Actin and Keratin are Binding Partners of the 1,25D$_3$-MARRS Receptor/PDIA3/ERp57

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Abstract: We have shown that the 1,25D$_3$-MARRS receptor is necessary for the rapid, pre-genomic effects of 1,25(OH)$_2$D$_3$ on phosphate and/or calcium absorption in chick intestines. However, a clear understanding of the proteins involved in the signaling mechanisms by which the 1,25D$_3$-MARRS receptor facilitates 1,25(OH)$_2$D$_3$-mediated phosphate or calcium uptake, as well as other cellular effects, is still under investigation. We used co-immunoprecipitation studies and mass spectroscopy to identify actin and keratin as proteins that interact with the 1,25D$_3$-MARRS receptor. Using confocal microscopy, we visualized 1,25(OH)$_2$D$_3$-MARRS receptor localizations relative to actin and/or keratin distribution in chick enterocytes. Cells cultured in media containing phenol red had the 1,25D$_3$-MARRS receptor and actin localized largely in the nucleus, which was dispersed upon addition of (OH)$_2$ 1,25(OH)$_2$D$_3$. In the absence of phenol red, staining was cytoplasmic. Addition of steroid caused diminished staining at 10 s and 30 s, with a return of intensity between 1 and 5 min. Nuclear staining was observed after 1 min. We found that F-actin concentrations are maximal when 1,25D$_3$-MARRS receptor localizations within enterocytes are low suggesting that cyclical conversions of F-actin to G-actin are involved in the 1,25(OH)$_2$D$_3$-mediated redistribution of the 1,25D$_3$-MARRS receptor within the cell. We also found that keratin distribution remains constant with 1,25(OH)$_2$D$_3$ exposure when F-actin depolymerizes into G-actin, which suggests that actin and keratin work in concert to facilitate hormone-mediated redistribution of the 1,25D$_3$-MARRS receptor. We subsequently investigated whether the cyclical redistribution was related to either 1,25(OH)$_2$D$_3$-stimulated phosphate or calcium uptake, but no congruent pattern was found.

INTRODUCTION

Recently identified as a plasma membrane receptor for 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], the 1,25D3-MARRS (membrane associated rapid response steroid-binding) receptor has been biochemically characterized and identified as a key player in facilitating the rapid, pre-genomic effects of 1,25(OH)2D3-stimulated phosphate uptake in chick enterocytes [1, 2] mouse enterocytes [3, 4] as well as chick kidney cells [5] In addition, the 1,25D3-MARRS receptor also facilitates 1,25-(OH)2D3-stimulated calcium uptake, transport and/or extrusion in chick [6-9] or mouse enterocytes [6, 7, 10, 11] by linking them to a complex array of membrane-initiated cell signaling events that include 1,25(OH)2D3-stimulated activation of the protein kinase C pathway, the cAMP pathway, as well as the MAP kinase pathway [12].

While the membrane-initiated steroid signaling events involved in activation of the 1,25D$_3$-MARRS receptor to elicit "pre-genomic" cellular responses are well documented, investigators have identified that the 1,25D$_3$-MARRS receptor also translocates to the nucleus [13] to induce genomic effects. Jia and Nemere [14] first identified that binding of 1,25(OH)$_2$D$_3$ to the 1,25D$_3$-MARRS receptor induces redistribution of the receptor from the plasma membrane into subcellular organelles that include the nucleus in chick brain and kidneys. Nemere *et al.* [13] have also demonstrated that hormone binding also induces the 1,25D$_3$-MARRS receptor redistribution to the nu-

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cleus in chick enterocytes. In addition, Wu *et al.* [15] have reported that $1,25(OH)_2D_3$ stimulates subcellular redistribution of the $1,25D_3$-MARRS receptor from the plasma membrane to bind to a transcription factor, NF kappa B, in the nuclei of leukemia cells.

In this report, we confirmed that the $1,25D_3$-MARRS receptor is present in the plasma membrane as well as cytoplasmic and nuclear extracts. We also identified that both actin and keratin are bound to the $1,25D_3$-MARRS receptor and that $1,25(OH)_2D_3$ stimulates changes in the intracellular redistribution of $1,25D_3$-MARRS receptor interactions with both actin and keratin.

EXPERIMENTS

Animals and Surgical Procedures

All surgical procedures were approved by the Institutional Animal Use and Care Committee at Utah State University (Logan, UT). White leghorn cockerels (Privett Hatchery; Portales, NM) were obtained on the day of hatch and raised for 3-7 wks on a commercially available vitamin D-replete diet (Nutrena Feeds; Murray, UT). On the day of use, chicks were anesthetized with chloropent (0.3 ml/100g body weight), the duodenal loop surgically removed to ice-cold 0.9% saline solution and chilled for 15 min. The pancreas was excised from the duodenal loop and discarded. For basal lateral membrane isolation, the duodenal loop was slit longitudinally and the intestinal mucosa collected by scraping into homogenization media [16]. For cell isolation, the duodenal loop was everted and rinsed in chilled saline solution.

Preparation of Basal Lateral Membranes (BLM)

The basal lateral membrane fractions of chick enterocytes were used as a positive control in co-immunoprecipitation studies to determine if the $1,25D_3$-MARRS receptor forms heterodimers either in the absence or presence of hormone. The basal lateral membranes were isolated from subcellular fractions prepared as reported previously [17, 18]. Duodenal loops were chilled in physiological saline for 15 min, prior to removal of the pancreas. The intestinal segment was slit longitudinally, rinsed in additional ice cold saline, and the mucosa collected by scraping. Intestinal mucosa

was disrupted in 40 ml of homogenization medium (250 mM sucrose, 5 mM histidine, 5 mM imidazole, 2 mM EGTA, pH 7.0) with a Dounce homogenizer (Kontes Co.; Vineland, NJ) and a Teflon pestle. The whole homogenate was first subjected to differential centrifugation to obtain the 20,000 x g postnuclear pellet containing intracellular organelles and BLM. Intracellular organelles were separated from BLM by centrifugation in Percoll (Sigma Aldrich; St. Louis, MO) density gradients. Eighteen fractions were collected from the gradient (52 drops each). Fractions 16-18 contained BLM as judged by marker enzyme analyses (Na^+,K^+ ATPase) [17-19] were pooled, and the Percoll removed by ultracentrifugation [18]. BLM were stored at -20°C until used.

Cell Isolation

The chick enterocytes were isolated with citrate chelation media [20, 21] adjusted to pH 5.0 to promote viability and retention of morphology [22, 23]. The cells were collected by low speed centrifugation (500 x g, 5 min, 4°C), resuspended in a small volume of Gey's balanced salt solution (GBSS, containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.84 mM $NaHPO_4$, 1.03 mM $MgCl_2 \cdot 6H_2O$, 0.28 mM $MgSO_4 \cdot 7H_2O$, 0.9 mM $CaCl_2$, pH 7.3), and transported on ice to the Center for Integrated Biosystems (Utah State University; Logan, UT). Cell suspensions were either cultured or used in co-immunoprecitation studies.

Cell Cultures

Two-ml aliquots of cell suspension were pipetted into 100 x 15 mm plastic Petri dishes (Falcon, Scientific Products; Franklin Lakes, NJ) containing 6 ml of RPMI-1640 medium with or without phenol red (Hyclone; Logan, UT); 2.05 mM L-glutamine (Sigma Aldrich; St. Louis, MO) was added to RPMI-1640 medium without phenol red. Cells were then cultured for 18-20 or 72 hr with antibiotics [100 units/ml penicillin, 100 mg/ml streptomycin (both from Sigma Chemical Co; St. Louis, MO)] prior to uptake studies.

Calcium or Phosphate Uptake in Cultured Cells

Chick enterocytes were cultured 18-20 hr or 72 hr in RPMI-1640 containing phenol red. Phosphate or calcium uptake studies were performed in the presence or absence of 300 pM $1,25(OH)_2D_3$ to determine the effects of hormone on phosphate or

calcium uptake in cultured enterocytes. The media were aspirated and replaced with GBSS-0.1% BSA. After each dish was exposed to an additional volume of GBSS with radionuclide [2 μCi/ml $H_3{}^{32}PO_4$ or 1 μCi/ml $^{45}CaCl_2$ final concentration (Perkin Elmer; Boston, MA)] for 1 or 5 min, dishes for the -1 or -5 min timepoints were washed three times with 4 ml of ice cold GBSS, and treated with lysis buffer (10 mM Tris, 1.5 mM EDTAm 1 mM dithiothreitol [TED]) containing 0.1% Triton X-100). The remaining dishes were treated with 0.01% ethanol for vehicle controls or 300 pM $1,25(OH)_2D_3$. After 10 s, 30 s, 1, 3, 5, 7, or 10 min the media were aspirated and the cells washed and then lysed in preparation for protein determination by the Bradford assay (BioRad; Hercules, CA) and radionuclide measurement by liquid scintillation spectrophotometry.

Protein Determination

Colorimetric analysis of intestinal epithelial cell lysates was determined by Bradford assay (Bio-Rad; Hercules, CA) at 595 nm. The protein content of lysed cells was quantified using bovine γ-globulin as the standard.

Nuclear and Cytoplasmic Extracts

Nuclear and cytoplasmic extracts of chick intestinal cells were used in co-immunoprecipitation studies to identify the possible effects of $1,25(OH)_2D_3$ on the $1,25D_3$-MARRS receptor and its heterodimers. Prior to nuclear and cytoplasmic extraction, whole chick intestinal cells were incubated with or without 300 pM $1,25(OH)_2D_3$ for 1 or 5 min. Nuclear and cytoplasmic extracts were then collected according to the Nuclear Extract Kit protocol (Active Motif; Carlsbad, CA). Four hundred-μl aliquots of cells treated with vehicle or 130 pM or 300 pM $1,25(OH)_2D_3$ were pipetted into 8 ml phosphate buffered saline (PBS) containing phosphatase inhibitors and centrifuged 500 x g for 5 min. The supernatant was discarded and the cell pellets were resuspended in hypotonic buffer. The resuspended cells were incubated for 15 min at 4°C. Detergent was added to the cells and the suspension vortexed. The suspension was centrifuged at 14,000 x g for 30 sec at 4°C. The supernatant containing the cytoplasmic extract was stored at -20°C until analyzed by SDS-PAGE followed either by silver staining or by Western analyses with either the antibody against the $1,25D_3$-MARRS receptor (Ab099 or Ab593), antibody against β-actin (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA), or antibody against keratin 2 (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA). The pellet containing the nuclear fraction was resuspended in complete lysis buffer and vortexed. The suspension was incubated for 30 min, 4°C on an orbital shaker at 150 rpm and centrifuged 14,000 x g for 10 min at 4°C. The supernatant containing the nuclear extract was stored in coupling buffer (BupH Modified Dulbecco's PBS; 0.14 M NaCl, 0.008 M $NaPO_4$, 0.002 M KPO_4, and 0.01 M KCl, pH 7.4) at -20°C until analyzed by SDS-PAGE followed either by silver staining or by Western blot analyses as described below.

In an alternate nuclear and cytoplasmic extraction protocol involving chromatin immunoprecipitation, 1 ml aliquots of cell resuspension ($\approx$ 2.2 x 10^7 cells) were incubated with vehicle or hormone [130 pM, 300 pM, or 650 pM $1,25(OH)_2D_3$] diluted into 1 ml GBSS-0.1% bovine serum albumin (BSA). After 1 or 5 min, 4 ml ice cold GBSS was added and each tube was placed on ice. Each sample was centrifuged at 1000 x g for 5 min at 4°C, the supernatant decanted, the tube swabbed while still in the inverted position, and the pellet resuspended in 500 μl reagent grade water with 5 μl protease inhibitor cocktail and 5 μl phenylmethanesulfonyl fluoride [(PMSF); Fluka Analytical, St. Gallen, Switzerland]. Nuclear and cytoplasmic extracts were then collected according to the chromatin immunoprecipitation protocol as outlined in Chang *et al.* [24]. A final concentration of 1% formaldehyde was added to each resuspension and the solution was incubated on a shaking platform for 25 min at 23°C; 0.125 M glycine was added to each sample and then rocked for an additional 10 min and centrifuged at 10,000 x g for 5 min at 4°C. Each pellet was washed twice with 1 ml ice cold phosphate buffered saline (PBS) and 10 μl 100 mM PMSF and centrifuged at 1000 rpm (96 x g) for 5 min at 4°C. The pellets were resuspended in 400 μl cell lysis buffer [containing 5 mM PIPES at pH 8.0, 85 mM KCL, 0.5% nonidet (NP-40, USB Corporation; Cleveland, OH), and reagent grade water], 4 μl PMSF and 4 μl protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO). Each pellet was homogenized with 60 strokes on ice. After adding 25 μl NP-40, the suspensions were vortexed for 25 seconds and the cell resuspensions checked under the microscope for cell lysis. Once the cells were properly lysed, each sample was centrifuged at 5000 rpm (2,404 x g)

for 5 min at 4°C; the supernatant containing the cytoplasmic extract was stored at 4°C until used in co-immunoprecipitation studies and/or SDS-PAGE and silver staining. The pellet containing the nuclei was resuspended in 400 μl nuclei lysis buffer (containing 50 mM Tris-Cl at pH 8.1, 10 mM EDTA, 1% SDS, and reagent grade water), 4 μl PMSF, and 4 μl protease inhibitor cocktail and incubated on ice for 10 min. The samples containing chromatin were sonicated to previously determined 300-600 base pairs and centrifuged at 14,000 rpm (18,645 x g) for 10 min at 4°C. The supernatant containing the nuclear extract was removed to a new tube with 4 μl PMSF and 4 μl protease inhibitor cocktail.

Co-Immunoprecipitation with Ab099 (or Ab593)

Co-immunoprecipitation (Co-IP) studies were performed using a highly specific polyclonal antibody (Ab099 or Ab593) generated by the multiple antigenic peptide format to the N-terminal sequence of the $1,25D_3$-MARRS receptor. The basic protocol for co-precipitating proteins with protein A/G-sepharose beads was adapted from [25]. Prior to experimentation, 2 ml recombinant protein A-sepharose 4B bead slurry (Zymed Laboratories/Invitrogen; Carlsbad, CA) was blocked by combining 10 μl sonicated and boiled calf thymus DNA (Sigma-Aldrich; St. Louis, MO) at 100 μg/ml concentration and 10 μl bovine serum albumin (Sigma-Aldrich; St. Louis, MO) at 100 μg/ml concentration. The slurry was rotated 24 hrs at 4°C and then washed 3 times with dialysis buffer (containing 2 mM EDTA, 50 mM Tris-Cl pH 8.0, 0.2% Sarkosyl, and reagent grade water. The blocked sepharose beads were then centrifuged 5000 rpm at 4°C for 5 min; the supernatant was discarded and the blocked sepharose beads were equilibrated 1:1 with dialysis buffer and 0.02% NaN$_3$ (Sigma-Aldrich; St. Louis, MO) and stored at 4°C until experimentation.

As a positive control for co-immunoprecipitation studies, BLM were incubated with 130 pM, 300 pM, or 600 pM $1,25(OH)_2D_3$ for 2-5 hrs. After incubation of BLM samples with conjugated Ab099 (or Ab593), one volume of 20 mM CHAPSO (a zwitterionic detergent; Sigma-Aldrich Chemical Co., St. Louis, MO) was added to achieve a final concentration of 10 mM CHAPSO (critical micellar concentration which has been shown to solubilize the $1,25D_3$-MARRS receptor)

[29]. Samples were analyzed using SDS polyacrylamide-gel electrophoresis (SDS-PAGE) and were either silver stained or used for a Western blot (see below) to identify specific protein bands.

For the cytoplasmic or nuclear extracts, one hundred μl of each sample were pipetted into separate microcentrifuge tubes. (For the control samples, the negative control contained dialysis buffer only and a positive control contained samples and antibody only.) Upon the addition of 1-2 μg of Ab099 (or Ab593) to each sample, the microcentrifuge tubes were incubated on a rotating platform at 4°C for 3 hours. Then, 50 μl of blocked sepharose beads were added to each sample and rotated at 4°C for an additional 2 hours. The samples were centrifuged at 5000 rpm (2,404 x g) for 5 min at 4°C. Each pellet was washed twice with 1 ml dialysis buffer, 10 μl PMSF and 10 μl protease inhibitor cocktail, incubated on a rotating platform for 3 min at 4°C after each wash, and subsequently centrifuged at 5000 rpm (2,404 x g) for 5 min at 4°C. Each pellet was then washed four times with 1 ml immunoprecipitation (IP) wash buffer (containing 100 mM Tris-Cl at pH 9.0, 500 mM LiCl, 1% NP-40 and 1% deoxycholic acid), 10 μl PMSF, and 10 μl protease inhibitor cocktail. After the final wash, the samples were centrifuged again and the last traces of buffer (supernatant) were removed using gel tips. The sepharose beads containing the co-immunoprecipitated complexes were incubated in 75 μl elution buffer (containing 50 mM NaHCO$_3$, 1% SDS, and reagent grade water) on a rotating platform for 30 min at 23°C, centrifuged at 5000 rpm (2,404 x g) for 5 min, and the supernatants containing the co-IP isolates were transferred to clean microcentrifuge tubes. This elution step was repeated to obtain a second eluant. Both eluants were combined and analyzed by SDS-PAGE with silver staining or Western blot. Additionally, the eluants containing the co-immunopreci-pitated protein complexes were analyzed by mass spectrometry at the Center for Integrated Biosystems (Utah State University, Logan, UT).

Sodium Dodecyl Sulfate Polyacrylamide-Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins present in cell lysates or cytoplasmic or nuclear extracts. Protein samples (5-30 μg/well) were separated on 8% (wt/vol) SDS-PAGE gels with 5% stacking gels. After separation using SDS-PAGE, proteins were visualized by silver staining or Western blot analysis.

Western Blot Analyses

Western blot analyses were used to determine concentration of the immunoreactive levels of the $1,25D_3$-MARRS receptor, β-actin, or keratin in chick enterocytes in the presence or absence of 300 pM $1,25(OH)_2D_3$. Proteins were transferred from the SDS-PAGE gels to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Fisher Scientific) by the use of a Trans-Blot SD Semidry transfer cell (Bio-Rad Laboratories, Inc.) and Western analyses were performed. To avoid nonspecific binding, the membrane was soaked for 1h at 37°C in blocking solution [0.5% nonfat dry milk in phosphate buffered saline (PBS; 0.9% NaCl and 10 mM Na_2HPO_4, pH 7.4)], followed by washing three times for 5 min each time with washing solution [0.1%(wt/vol) BSA in Tris-buffered saline (TBS; 0.9% NaCl in 20 mM Tris-HCl, pH 7.4)], and incubation with primary antibody overnight at 4°C -either Ab099/Ab593 (rabbit anti-$1,25D_3$-MARRS protein N-terminal peptide; dilution 1:500 in 1% BSA and 0.05% Tween 20 in TBS), antibody against β-actin (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA), or antibody against keratin 2 (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA). After three additional washes, the membrane was incubated with secondary antibody in 1% BSA and 0.05% Tween 20 in TBS for 2 hr at room temperature and then washed as previously indicated. [Secondary antibodies used were either alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma; St. Louis, MO) for chromogenic visualization, or horseradish peroxidase-conjugated goat anti-rabbit IgG for chemiluminescent visualization (GE Healthcare Life Sciences; Piscataway, NJ). Immunoreactive bands were visualized with the chromogens 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Sigma; St. Louis, MO) or ECF substrate using the ECL Western Blotting kit (GE Healthcare Life Sciences; Piscataway, NJ). Relative amounts of the $1,25D_3$-MARRS receptor, β-actin, or keratin were quantified with the Kodak 2000R Imager using Kodak 1D Image Analysis Software.

Confocal Microscopy

For co-immunoprecipitation studies, successful identification of protein isolates interacting with the $1,25D_3$-MARRS receptor was verified by observing co-localization within cells. Intestinal epithelial cells were seeded onto coverslips placed in 35 mm Petri dishes and allowed to adhere over-night. The following morning, media were replaced with GBSS and cells treated either with vehicle or hormone for 1 or 5 min. At the end of this time, media were aspirated and the coverslips were gently washed with PBS. The coverslips were incubated in 3% paraformaldehyde, 3% sucrose in PBS and fixed for 30 min. After washing with PBS, cells were permeabilized with 0.15% Triton X-100 in PBS for 5 min and again washed. Coverslips were then overlayed with 5% normal rabbit in RPMI 1640 and washed with PBS. Coverslips were then overlayed with serum primary antibody [1/500 in 0.1% BSA-PBS for Ab099/Ab593, β-actin antibody, or keratin 2 antibody (Santa-Cruz Biotechnology, Inc; Santa Cruz, CA) for 30 min], washed, and then incubated with fluorescently-tagged secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Gove, PA). After washing with PBS, the coverslips were incubated in 100 nM tetramethylrhodamine B isothiocyanate (TRITC) labeled phalloidin or fluorescein isiothiocyanate (FITC) labeled phalloidin (Sigma-Aldrich). After the final wash, the coverslips were placed over mounting media (10% 1 M TRIS: 90% glycerol, pH 8.0) on a microscope slide, and sealed for subsequent confocal microscopy analysis.

Statistical Analysis

Values are expressed as mean ± SEM for the number of independent experiments indicated in the figure legends. The data were analyzed for significance using repeated measures, 2-way ANOVA (SigmaStat 3.1 and SigmaPlot 9.01) or the Student's *t*-test; significant differences were determined with a 95% probability (*$P<0.05$), unless otherwise indicated.

RESULTS

$1,25D_3$-MARRS Coimmunoprecipitates with Actin and Keratin in Chick Enterocytes

It has already been shown that the $1,25D_3$-MARRS receptor mediates rapid, $1,25(OH)_2D_3$-stimulated phosphate uptake in both chick and mouse enterocytes [3, 4] as well as $1,25(OH)_2D_3$-stimulated calcium uptake [8, 10].

In addition, investigators have demonstrated that the $1,25D_3$-MARRS receptor translocates from the baso-lateral membrane of chick intestines to other cellular compartments that include the nucleus upon $1,25(OH)_2D_3$ exposure [13]. In order to

identify possible protein-protein interactions involved in $1,25(OH)_2D_3$-stimulated translocation of the $1,25D_3$-MARRS receptor, we performed co-immunopre-cipitation studies in chick enterocytes using a highly specific polyclonal antibody (Ab099 or Ab593) generated against the N-terminal sequence of the $1,25D_3$-MARRS receptor from chick enterocytes exposed either to vehicle or 300 pM $1,25(OH)_2D_3$. Upon mass spectroscopic analysis of protein isolates and Edman degradation, both keratin and actin were identified as binding partners of the $1,25D_3$-MARRS receptor in the absence and presence of hormone (data not shown). Western blot analysis of protein isolates using chemiluminescent visualization confirmed that both actin (Fig. **1A**) and keratin (Fig. **3A**) were pulled down with the $1,25D_3$-MARRS receptor in both the absence and presence of hormone. Quantification of the concentration of actin pulled down revealed that the cytoplasmic extract in the absence of hormone contained the highest concentration of the $1,25D_3$-MARRS receptor: actin complex (Fig. **1B**); however, quantification of the concentration of keratin pulled down in the $1,25D_3$-MARRS receptor: keratin complex revealed no differences in either the cytoplasmic or nuclear extracts in the absence or presence of hormone (Fig. **3B**). We then proceeded to identify whether or not actin and keratin co-immunoprecipitated out the $1,25D_3$-MARRS receptor. We found through Western blot analysis and chemiluminescent visualization that while both actin (Fig. **2A**) and keratin (Fig. **4A**) pulled down the $1,25D_3$-MARRS receptor, quantification of protein isolates containing either the actin: $1,25D_3$-MARRS receptor complex (Fig. **2B**) or the keratin: $1,25D_3$-MARRS receptor complex (Fig. **4B**) revealed no differences observed in either cell extract in the absence or presence of $1,25(OH)_2D_3$.
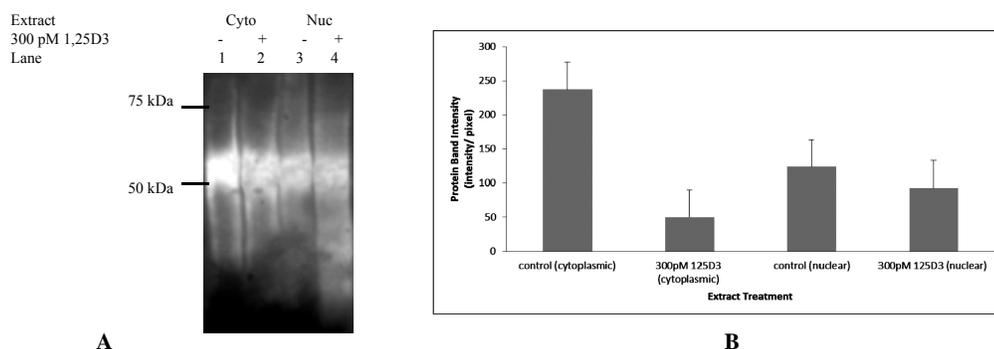
Fig. (1). A-B. β-actin co-immunoprecipitates with $1,25D_3$MARRS prior to and after 300 pM $1,25(OH)_2D_3$ exposure. Enterocytes were treated with the vehicle ethanol or with 300 pM $1,25(OH)_2D_3$ for 5 min prior to co-immunoprecipitation studies. Co-immunoprecipitates were isolated from cytoplasmic (A, Cyto, lanes 1-2) and nuclear (A, Nuc, lanes 3-4) extracts of isolated chick intestinal cells using Ab593 against $1,25D_3$MARRS. Western blotting using antibody against β-actin with chemiluminescent visualization (**1A**) revealed that β-actin co-immunoprecipitates with $1,25D_3$MARRS prior to and after 300 pM $1,25(OH)_2D_3$ exposure and the concentration of β-actin co-immunoprecipitate is highest in untreated cytoplasmic extracts (**1B**).
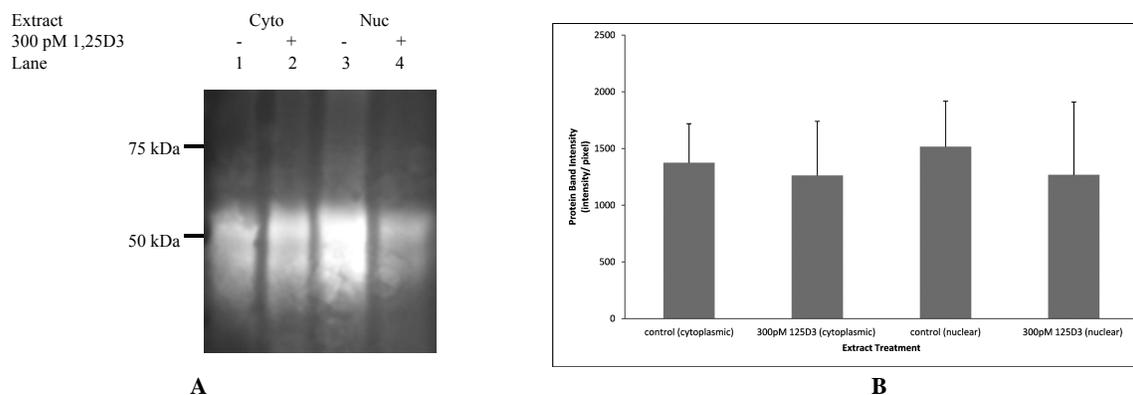
Fig. (2). A-B. $1,25D_3$MARRS co-immunoprecipitates with β-actin prior to and after 300 pM $1,25(OH)_2D_3$ exposure. Enterocytes were treated with the vehicle ethanol or with 300 pM $1,25(OH)_2D_3$ for 5 min prior to co-immunoprecipitation studies with β-actin. Western blotting using antibody against β-actin with chemiluminescent visualization (**2A**) revealed that $1,25D_3$MARRS co-immunoprecipitates with β-actin prior to and after 300 pM $1,25(OH)_2D_3$ exposure in both cytoplasmic and nuclear extracts (**2B**).
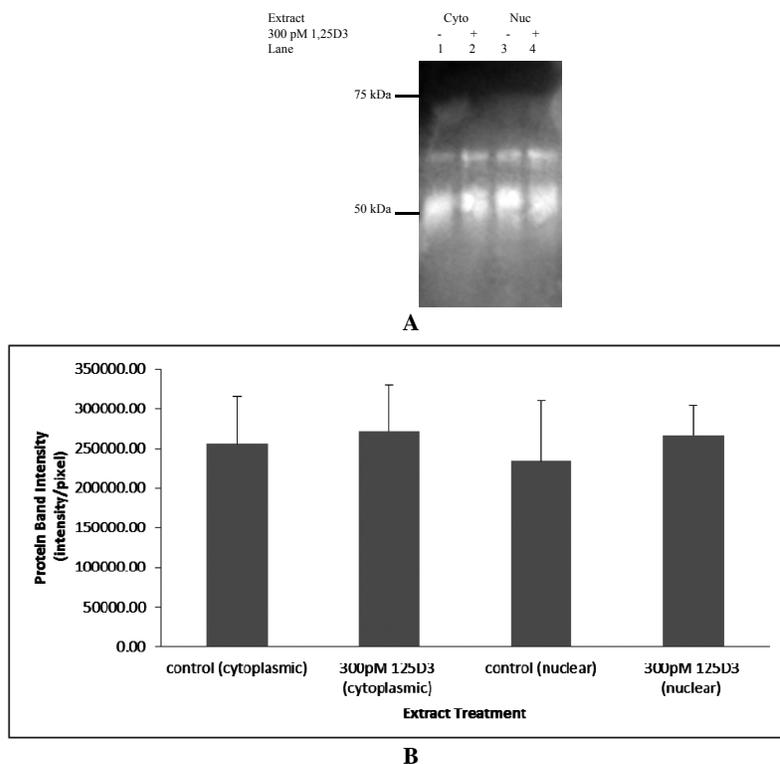
Fig. (3). A-B. Keratin co-immunoprecipitates with 1,25D$_3$MARRS prior to and after 300 pM 1,25(OH)$_2$D$_3$ exposure. Enterocytes were treated with the vehicle ethanol or with 300 pM 1,25(OH)$_2$D$_3$ for 5 min prior to co-immunoprecipitation studies with 1,25D$_3$MARRS. Co-immunoprecipitates were isolated from cytoplasmic (Cyto, lanes 1-2) and nuclear (Nuc, lanes 3-4) extracts of isolated chick intestinal cells using antibody against keratin. Western blotting using antibody against keratin with chemiluminescent visualization (**3A**) revealed that keratin co-immunoprecipitates with 1,25D$_3$MARRS prior to and after 300 pM 1,25(OH)$_2$D$_3$ exposure (**3B**).
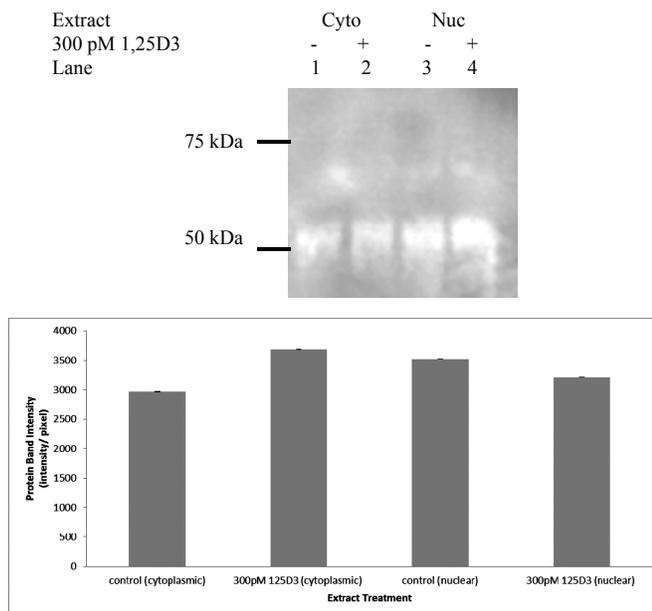
Fig. (4). A-B . 1,25D$_3$MARRS co-immunoprecipitates with keratin prior to and after exposure to 1,25(OH)$_2$D$_3$. Enterocytes were treated with the vehicle ethanol or with 300 pM 1,25(OH)$_2$D$_3$ for 5 min prior to co-immunoprecipitation studies with antibody against keratin. Western blotting using antibody against 1,25D$_3$MARRS with chemiluminescent visualization (**4A**) revealed that 1,25D$_3$MARRS co-immunoprecipitates with keratin prior to and after 300 pM 1,25(OH)$_2$D$_3$ exposure in both cytoplasmic and nuclear extracts (**4B**).
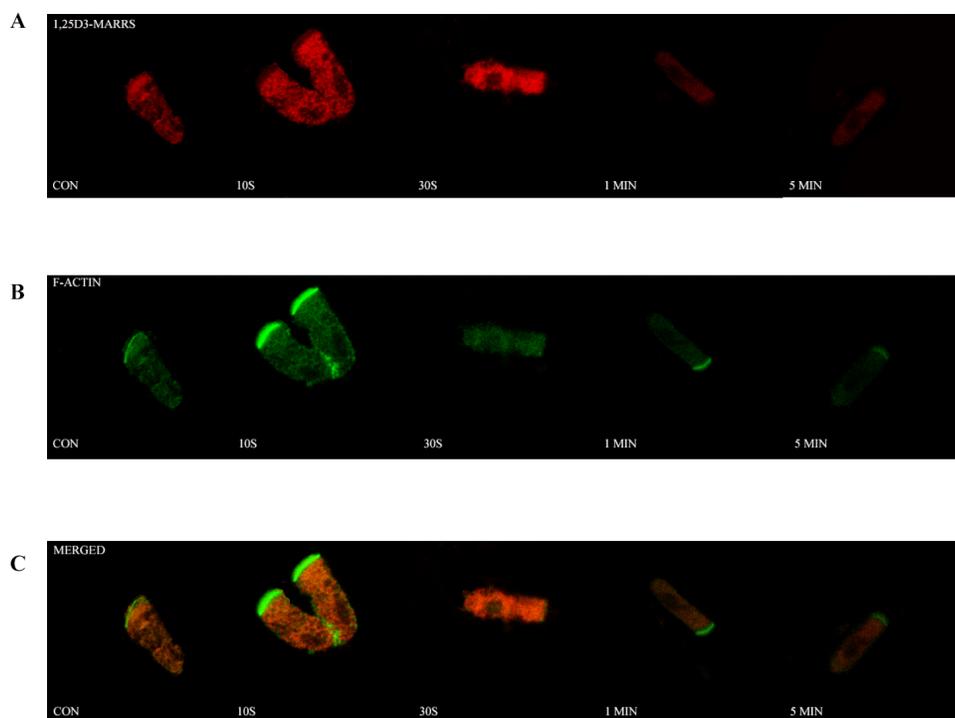
Fig. (5). A-C. Phenol red induces immunocytochemical localizations of 1,25D$_3$-MARRS (**A**) and F-actin (**B**) in chick enterocytes within seconds after exposure to vehicle (0.01% ethanol; CON) or 300pM 1,25D$_3$ (hormone; 10 sec -5 min). 1,25D$_3$ stimulates intracellular redistribution of 1,25D$_3$-MARRS receptor that is maximal within 30 s and cyclic polymerization of F-actin that is maximal within 10s (**C**, merged images).
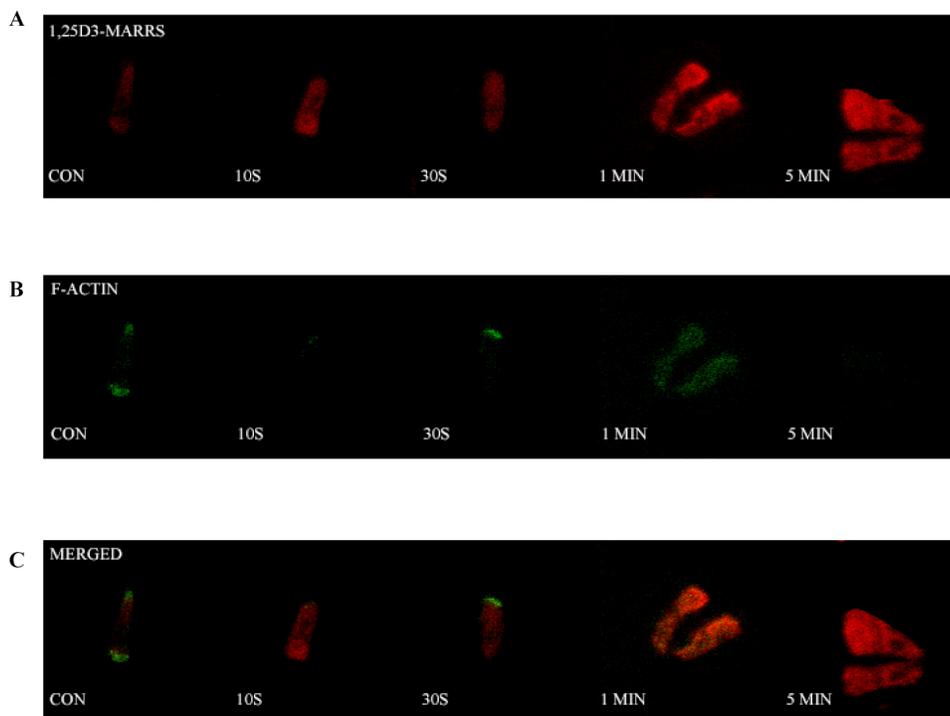
Fig. (6). A-C. Immunocytochemical localizations of 1,25D$_3$-MARRS (**A**) and F-actin (**B**) in chick enterocytes after exposure to vehicle (0.01% ethanol; CON) or 300pM 1,25D$_3$ (hormone; 10 sec -5 min). 1,25D$_3$ stimulates intracellular redistribution of 1,25D$_3$-MARRS receptor that is maximal within 5 min and cyclic polymerization of F-actin that is maximal within 1 min (**C**, merged images).
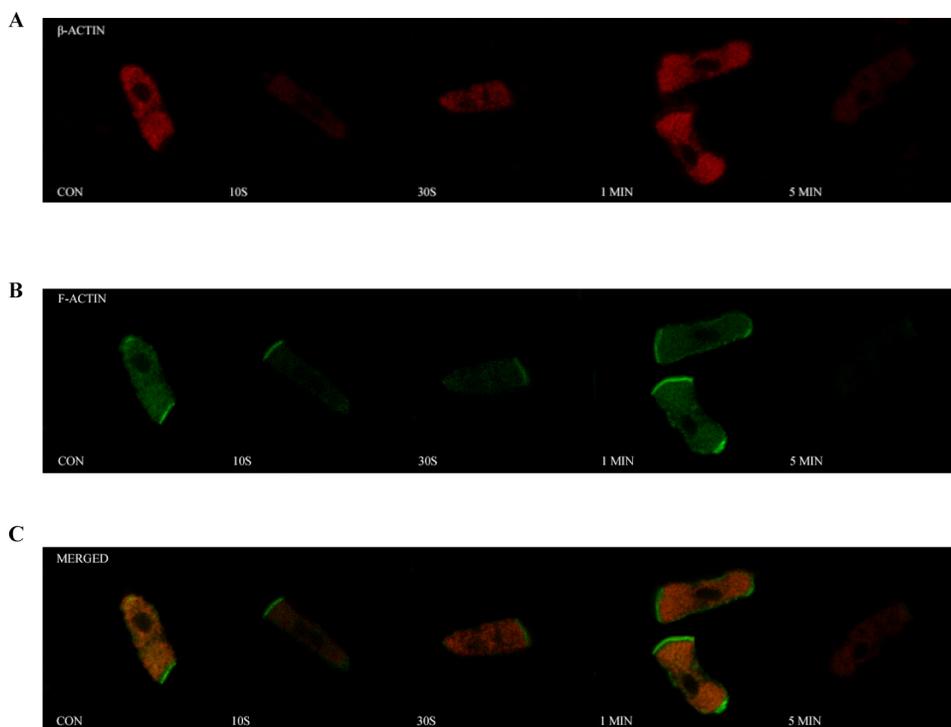
Fig. (7). A-C. Immunocytochemical localizations of β-actin (**A**) and F-actin (**B**) in chick enterocytes after exposure to vehicle (0.01% ethanol; CON) or 300pM 1,25D$_3$ (hormone; 10 sec -5 min). 1,25D$_3$ stimulates intracellular redistribution of β -actin that parallels cyclic polymerization of F-actin (**C**, merged images).

1,25(OH)$_2$D$_3$ Stimulates Cellular Redistribution of the 1,25D$_3$-MARRS Receptor and Cytoskeletal Components (Actin and Keratin)

Confocal imaging of cultured chick enterocytes was used to visualize cellular redistribution of the 1,25D$_3$-MARRS receptor, actin and keratin after hormone exposure. Initially we used cells cultured in media containing phenol red, and found labeling of both the 1,25D3-MARRS receptor and actin in the nucleus (Fig. **5**), which dispersed after hormone treatment (Fig. **5**). For the remainder of the experiments with microscopy, media without phenol red were used. Using a highly specific polyclonal antibody (Ab099 or Ab593) generated against the N-terminal sequence of the 1,25D$_3$-MARRS receptor as the primary antibody and a fluorescein-linked secondary antibody, we demonstrated that cellular redistribution of the 1,25D$_3$-MARRS receptor occurred within 10s of exposure to 1,25(OH)$_2$D$_3$ and was maximal by 30s (Fig. **6A**). We simultaneously used a phalloidin stain to track changes in filamentous actin (F-actin) and found that, upon hormone exposure, F-actin was concentrated in the brush border membranes of the enterocytes within 10s (Fig. **7B**) where nutrients such as vitamin D, calcium and phosphate are ab-

sorbed into the intestinal lumen. These observations illustrate that 1,25(OH)$_2$D$_3$ stimulated intracellular redistribution of the 1,25D$_3$-MARRS receptor. We also found that 1,25(OH)$_2$D$_3$ simultaneously induced cyclical conversions of F-action to globular actin (G-actin) (Fig. **7C**) that were involved in the cytoskeletal transport of proteins and/or nutrients across intestinal cells. We then proceeded to use the antibody against β-actin as the primary antibody and also observed that cytoskeletal arrangements of both G-actin and F-actin were cyclical in the presence of hormone (Fig. **7A-C**).

Similar studies were conducted using an anti-keratin 2 antibody. As shown in Fig. (**8**), keratin staining diminished rapidly 10 sec after hormone, became faintly visible 1 min after steroid, and returned to control levels at 5 min.

After observing these 1,25(OH)$_2$D$_3$-mediated oscillations in cultured, adherent cells, studies were undertaken to determine if they might be correlated to steroid-induced phosphate or calcium uptake. (Fig. **9**) depicts the results of phosphate uptake experiments in which adherent cells were treated with vehicle or 300 pM 1,25(OH)$_2$D$_3$ for
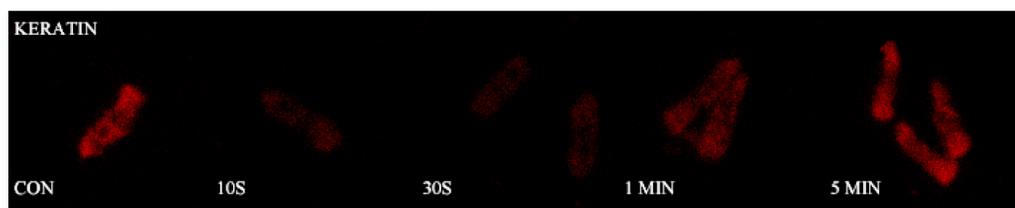
Fig. (8). Immunocytochemical localizations of keratin in chick enterocytes after exposure to vehicle (0.01% ethanol; CON) or 300pM $1,25D_3$ (hormone; 10 sec -5 min). $1,25D_3$ stimulates intracellular redistribution of keratin that parallels cyclic polymerization of F-actin.
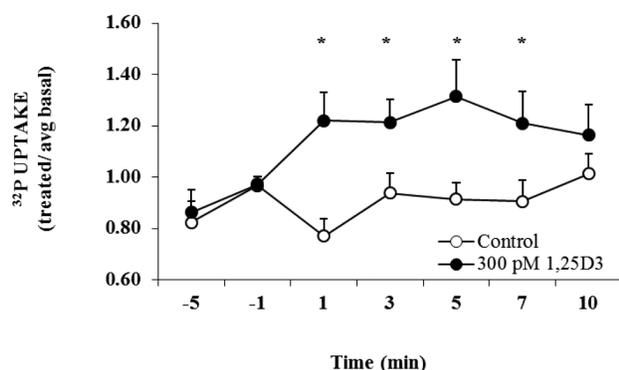
Fig. (9). $1,25(OH)_2D_3$ stimulates $^{32}PO_4$ uptake in isolated intestinal epithelial cells within 1 min. Primary cell cultures of chick enterocytes were incubated 18 hr prior to treatment with the vehicle ethanol (○-○) or with 300 pM $1,25(OH)_2D_3$(●-●). Significant increases in $1,25(OH)_2D_3$-mediated phosphate uptake were observed at 1, 3, 5 and 7 min when compared to control values. Values represent means ± SEM (n=12). Significant differences (*$P<0.05$) were determined using the unpaired Student's t-test.

time course studies. The steroid induced a significant uptake at 1 min after addition, and uptake remained elevated until 7 min after hormone. Thus, no correlation could be found between hormone-stimulated phosphate uptake and oscillations in

$1,25D_3$-MARRS receptor staining, or actin staining. As previously reported [8] $1,25(OH)_2D_3$ had no visible effect on ^{45}Ca uptake in freshly isolated cells because uptake is balanced by efflux. As shown in Fig. (**10**), there was a marginal effect after an 18 h culture period, but a significant increase in radionuclide uptake only occured after 72 h of culture (Fig. **11**). However, there again was no correlation with hormone-mediated changes in staining.

DISCUSSION

Co-immunoprecipitation studies identified actin and keratin as binding partners of the $1,25D_3$-MARRS receptor according to both mass spectroscopy and Western analysis. These binding partners are similar to those found by Cicchillitti *et al.* in HeLa cells [26] but different than those reported by Clark *et al.* [27] who found that the $1,25D_3$-MARRS receptor colocalized with NFκB in promyleocytic leukemia cells. This suggests that binding partners for the $1,25D_3$-MARRS receptor may be tissue and/or species specific.

Fig. (10). $1,25(OH)_2D_3$ stimulates ^{45}Ca uptake in isolated intestinal epithelial cells within 3 min. Primary cell cultures of chick enterocytes were incubated 18 hr prior to treatment with the vehicle ethanol (○-○) or with 300 pM $1,25(OH)_2D_3$(●-●). Significant increases in 300 pM $1,25(OH)_2D_3$-mediated calcium uptake was observed at 3 min when compared to controls. Values represent means ± SEM (n=10). Significant differences (*$P<0.10$) were determined using the unpaired Student's t-test.

Fig. (11). 1,25$(OH)_2D_3$ stimulation of ^{45}Ca uptake is delayed in isolated chick intestinal epithelial to 7 min after 72 hr culture. Primary cell cultures of chick enterocytes were incubated 72 hr prior to treatment with the vehicle ethanol (○-○) or with 300 pM 1,25$(OH)_2D_3$(●-●). Significant increases in 300 pM 1,25$(OH)_2D_3$-mediated calcium uptake was delayed to 7 min when compared to controls. Values represent means ± SEM (*n*=12). Significant differences (*$P < 0.05$) were determined using the unpaired Student's *t*-test.

Our initial experiments on localization of the 1,25D_3-MARRS receptor in intestinal epithelial cells cultured in phenol red indicated a nuclear localization without addition of steroid hormone. These are essentially the same findings as those by Barsony *et al.* [28] using dermal fibroblasts to study the localization of the classical vitamin D receptor (VDR). This, plus the observation that the 1,25D_3-MARRS receptor dispersed to the cytoplasm upon treatment with hormone, provided the incentive to perform the remainder of the studies with cells cultured in media without phenol red. Under these conditions, 1,25$(OH)_2D_3$ caused redistribution of the receptor to the nucleus within 30 sec and became maximal at 5 min. The oscillatory nature of the staining led us to examine whether it could be correlated with either phosphate or calcium uptake. While the steroid hormone significantly stimulated phosphate uptake in freshly isolated cells, stimulation of calcium uptake required culturing the cells for 18-72 h in order to diminish PK C activity [9]). However, there was no correlation between ion uptake and cyclical distribution of the 1,25D_3-MARRS receptor or actin polymerization. The reason for the cyclical changes remains to be determined.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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