRET, whereas those with <10% increase in FRET was considered negative for FRET. Multiple ROIs were selected to obtain FRET.

Positive and negative controls. For positive controls, samples were treated with rabbit anti-Cav1.2 subunit primary antibodies and then with a 1:1 mixture of Cy3- and Cy5-conjugated Fab fragments of donkey anti-rabbit secondary antibodies. The acceptor photobleaching approach was utilized to detect whether FRET occurred between the two secondary antibodies, and FRET efficiency was calculated. Positive controls also were conducted with anti-β2-subunit and anti-ahnak primary antibodies. For negative controls, we used 1) secondary, but no primary, antibodies, 2) nonimmune IgGs diluted at equivalent concentrations to replace the primary antibodies, 3) donor (Cy3) or acceptor (Cy5) single-labeled cells, and 4) substitution of rabbit anti-osteopontin (OPN) primary antibody for the anti-Cav1.2 subunit and anti-ahnak primary antibodies. OPN was selected, inasmuch as it is abundant in the secretory pathway for these cells but should never associate with the β2-subunit in the cytoplasm.

Intracellular Ca2+ measurements. Cells were loaded with fura 2 for 30 min, as described previously (46). Loaded osteoblasts were rinsed and incubated for an additional 15 min with HBSS alone to allow complete deesterification of the fluorescent indicator. Fura 2 fluorescence was recorded with a DeltaRam spectrofluorometer (Photon Technology International, Lawrenceville, NJ). Fura 2 fluorescence was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The ratio of fluorescence at 340 nm to fluorescence at 380 nm was calculated every second and recorded via desktop computer. The measurements were automatically corrected for autofluorescence by measurement of the fluorescence of the cells not loaded with fura 2-AM. When indicated, inhibitors were added before transfer to hypotonic solution.

RESULTS

Coimmunoprecipitation of Cav1.2 and β-subunits. To determine which β-subunit(s) form(s) direct association with the Cav1.2 subunit in the functional channel complexes of MC3T3-E1 cells, coimmunoprecipitation and subsequent Western blot experiments were performed. The molecular weight of β-subunits varies between 50 and 70 kDa (18). Our findings revealed that the auxiliary β2- and β3-subunits could be coimmunoprecipitated with the Ca1.2 subunits from the total protein extracts of mouse osteoblasts (Fig. 1), but no band was observed for β1- and β4-subunits under these same conditions (not shown). When nonimmune rabbit IgG replaced anti-Cav1.2 antibody (Fig. 1) or protein A-agarose was used after the primary antibodies were omitted (not shown), no coimmunoprecipitated product was found. This demonstrated the specificity of the antibodies used in the experiments and showed that, if present, β2- or β3-subunits are capable of associating with the Ca1.2 subunit in osteoblastic cells.

FRET identifies interactions between L-type Ca1.2 and β2-subunits. Indirect immunofluorescence was used with Cy3-labeled Ca1.2 subunits for the donor and Cy5-labeled β2-subunits for the acceptor. ROI 1, selected from the bleached area along the cell plasma membrane, exhibited low Cy5 (acceptor) fluorescence intensity over time with a significant increase of the dequenched Cy3 (donor) fluorescence intensity.
Dynamic aspects of VSCC complexes in osteoblastic cells. In untreated or vehicle-treated MC3T3-E1 cells, actin fila-
ments, visualized by fluorescent dye-conjugated phalloidin, arranged themselves into tight bundles or organized networks throughout the cytosol (Fig. 6A). After 30 min of treatment with cytochalasin D, bundles and networks of actin filaments were visibly broken into short segments aligned along their original vectors with the simultaneous appearance of numerous punctate fluorescent spots in the cytoplasm (Fig. 6B).

Acceptor photobleaching was performed to assess FRET in VSCC complexes in MC3T3-E1 cells after cytochalasin D treatment. In the bleached area (ROI 1), fluorescence intensity of the Cy3 donor went up when the acceptor fluorescence was eliminated by bleaching (Fig. 6C). Curves B and C, representing donor and acceptor signals in the unbleached area (ROI 2), remained unchanged (Fig. 6C). These findings clearly indicate that actin disassembly did not disrupt the integrity of the two L-type VSCC subunits, Ca\textsubscript{1.2} and β\textsubscript{2}. $E_{\text{avg}}$ between Ca\textsubscript{1.2} and β\textsubscript{2}-subunits in the cytochalasin D-treated cells was $14.56 \pm 0.06\%$ (acceptor photobleaching method) and $16.41 \pm 0.04\%$ (sensitized emission method; Table 2).

Similarly, we investigated whether cytochalasin D would affect the association between L-type VSCCs and ahnak. The data shown in Fig. 6D clearly indicate that although the actin cytoskeleton was disrupted, the association between the β\textsubscript{2}-subunit of the Ca\textsubscript{1.2} channel and ahnak remained intact. The fluorescence intensity of the donor went up when the acceptor fluorescence was eliminated by bleaching in the bleached area (ROI 1). $E_{\text{avg}}$ between the L-type Ca\textsubscript{1.2} channel and ahnak in cytochalasin D-treated cells was $18.85 \pm 0.02\%$ (acceptor photobleaching method) and $16.40 \pm 0.03\%$ (sensitized emission method; Table 2).

Knockdown of ahnak protein by siRNA. We next used siRNA to knock down the expression of ahnak in osteoblasts. We used specific sheep anti-ahnak protein antibody to stain the cells at 48 h after transfection. The immunostaining signal was significantly reduced in most of the transfected cells (Fig. 7C) compared with the untreated (Fig. 7A) and Oligofectamine-treated (Fig. 7B) control cells. The siRNA-transfected cells also were analyzed by Western blotting at 24, 48, 72, and 96 h after transfection for protein expression level. As shown in Fig.

Table 1. Corrected FRET efficiency between L-type Ca\textsubscript{1.2} and β\textsubscript{2}-subunits in growth-phase MC3T3-E1 cells using acceptor photobleaching and sensitized emission methods

<table>
<thead>
<tr>
<th>Method</th>
<th>(n = 26)</th>
<th>E\textsubscript{avg}</th>
<th>(n = 180)</th>
<th>E\textsubscript{avg}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acceptor Photobleaching</td>
<td>Range, %</td>
<td>8.89–34.36</td>
<td>10.32–39.94</td>
<td>17.71 ± 0.01</td>
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<td>Sensitized Emission</td>
<td></td>
<td>17.17 ± 0.01</td>
<td>20.45 ± 0.07</td>
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</table>

Fluorescence resonance energy transfer (FRET) efficiency was determined by Carl Zeiss LSM FRET Macro software (version 1.5). $E_{\text{avg}}$, average efficiency (means ± SD); n, number of regions of interest.
D, ahnak protein expression was strikingly reduced in 48-h-posttransfected cells (>80% knockdown on the protein level), whereas the ahnak protein levels in cells transfected with scrambled siRNA were comparable with those in the original untreated cells at all time points. Thus siRNA successfully inhibited the expression of ahnak protein in transfected cells in a time-dependent fashion. All subsequent siRNA studies were performed at 48 h.

Knockdown of ahnak protein leaves the cytoskeleton intact but negatively influences Ca\textsuperscript{2+} influx in osteoblastic cells. siRNA inhibition of ahnak did not disrupt the structure of actin filaments, as indicated by immunostaining with FITC-labeled phalloidin (Fig. 8C), compared with untreated (Fig. 8A) or Oligofectamine-treated (Fig. 8B) controls. The results indicated that Ca\textsubscript{1.2} and β\textsubscript{2}-subunits and ahnak form a multicomponent complex that functions in the Ca\textsuperscript{2+} signal independent of the integrity of the actin-based cytoskeleton in bone cells. Using fura 2 as a Ca\textsuperscript{2+} indicator, we further demonstrated that Ca\textsuperscript{2+} influx across the plasma membrane in response to hypotonic swelling was significantly impaired by ahnak protein knockdown by siRNA (P < 0.05; Fig. 8D). Although cytochalasin D effectively disrupted the actin filaments, it did not decrease the total Ca\textsuperscript{2+} influx across the plasma membrane in response to hypotonic conditions. This indicates that the stability of functional complexes, including the Ca\textsubscript{1.2} and β\textsubscript{2}-subunits and ahnak, does not require association with the actin cytoskeleton for ion flux but is critically dependent on the presence of ahnak. Interestingly, we found that inclusion of the L-type VSCC inhibitor nifedipine (1 μM) blocked 45% of the total Ca\textsuperscript{2+} response to hypotonic swelling, whereas addition of the mechanosensitive channel inhibitor Gd\textsuperscript{3+} (5 μM) blocked 42% of the total response (Fig. 8). Thus knockdown of ahnak led to the disruption of Ca\textsuperscript{2+} influx through L-type VSCCs and the mechanosensitive channel.

Fig. 3. FRET image of L-type Ca\textsubscript{1.2} and β\textsubscript{2}-subunits analyzed by sensitized emission method in MC3T3-E1 cells. A: dual-channel image with Cy3-labeled L-type VSCC Ca\textsubscript{1.2} subunit (donor) and Cy5-labeled β\textsubscript{2}-subunit (acceptor). B: sensitized emission map showing FRET between Ca\textsubscript{1.2} and β\textsubscript{2}-subunits along the plasma membrane and in the cell interior of MC3T3-E1 cells but nearly absent FRET in the nuclei (arrow shows >40% increase of F). C: dual-channel image with Cy3-labeled β\textsubscript{2}-subunit (donor) and Cy5-labeled osteopontin (OPN; acceptor). D: representative sensitized emission map indicating no direct energy transfer between OPN and β\textsubscript{2}-subunits.

Fig. 4. Ahnak protein expression and subcellular localization in growth-phase MC3T3-E1 cells and long bone. A: ahnak was detected by Western blotting as ~700-kDa protein product. Lane 1, sheep anti-ahnak primary antibody; lane 2, rabbit anti-ahnak primary antibody; lane 3, negative control using peptide block that demonstrated specificity of anti-ahnak primary antibodies. B: ahnak (green) was primarily located along the cell membrane, with diffuse intracellular staining. Nuclei (red) were counterstained with ToPro-3. C: negative controls were performed with nonimmune sheep IgG replacing primary antibodies. D and E: immunostaining of ahnak in mouse femur. Red stain shows ahnak; green is a nuclear counterstain; areas of overlap appear yellow. Arrows in D and E indicate chondro-osseous junction, with cartilage at right. Note labeling of osteoblasts lining bone surfaces at left of chondro-osseous junction.
ahnak had a more pronounced effect on Ca\(^{2+}\) entry than did blocking of L-type VSCCs or Gd\(^{3+}\) alone.

**DISCUSSION**

In cells of the osteoblastic lineage, intracellular Ca\(^{2+}\) signals coordinate cell behavior, which translates into systemic control of Ca\(^{2+}\) homeostasis and maintains normal bone mineral density. Calcitropic hormones, including 1,25-dihydroxyvitamin D\(_3\), parathyroid hormone, and TNF\(_\alpha\) family members, can alter intracellular Ca\(^{2+}\) homeostasis. The key cell surface sensor of mechanical transduction in the osteoblast is the L-type VSCC. Thus, understanding the structure and function of VSCCs in osteoblasts is central to linking Ca\(^{2+}\) signals at the cellular level to overall regulation of bone formation.

Previous work from our laboratory clarified the major types of VSCCs expressed in rodent osteoblasts during various stages of differentiation (5, 48); however, it remained unclear which auxiliary subunits coassemble with the major subunit, Cav1.2, and where assembly takes place. Traditional approaches, including affinity chromatography and coimmunoprecipitation, can reveal information about the ability of proteins to associate but do not provide information about where protein interactions occur in their natural cellular environment. FRET analysis of protein association in intact cells provides this information by virtue of a nonradioactive energy transfer that occurs via a dipole-dipole interaction between an excited donor fluorophore and another acceptor fluorophore when they are sufficiently close (15). Thus this technique provides nanometer resolution, well beyond the optical resolution of a light microscope (30, 47).

### Table 2. Corrected FRET efficiency among L-type Cav1.2 and \(\beta_2\)-subunits and ahnak protein in growth-phase MC3T3-E1 cells using acceptor photobleaching and sensitized emission methods

<table>
<thead>
<tr>
<th>Condition</th>
<th>(\beta_2)-Subunit and Ahnak</th>
<th>Cav1.2 and (\beta_2)-subunit</th>
<th>(\beta_2)-Subunit and ahnak</th>
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<tr>
<td><strong>Acceptor photobleaching method</strong></td>
<td>Range, %</td>
<td>7.70–18.70</td>
<td>6.04–27.13</td>
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<td></td>
<td>(E_{\text{avg}}), %</td>
<td>12.05±0.01</td>
<td>14.56±0.06</td>
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<tr>
<td><strong>Sensitized emission method</strong></td>
<td>Range, %</td>
<td>11.17–41.56</td>
<td>10.41–21.09</td>
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<tr>
<td></td>
<td>(E_{\text{avg}}), %</td>
<td>26.56±0.08</td>
<td>16.41±0.04</td>
</tr>
</tbody>
</table>

FRET efficiency was determined by Carl Zeiss LSM FRET Macro software (version 1.5).
Our data revealed that β₂- or β₃-subunits could be coimmunoprecipitated with the Ca₁.₂ subunit from whole cell lysates made from MC3T3-E1 cells. Neither β₁- nor β₄-subunits were detected in association with Ca₁.₂. Indirect labeling was used to investigate assembly and trafficking of Ca²⁺ channel subunits in osteoblasts without the effects that transfection of fluorescent protein vectors might have on normal protein interactions. The acceptor photobleaching method using specific antibodies to the β₂- or Ca₁.₂ subunit indicated a close association between these two subunits in intact cells. The sensitized emission analysis confirmed the association between Ca₁.₂ and β₂-subunits, with $E_{avg}$ of $\sim$20%.

FRET data indicated that β₂-subunits likely associated directly with the pore-forming Ca₁.₂ subunit at the cell membrane, where the functional VSCC resides. Other findings provided direct evidence that Ca₁.₂ and β₂-subunits initiate assembly in the intracellular pool, presumably around the ER and Golgi complexes, after each subunit is synthesized in the appropriate intracellular compartment: the ER for the Ca₁.₂ subunit and the cytoplasm for the β₂-subunit. It is accepted that the newly synthesized β₂-subunit interacts with Ca₁.₂ at the cytoplasmic side of the ER, blocking the ER-retention motif and allowing the channel complex to traffic to the plasma membrane (7). It thus appears that expression of the functional L-type Ca²⁺ channel is regulated in osteoblasts, as in cardiac cells, by assembly and membrane trafficking of the channel subunits. In the case of hormone and mechanosensitive osteoblasts, the appropriate endocrine or mechanical signaling can modulate the assembly and subsequent trafficking of the α₁β complexes to the cell membrane, thus providing a mechanism for regulating the functional VSCC expression level and Ca²⁺ permeability at the plasma membrane.

Ahnak is a giant scaffolding protein with multiple functions, not all of which are known. Ahnak has been identified in various tissues, including brain, cardiac, smooth skeletal muscle, T lymphocytes, skin, and placenta (19, 22, 26, 28, 38). The expression of ahnak protein in osteoblasts has not been reported, although ahnak may participate in regulating Ca²⁺ signaling in bone-forming cells. Database searches of the human genome reveal a second AHNAK gene with an open reading frame on chromosome 14q32, named “AHNAK 2,” encoding a 600-kDa protein (32). It is not known whether the presence of a second highly related protein explains our observation of a second band below 700 kDa in the Western blot detected with our sheep antibody. Immunolabeling
indicated that ahnak preferentially distributed along the plasma membrane in osteoblasts, consistent with its ability to directly interact with the Ca\(^{2+}\) channel \(\beta_2\)-subunit at the cell membrane (27). The presence of ahnak in the cytoplasmic compartment, as well as along the membrane, is consistent with previous reports (42, 52). It has also been proposed that ahnak protein is localized within the lumen of certain cytoplasmic vesicles, where it may participate in Ca\(^{2+}\)-dependent exocytosis (8, 37). Given the highly secretory nature of osteoblasts, it is intriguing to speculate that the ahnak-VSCC complex might have a similar function in osteoblasts, which secrete matrix proteins (20) and signaling molecules such as ATP (21) in response to hormonal and mechanical stimuli. It also would be of interest to determine whether ahnak plays a direct role in membrane targeting or recycling of VSCC complexes.

Ahnak further has been shown to be required for plasma membrane insertion of the L-type Ca\(^{1.1}\) (\(\alpha_{1S}\)) channel in T cells, presumably through its interaction with the associated \(\beta\)-subunit (38). Ahnak-deficient T cells demonstrated a reduced Ca\(^{2+}\) influx after antigen binding to T cell antigen receptor, with poor activation of downstream nuclear factor of activated T cells signaling pathway, indicating the essential role of ahnak in T cell Ca\(^{2+}\) signaling. This finding is consistent with our findings in osteoblasts, indicating that ahnak may play an important role in controlling Ca\(^{2+}\) influx across the cell membrane, which directly influences intracellular Ca\(^{2+}\) concentration and Ca\(^{2+}\)-mediated signaling pathways.

Ahnak was found to associate with the L-type VSCCs via the accessory \(\beta_2\)-subunit, but not with the \(\alpha_1\)-subunit, consistent with observations in cardiac tissues and T cells (24, 45). Because the \(\beta_2\)-subunit associates with the ahnak protein at a site independent of \(\beta\)-interaction domain (1), it will not interfere with the interaction between Ca\(_{1.2}\) and \(\beta\)-subunits. Given its extraordinary size, its apparent plasma membrane distribution, and its ability to interact with multiple \(\beta\)-subunits from Ca\(^{2+}\) channels, ahnak can participate in multiple functions: organizing cell surface receptors, clustering ion channels, securing adhesion molecules and intracellular enzymes in focal adhesions, and bridging L-type Ca\(_{1.2}\) VSCCs to the cytoskeleton to modulate various intracellular signaling pathways (27). Our data demonstrating that knockdown of ahnak severely impaired the ability of osteoblasts to support Ca\(_{1.2}\)-mediated entry of Ca\(^{2+}\) in response to hypotonic swelling indicate that one key function of ahnak in bone-forming cells involves the stabilization of plasma membrane VSCC complexes. The finding that knockdown of ahnak more severely impaired Ca\(^{2+}\) uptake than did blockade of L-type channels or mechanosensitive channels alone (46) suggests that ahnak may regulate more than one pathway for Ca\(^{2+}\) entry into osteoblasts.

Actin organization has been reported to modulate the electrophysiological activities of some VSCCs (34). For example, disruption of actin filaments by cytochalasin D dramatically reduced the L-type Ca\(^{2+}\) current in vascular smooth muscle cells (40). In our study, FRET analysis of VSCC subunit associations in cytochalasin D-treated osteoblastic cells showed that actin cytoskeletal disruption did not interfere with the functional associations between L-type Ca\(_{1.2}\) and \(\beta_2\)-subunits, indicating that this formed complex is stable in osteoblasts. The only molecule reported to interfere with the assembly of the two Ca\(^{2+}\) channel subunits is a small GTPase, Gem, which directly blocks the \(\alpha_1\)-\(\beta\)-binding site (54). Many biologically important functions of osteoblasts are Ca\(^{2+}\) dependent, including cell movements powered by actin cytoskeleton disassembly and reassembly (9) and key events in mitosis (11). It thus makes sense that osteoblasts maintain the ability to allow regulated Ca\(^{2+}\) influx under conditions of cytoskeletal...

Fig. 7. Small interfering RNA (siRNA) significantly reduced ahnak protein expression. Ahnak (green) was primarily located by immunostaining along the cell membrane with diffuse cytoplasmic staining in untreated (A) and Oligofectamine-treated (B) MC3T3-E1 cells. siRNA significantly reduced ahnak protein expression, indicated by decreased ahnak (green) immunostaining after 48 h (C). Ahnak protein level was dramatically knocked down 48 h after siRNA treatment, as indicated by Western blotting, whereas cells transfected with nonspecific scrambled siRNA did not show ahnak expression changes (D). Lane 1, untreated control; lane 2, 24 h after transfection; lane 3, 48 h after transfection; lane 4, 72 h after transfection; lane 5, 96 h after transfection.
disassembly and, further, that, at least in osteoblastic cells, effects of Ca\(^{2+}\) signals on the cytoskeleton are downstream of the action of the VSCC.

In summary, in the present study, we demonstrated that the major VSCC in differentiating osteoblasts, the L-type Ca\(_{1.2}\), assembles with \(\beta_2\)-subunits and the giant ahnak protein to form a stable multisubunit complex. Importantly, the channel complex resists disassembly and remains functional and capable of supporting Ca\(^{2+}\) influx during cytoskeletal disruption as long as ahnak is present. This finding has major implications for regulation of Ca\(^{2+}\) movement in osteoblasts undergoing dynamic movements, such as those that occur during cell division or cell migration, when dynamic changes in cytoskeletal organization occur.

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Present addresses: Y. Shao, Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104; K. Akanbi, Biomerieux, Inc., 100 Rodolphe St., Durham, NC 27712.

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