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Spectrin Localization in Osteoclasts: Immunocytochemistry, Cloning, and Partial Sequencing

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Abstract The presence of spectrin was demonstrated in chick osteoclasts by Western blotting and light and electron microscopic immunolocalization. Additionally, screening of a chick osteoclast cDNA library revealed the presence of α-spectrin. Light microscope level immunocytochemical staining of osteoclasts in situ revealed spectrin staining throughout the cytoplasm with heavier staining found at the marrow-facing cell margin and around the nuclei. Confocal microscopy of isolated osteoclasts plated onto a glass substrate showed that spectrin encircled the organelle-rich cell center. Nuclei and cytoplasmic inclusions were also stained and the plasma membrane was stained in a nonuniform, patchy distribution corresponding to regions of apparent membrane ruffling. Ultracytochemical localization showed spectrin to be found at the plasma membrane and distributed throughout the cytoplasm with especially intense staining of the nuclear membrane and filaments within the nuclear compartment. J. Cell. Biochem. 71:204–215, 1998.

Key words: osteoclast; spectrin; membrane skeleton; bone; bone resorption

Osteoclasts are large, multinucleated, bone-resorbing cells that display both a morphological and a functional polarity when attached to bone. The most striking feature of an osteoclast engaged in bone resorption is the ruffled border, a specialized region of the plasma membrane consisting of extensive cytoplasmic processes and overlying the resorption lacunae. Surrounding the ruffled border and tightly apposed to the bone surface is the clear zone, an organelle-free region containing actin filaments [King and Holtrop, 1975] and other cytoskeletal elements such as α-actinin and vinculin [Marchisio et al., 1984; Lakkakorpi and Väänänen, 1991]. The clear zone functions in cell adhesion, via receptors in the integrin family, and in sealing off the extracellular space from the resorptive area, thereby creating a specialized microenvironment in the resorption lacunae [Davies et al., 1989; Teti et al., 1991; Aubin, 1992]. The cytoplasm interior to the ruffled border possesses an abundance of vesicles containing lysosomal enzymes [Baron et al., 1988] and a nuclear region surrounded by multiple Golgi complexes and numerous mitochondria [for reviews see Baron et al., 1993; Teitelbaum et al., 1996]. Acid secretion from the ruffled border region requires ion-generating and ion-transport systems to be present in the cytosol and in several distinct plasma membrane domains [Baron et al., 1993; Gay, 1996].

Cells of multicellular organisms possess a multicomponent, spectrin-based membrane skeleton located just beneath and tethered to the plasma membrane. The organization of the membrane skeleton is similar in both erythroid and nonerythroid cells. In red blood cells, the membrane skeleton confers the properties of strength, elasticity, and deformability that are required to withstand the tremendous shear forces encountered in the circulation and to successfully traverse the microvasculature. De-

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fects in the membrane skeleton result in severe hemolytic anemia in mice and humans [Bodine et al., 1984; Peters and Lux, 1993; Lux and Palek, 1995]. In nonerythroid cells, the membrane skeleton functions in the establishment and maintenance of the polarized distribution of integral membrane proteins, including ion channels, transporters, and cell adhesion molecules [Peters and Lux, 1993]. Additionally, it is proposed to be involved in regulating secretory vesicle - plasma membrane interaction during secretion [Bennett, 1990; Bennett and Lambert, 1991]. The major component of the membrane skeleton, spectrin, exists as tetramers of α and β subunits, which are crosslinked into a two-dimensional array by short actin filaments and linked to transmembrane proteins in the overlying plasma membrane [for review see Bennett and Gilligan, 1993]. Ankyrin is the membrane skeleton protein that serves as the major linker molecule between the spectrin lattice and the plasma membrane.

Although spectrin is often found uniformly to line the cytoplasmic face of the cell membrane, numerous exceptions to this pattern exist. Spectrin has been localized to the cytoplasm, Golgi complex, endosomes, and nucleus of various cell types [Zagon et al., 1986; Lee et al., 1988; Fujimoto and Ogawa, 1989; Bachs et al., 1990; Gundersen et al., 1991; Beck et al., 1994; Devarajan et al., 1996]. Lymphocytes possess spectrin in cytoplasmic aggregates and in membrane-associated forms [Lee et al., 1988; Black et al., 1988]. In neural tissue, it is localized along the microtubules [Shimo-oka and Atsumi, 1986; Zagon et al., 1986] and around cytoplasmic organelles [Zagon et al., 1986]. In the principal cells of the kidney collecting duct, spectrin is found associated with the basolateral membrane and also diffusely scattered throughout the cytoplasm [Fujimoto and Ogawa, 1989]. The distribution of spectrin is often dynamic, undergoing rearrangement upon stimulation in cells such as adrenal chromaffin [Perrin and Aunis, 1985; Fujimoto and Ogawa, 1989] , gastric parietal [Mercier et al., 1989], parotid acinar [Perrin et al., 1992], T lymphocytes [Gregorio et al., 1992], and platelets [Fox et al., 1993].

This report demonstrates the presence of spectrin in osteoclasts by Western blotting, immunocytochemistry, and cloning and partial sequencing. Study of the distribution of spectrin in osteoclasts and its interaction with various membrane components may provide important information regarding the mechanism by which these cells establish their polarized orientation on the bone surface.

MATERIALS AND METHODS

Bovine pancreatic trypsin (3× crystallized) was obtained from Worthington Biochemicals (Freehold, NJ). Bovine serum albumin (BSA, Fraction V), calf serum (CS), fetal bovine serum (FBS), goat serum (GS), salmon calcitonin (CT), minimal essential medium (MEM, Eagle's modification), L-glutamine, type 1-S hyaluronidase, type 1-A collagenase, Nα-p-tosyl-L-lysine-chloromethylketone (TLCK) , poly-L-lysine, and Percoll were from Sigma (St. Louis, MO). Trypsin (bovine pancreatic, 3× crystallized) was from Worthington Biochemicals. MEM was modified by the addition of 26 mM L-glutamine, 2 mM $NaHCO_3$, 100 U/ml penicillin, 100 µg/ml streptomycin, and additional $CaCl_2$ (~0.03 g/L), KCl (~0.05 g/L), and NaCl (~0.08 g/L) to adjust osmolality to 310 mOsm. All other chemicals were reagent grade.

Cells and Embryos

The isolated osteoclast studies utilized rapidly growing broiler chicks (Peterson x Arbor Acre strain) obtained from the Metz Hatchery (Belleville, PA). Chicks were raised to 2.5 - 3 weeks of age on a normal chick starter diet by the Poultry Education and Research Center of Pennsylvania State University. Light and electron microscopic tissue studies were performed on Day 19 broiler chick embryos provided as a gift by Avian Farms International (Waterville, ME).

Antibodies

Rabbit antisera raised against a mixture of purified chicken erythrocyte α and β spectrin was purchased from Accurate Chemical & Scientific Corp (Westbury, NY). The secondary antibody, Cy3 conjugated goat antirabbit IgG, was purchased from Chemicon International (Temecula, CA). The 20 nm gold conjugated, goat antirabbit IgG was from British BioCell International (obtained through Ted Pella, Redding, CA). The biotinylated antirabbit IgG was from Kirkegaard and Perry (Gaithersburg, MD).

Cell Isolation and Culture

Osteoclasts were isolated from the endosteum of tibias from chickens that had been

injected subcutaneously with CT (30 mU/100 g body weight) to facilitate release of the cells from bone surfaces [Ali et al., 1984; Hunter et al., 1988]. The birds were sacrificed 60 minutes after CT injection. Tibias were aseptically removed, cleaned with sterile gauze to remove attached tendon and muscle, and split longitudinally. The bone marrow was lifted out with forceps and the bones were then placed in MEM + 10% heat inactivated CS and flushed by vigorous pipetting. The bone pieces were then subjected to a mild, three-stage, enzymatic digestion consisting of: (1) 0.05% hyaluronidase in MEM + 10% CS for 10 minutes and rinsed in MEM; (2) 0.03% trypsin in MEM for 20 minutes and rinsed in MEM + 10% CS; and (3) 0.1% collagenase + 0.0027% TLCK in MEM + 10% CS for 75 minutes and rinsed in MEM + 10% CS [May and Gay, 1997]. For the confocal microscopy experiments, the cell isolation procedure consisted of digestion in 0.03% trypsin in MEM for 20 minutes followed by rinsing in MEM + 10% CS.

All digestion steps were carried out in an incubator at 37°C, 5% CO_2, 95% air, and saturated humidity. The endosteal surfaces were then gently scraped with a rubber policeman in MEM + 10% CS. The released cells were filtered through 250 µm and 105 µm polypropylene meshes (Small Parts, Miami Lakes, FL) and centrifuged at 635 g for 10 minutes. The pellet was resuspended in MEM + 5% heat inactivated FBS. Approximately 4000–6000 osteoclasts were plated per well on to round, glass coverslips.

Immunoblotting for Spectrin Expression

Osteoclast preparations to be used for Western blotting analysis were further purified by Percoll gradient centrifugation [Bekker and Gay, 1990]. Following the three-stage isolation procedure described above, the cell suspension was resuspended in 10 ml of a 35% Percoll solution, 1.0 ml of normal Tyrodes was layered on top, and the mixture centrifuged at 700 g for 20 minutes at 4°C. The interface of the Percoll and Tyrodes solution was collected by aspiration, resuspended in Tyrodes buffer, and centrifuged at 765 g for 10 minutes at 4° C. The pelleted cells were then resuspended in a 0.01 M Tris, 0.05 M NaCl buffer, pH 7.6 containing a cocktail of phosphatase and protease inhibitors as previously described [Migliaccio et al., 1993], allowed to lyse for 30 minutes on ice and centrifuged at 23,600 g for 30 minutes at 4° C. The supernatant and pellet were solubilized in buffer [Laemmli, 1970], vortexed, boiled for 5 minutes and frozen in liquid nitrogen.

SDS-PAGE was performed using a 5% stacking/10% running gel [Laemmli, 1970] and prestained molecular weight standards (Bio-Rad Kaleidoscope standards: myosin, 202 kD; β-galactosidase, 133 kD; BSA, 71 kD; carbonic anhydrase, 41.8 kD; soybean trypsin inhibitor, 30.6 kD; lysozyme, 17.8 kD and aprotinin, 6.9 kD). Separated proteins were transferred in a Bio-Rad Trans Blot SD semidry apparatus to Immobilon P membranes (Millipore Corp., Bedford, MA) in 0.048 M TrisCl, 0.039 M glycine, pH 9.2, containing 20% methanol. Efficiency of transfer was monitored by the transfer of prestained markers. Filters were blocked in 5% BSA in TTBS (0.010 M TrisCl, 0.05% Tween-20, 0.150 M NaCl, pH 8.0) for a minimum of 1 hour, incubated for 1–2 hours with primary antibody (diluted 1:300 in TTBS plus 1% BSA), washed two times in TTBS, incubated 1 hour with goat antirabbit IgG alkaline phosphatase conjugate (Bio-Rad) at a dilution of 1:10,000 in TTBS with 1% BSA, and washed twice in TTBS. Bound antibody was visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate using a commercial kit (Bio-Rad). All incubations were at room temperature.

Immunocytochemistry

Tibias were harvested from 19-day chick embryos and immediately fixed in Bouin's fixative. The specimens were processed for paraffin embedding and sections were mounted on baked poly-L-lysine (Sigma) coated slides. For detection of spectrin, sections were deparaffinized and hydrated in Tris buffer (0.2 M TrisCl, pH 7.2), incubated with 2% BSA in the same Tris buffer for 30 minutes at room temperature, washed in Tris buffer containing 0.25% BSA, and incubated overnight in a humidified chamber at 4°C with rabbit antichicken RBC spectrin diluted 1:200 in Tris buffer containing 0.1% BSA. Control slides were incubated in buffer alone. The following day, the slides were washed in Tris buffer followed by detection of bound antispectrin using a commercial kit (Kirkegaard and Perry, Gaithersburg,MD). Briefly, slides were incubated for 45 minutes at room temperature with biotinylated antirabbit IgG, washed in Tris buffer, and incubated for 10–15 minutes at RT with alkaline phosphatase-

conjugated streptavidin. Positive reaction was seen as a red precipitate.

Confocal Microscopy

Spectrin immunocytochemistry was performed on osteoclasts after 4 days in culture when the cells were flattened and well spread as previously described [Hunter et al., 1989]. Briefly, coverslips were rinsed in PBS for 30 seconds and fixed in 4% paraformaldehyde in 0.01 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.2, for 10 minutes at room temperature. Coverslips were then rinsed in PBS (5 minutes, room temperature) and then rinsed in 0.01 M sodium phosphate buffer containing 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl and 0.1% gelatin, pH 7.5, for 10 minutes at room temperature. Antibody incubations (45 minutes, room temperature) were performed using equal volumes of the Triton X-100 /SDS buffer and the primary or secondary (Cy3 conjugate) antibodies diluted in PBS. After each antibody incubation, the coverslips were dipped one time in the Triton X-100/SDS buffer and then given three 5-minute rinses in that buffer at room temperature. After the last rinse, the coverslips were dipped one time in distilled water and mounted on glass slides using Fluoromount G. Negative controls consisted of coverslips incubated in buffer lacking the primary antibody followed by incubation in the Cy3 conjugated goat antirabbit IgG. Slides were examined with a Bio-Rad MRC 600 Laser Scanning Confocal microscope with an excitation wavelength of 568 nm. Images were obtained by making projections of individual optical z-sections (i.e., sections parallel to the coverslip) taken at 1 µm intervals through the cell. Confocal images were constructed using Bio-Rad software.

Immunoelectron Microscopy

Tibias were removed from day 19 chick embryos and immediately fixed in periodate-lysine-paraformaldehyde fixative (PLP) [McLean and Nakane, 1974] for 3.5 hours at room temperature. Tissue was demineralized for 3 days with daily changes of 0.1 M disodium EDTA , dehydrated in a graded ethanol series, embedded in LR White (Ted Pella, Redding, CA), and polymerized for 24 hours at 50°C.

Ultrathin sections mounted on nickel grids were incubated in 0.02 M glycine in 0.02 M Tris buffer containing 0.1 % BSA for 10 minutes at room temperature followed by incubation for 30 minutes in the Tris/BSA buffer to which 0.05% Tween 20 and 10% heat-inactivated NGS had been added. All antibody dilutions were made in the Tris/BSA/Tween buffer containing 1% heat-inactivated NGS. Incubation in the anti-spectrin antibody was for 15 hours at room temperature in a humidified chamber with gentle rocking followed by multiple rinses in the Tris/BSA/Tween 20 buffer. Incubation in goat-antirabbit IgG gold conjugate was for 1 hour with gentle rocking followed by another series of wash steps. Negative controls consisted of grids incubated in buffer lacking primary antibody followed by incubation in goat ant-rabbit IgG gold conjugate. Sections were counterstained with uranyl acetate and lead citrate and viewed with a Phillips CM-10 electron microscope.

cDNA Library Screening

Avian osteoclasts were isolated by the procedure outlined in Collin-Osdoby et al., [1991] and polyA mRNA was used to produce a lambda Zap II expression library [Sunyer et al., 1993]. The cDNA library was screened by plaque hybridization [Sambrook et al., 1989] using a chicken α-spectrin cDNA probe [Birkenmeier et al., 1985]. Hybridizations and posthybridization washes were performed at 65°C, as described in Peters et al. [1991]. Positive lambda Zap II clones were excised in vivo according to the manufacturer's directions, which generated subclones in the Bluescript SK(+) plasmid.

DNA Sequencing

Plasmid DNA was prepared by the cetyltrimethylammonium bromide (CTAB) method [Bel Sal., 1989] and sequenced using dye terminator chemistry on an ABI 373A stretch automated sequencer. The data were analyzed using Sequencher 3.0.

RESULTS

Spectrin Demonstration by Western Blotting

We demonstrated the presence of spectrin in osteoclasts by SDS-PAGE followed by Western blotting using a polyclonal antibody specific for both chicken α and β spectrin. Spectrin was found in both the supernatant and pellet fractions at positions indicating approximate molecular weights of 220 and 240 kDa (Fig. 1, Lanes A and B). A control blot run using buffer

in place of primary antibody showed no staining (Fig. 1, Lanes C and D). Additional support for the specificity of the primary antibody was provided by the immunoelectron microscopic staining of spectrin in chicken red blood cells (see Fig. 4A).

Immunocytochemistry

Immunocytochemistry of osteoclasts in situ. The immunocytochemical staining of osteoclasts in situ showed that spectrin is distributed throughout the cytoplasm with a heavier distribution on the marrow-facing surface (Fig. 2A,B, arrowheads). Staining was found to encircle the nuclei and other cytoplasmic inclusions (Fig. 2A,B, small arrows). Moderate staining was found in the clear zone (Fig. 2A, thick arrow). Control sections treated with buffer in place of primary antibody showed no staining (Fig. 2C).

Confocal microscopy of isolated osteoclasts. Fluorescent staining of spectrin in isolated osteoclasts plated onto glass is shown as a montage of confocal images of a single cell (Fig. 3). Spectrin is found abundantly in a generalized, filamentous pattern in the region of the cell closest to the glass substrate (Fig. 3A). Moving progressively away from the substrate, spectrin appears to encircle the cell center (Fig. 3C-F, open arrowheads). The nuclei are clearly stained (Fig. 3B,C, arrows) as are other cytoplasmic inclusions (Fig. 3B,C, small arrowheads). The plasma membrane is stained in a nonuniform, patchy distribution with more intense staining found in regions of apparently ruffling membrane (Fig. 3B-F, thick arrowheads). Control coverslips showed no background staining.

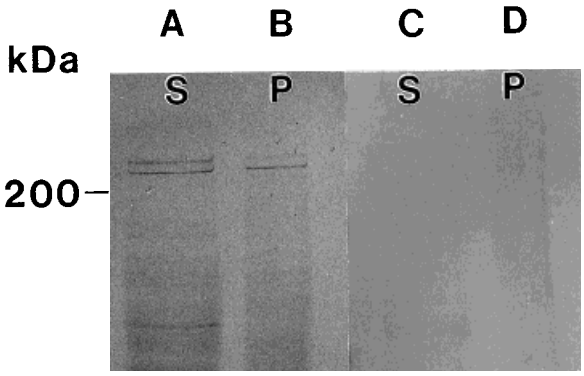

Fig. 1. Immunoblot demonstration of spectrin in lysate of isolated osteoclasts. Supernatant (S) and pellet (P) fractions obtained after lysis, dilution, and centrifugation were analyzed by SDS-PAGE, transferred to Immobilon P (Millipore Corp, Bedford, MA) and immunostained with polyclonal antispectrin (**lanes A,B**) or buffer in place of primary antiserum (**lanes C,D**). The position of myosin, the 200 kD molecular weight marker is indicated.

Immunoelectron microscopy. Immunogold staining of red blood cells in the marrow cavity served as positive staining controls and as further evidence of the specificity of the antibody. In red blood cells, the gold particles were consistently found in a submembranous pattern around the periphery of the cell (Fig. 4A, small arrows) and also in the nucleus (data not shown). Examination of both red blood cells and osteoclasts on the negative control grids showed no background staining.

Spectrin in osteoclasts was associated with membranous surfaces in the cytoplasm and at the cell surface. Staining revealed approximately parallel arrays of gold particles running through the cytoplasm and underlying the plasma membrane at both the bone-facing and marrow-facing surfaces (Fig. 4B, arrowheads). In addition, mitochondrial surfaces were commonly labeled (Fig. 4B, white arrows). Within the cytoplasm, occasional clusters of 3–4 gold particles were observed in close association with membranes or filaments (Fig. 4B, asterisks). Gold particles were also found to be associated with filaments in the actin-rich attachment zone (Fig. 5, arrowheads). Spectrin staining was especially intense within the nuclei. Gold particles were closely aligned with the nuclear membrane (Fig. 6A,B, arrowheads) and were found along filaments within the nuclear compartment (Fig. 6A,B, arrows). Nucleolar staining was also observed (Fig. 6B, white arrows).

cDNA Library Screening

To confirm the presence of spectrin in osteoclasts, an osteoclast cDNA library [Sunyer et al., 1993] was screened using an available chicken α-spectrin cDNA [Birkenmeier et al., 1985] as the hybridization probe. 1×10^6 recombinants were screened, and two identical 3.81 Kb clones were obtained and end-sequenced (1422 bp total). Comparison with known sequences (GenBank) confirmed >99% identity with chicken α-spectrin [Wasenius et al., 1989; GenBank accession number ×14519], thus establishing the presence of spectrin in chicken osteoclasts at the molecular level and confirming the antibody localization data.

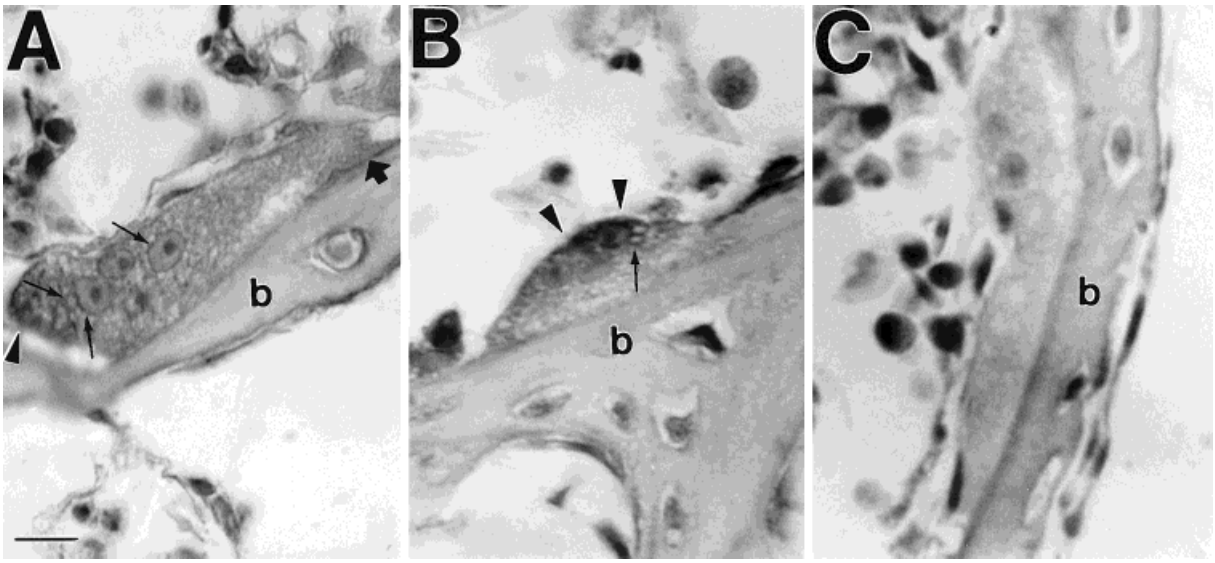

Fig. 2. Light microscope level immunostaining of osteoclasts in situ. Chick tibias were fixed in Bouin's fixative, embedded in paraffin, and stained with polyclonal anti-spectrin (**A,B**) or treated with buffer lacking the antibody (**C**). Heavier stain deposition was found along the marrow-facing surface of the cell (A and B, arrowheads) and encircling the nuclei and other cytoplasmic inclusions (A and B, small arrows). Moderate staining was found in the clear zone (A, thick arrow). b, denotes bone. Scale bar = 10 μm.

DISCUSSION

This study is, to our knowledge, the first report of spectrin identification and localization in osteoclasts. Spectrin was found in the supernatant and pellet fractions of isolated chick osteoclasts by Western blotting. Centrifugation at the speed employed yielded a supernatant fraction that would be expected to contain membrane derived from both plasma membrane and endoplasmic reticulum. The pellet fraction would contain any nonlysed cells, nuclei, mitochondria, lysosomes, and the insoluble cytoskeleton. Immunostaining revealed the association of spectrin with the plasma membrane and filaments and membranes within the cytoplasm and the nuclei.

Spectrin typically associates with integral membrane proteins via the membrane skeleton protein, ankyrin. Three ankyrin genes have been described from which an enormous diversity of protein isoforms are derived through tissue-specific alternative mRNA processing [Bennett and Lambert, 1991; Peters et al., 1995]. As a result, the polarized distribution of many different ankyrin ligands is attriibuted to association with ankyrin and, in turn, spectrin. Examples include the basolateral distribution of the anion exchanger (band 3, AE1) in kidney intercalated cells [Drenckhahn et al., 1985] and the Na^+K^+ATPase in kidney and parotid epithelial cells [Nelson and Veshnock, 1987; Morrow et al., 1989; Koob et al., 1990]. In retinal pigmented epithelium and choroid plexus, the Na^+K^+ATPase is limited to the apical membrane domain by association with the ankyrin/spectrin network [Gundersen et al., 1991; Alper et al., 1994; Huotari et al., 1995]. The voltage-gated Na^+ channel in brain is concentrated at the axon hillock, nodes of Ranvier and presynaptic terminals in association with the ankyrin/spectrin skeleton [Angelides et al., 1988; Srinivasan et al., 1988]. Other ion and amino acid transporters that have been found complexed to the spectrin skeleton include the H^+K^+ATPase in gastric parietal cells [Smith et al., 1993] and the hepatic system A amino acid transporter [Handlogten et al., 1996].

Light microscopic localization of spectrin in osteoclasts in situ revealed staining throughout the cell with greater intensity on the marrow-facing cell surface compared to the bone-facing surface (ruffled border). The marrow-facing plasma membrane of osteoclasts is proposed to possess both apical and basolateral domains [Salo et al., 1996, 1997; Nesbitt and Horton, 1997], whereas the ruffled border more closely resembles a lysosomal or endosomal membrane, as it secretes lysosomal hydrolases and acid [Blair et al., 1989]. The appearance of spectrin at both the marrow- and bone-facing

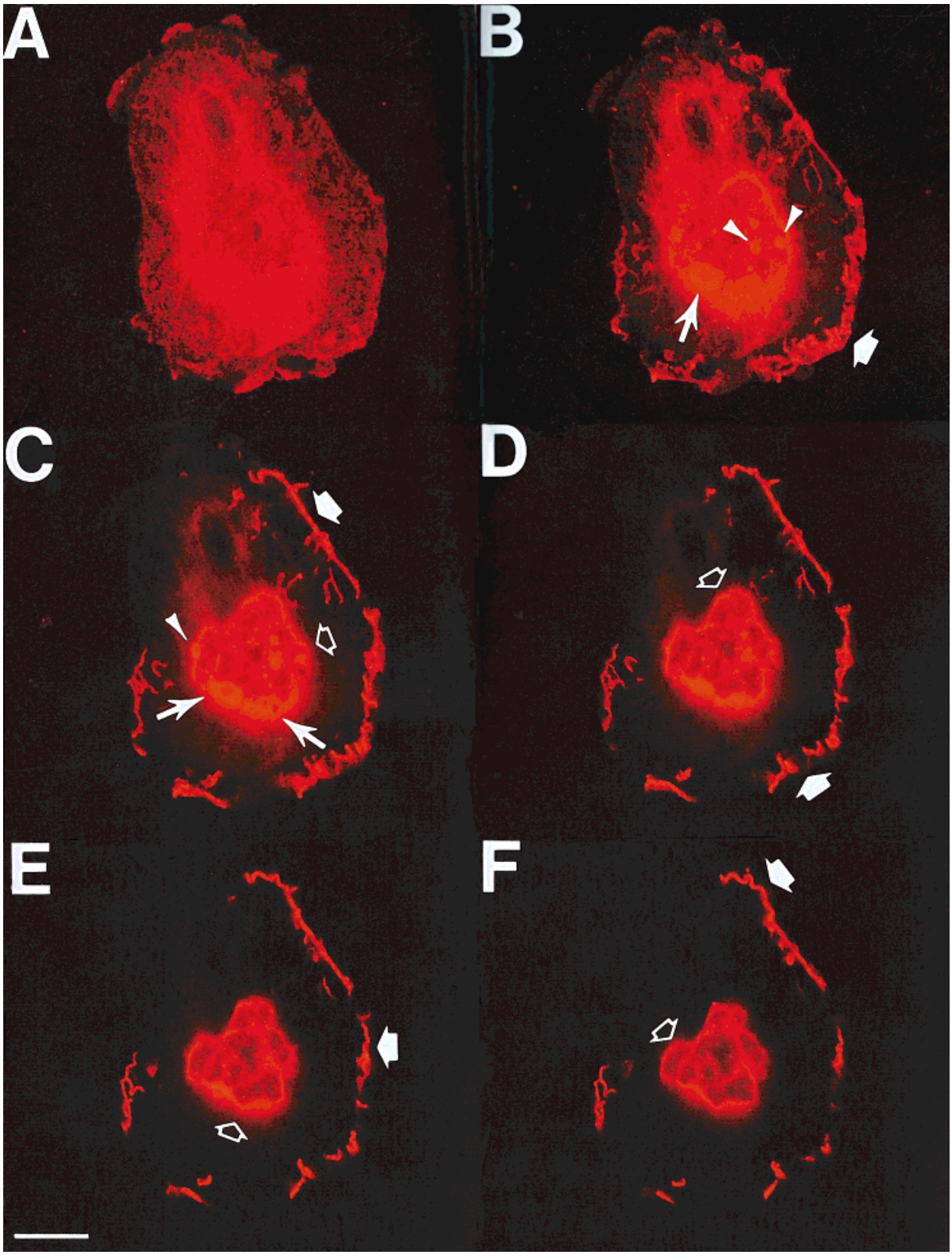

Fig. 3. Confocal laser scanning micrographs of immunofluorescent staining of spectrin in an isolated osteoclast plated onto a glass coverslip. Panel **A** shows the area of the cell in closest apposition to the substrate. Each subsequent panel is progressively further away from the substrate. Nuclear staining is evident (**B,C,** arrows) as is staining of other cytoplasmic inclusions (B,C, small arrowheads). Spectrin encircles the cell center (**C-F,** open arrowheads) and is found in a patchy distribution at the plasma membrane with more intense staining found in regions of membrane ruffling (B-F, thick arrowheads). Scale bar = 20 μm.

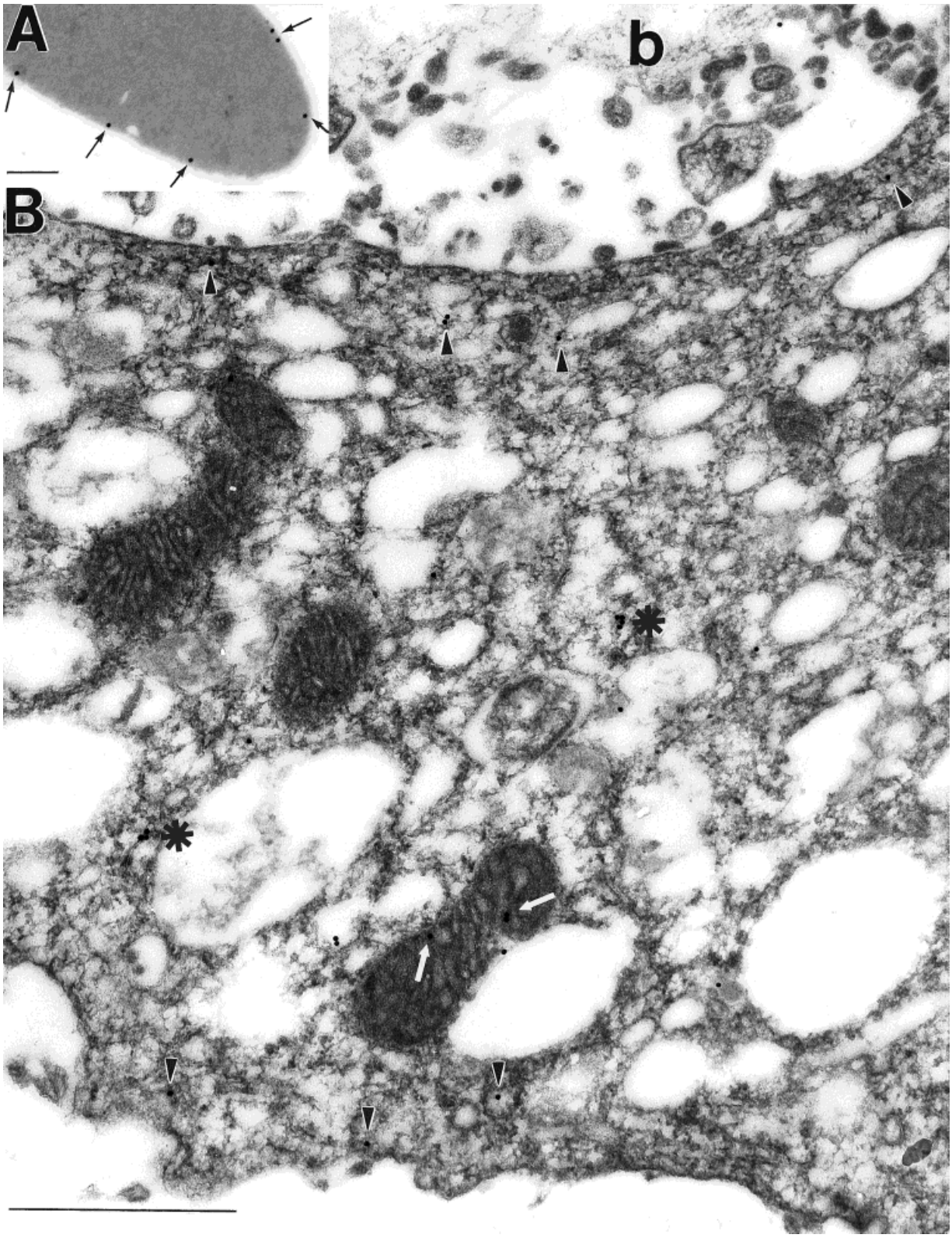

Fig. 4. Ultrastructural localization of spectrin in osteoclasts in situ. Chick embryo tibias were fixed, embedded in LR White, stained with polyclonal antispectrin, and visualized with gold conjugated to goat antirabbit IgG. A portion of a red blood cell showing typical submembranous spectrin staining is shown (**A**, small arrows). Spectrin in osteoclasts was found throughout the cytoplasm and underlying the plasma membrane at both the bone-facing and marrow-facing surfaces (**B**, arrowheads). Bone is indicated by b. Mitochondria were also labeled (B, white arrows). Clusters of gold particles were observed in close association with membranes or filaments (B, asterisks). Scale bars = 0.2 μm in A, 1 μm in B.

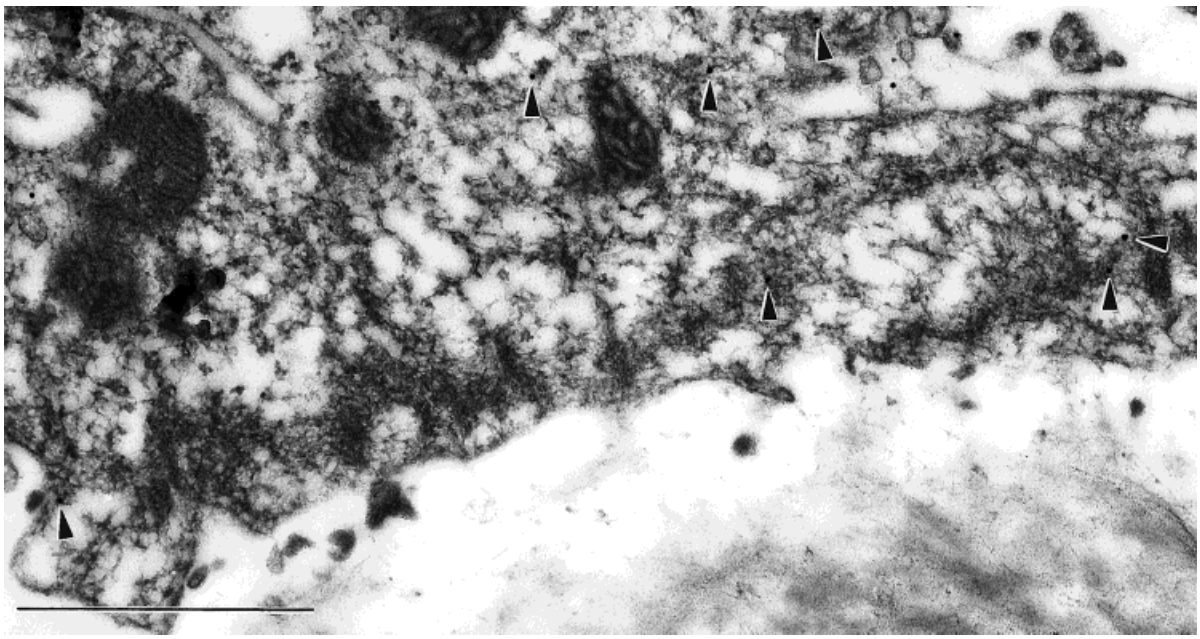

Fig. 5. Ultrastructural localization of spectrin in osteoclast clear zone. Gold particles were found in association with filaments in the actin-rich attachment zone (arrowheads). Scale bar = 1 μm.

surfaces is similar to the pattern observed in hepatic cells in which spectrin has been found at the basolateral and canalicular membrane surfaces [Falchetto et al., 1990; Handlogten et al., 1996].

Confocal imaging of immunofluorescent spectrin staining in isolated osteoclasts plated onto glass coverslips revealed staining of nuclei and cytoplasmic inclusions. α-Spectrin has been localized in rat liver nuclear matrix, nuclear pores, and the nuclear envelope and is suggested to be part of an intranuclear contractile system [Bachs et al., 1990]. Within osteoclasts, electron microscopic immunogold staining indicated the presence of spectrin within the nucleus and associated with the nuclear membrane. The location of nuclear pores could not be resolved with the fixation method employed in this study.

The most intriguing finding from the confocal staining was the apparent encirclement of the organelle-rich cell center by spectrin. A homolog of erythrocyte β-spectrin has been co-localized with markers of the Golgi complex in a variety of cell types [Beck et al., 1994]. Osteoclasts possess numerous Golgi complexes that are arrayed around the multiple nuclei in the cell center. An avenue of further investigation will be to determine whether the observed staining was due to detection of Golgi-associated spectrin.

The fluorescent confocal imaging also revealed irregularly distributed membrane staining that appeared to be at sites of membrane ruffling. Spectrin is present in locomoting fibroblasts, along the leading edge of the leading lamella and in actively protruding sites that are suggested to represent evolving focal adhesions [Merilaien et al., 1993; Sormunen and Lehto, 1995]. Osteoclasts possess podosomes, attachment structures related to focal adhesions, that are less tightly associated with the substratum and are highly dynamic; able to change size, location, and appearance over a time course of several minutes [Kanehisa et al., 1990;Lakkakorpi and Väänänen, 1991]. Osteoclasts are highly motile, dynamic cells that undergo significant shape and adherence changes throughout a cycle involving attachment, resorption, inactivation, and movement to another site of resorption [Fukushima et al., 1991]. They require a mechanically robust membrane skeleton that is flexible enough to accommodate shape changes and motility. In addition, membrane skeleton involvement in signal transduction is postulated to be a component of such a dynamic system. Human carcinoma A-431 cells, when stimulated with epidermal growth factor, respond with rapid phosphorylation of spectrin serine residues and re-distribution of spectrin to sites of membrane ruffling [Bretscher, 1989]. In lymphocytes, spectrin redistributes from a generalized plasma membrane pattern to a cytoplasmic aggregate upon stimulation of the T cell receptor-activated sig-

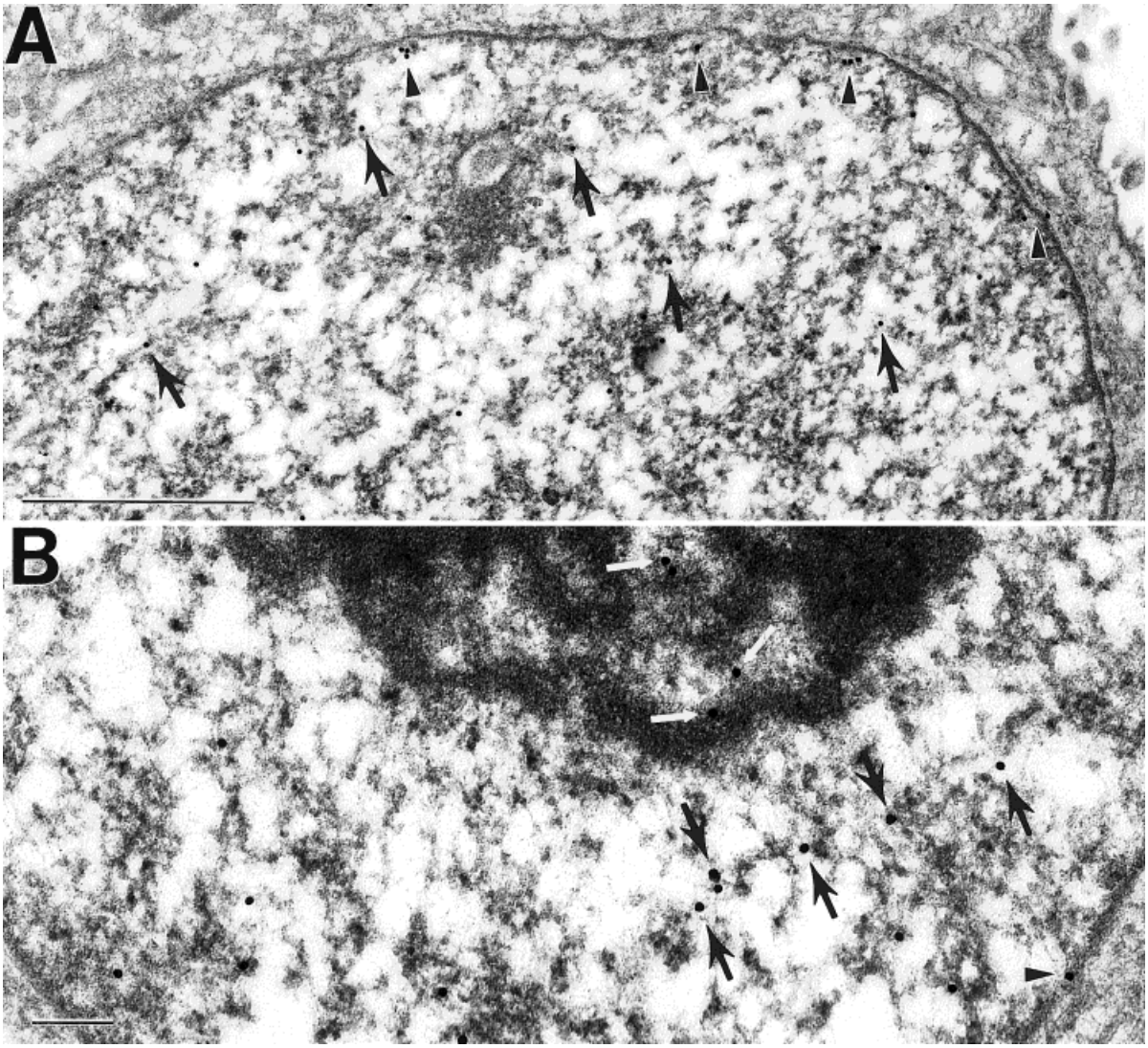

Fig. 6. Ultrastructural localization of spectrin in osteoclast nuclei. Gold particles were found in close association with nuclear membranes (**A,B**, arrowheads) and along filaments within the nucleus (A and B, arrows). Nucleolar staining was also observed (B, white arrows). Scale bars = 1 μm in A, 0.2 μm in B.

nal transduction pathway leading to protein kinase Cβ activation [Gregorio et al., 1992, 1994]. Gastric parietal cells stimulated to secrete acid, re-distribute both spectrin and the H^+K^+ATPase to the apical domain to form a secretory canaliculus [Mercier et al., 1989].

The results of this study raise a number of interesting questions that will be explored in future studies. For example, the determination of the β-spectrin isoform will reveal information about the actin and ankyrin binding potential of the molecule. Determining the presence of other membrane skeleton components will be needed before a detailed model of the osteoclast membrane skeleton can be constructed. Although we have partially cloned ANK1 from chick osteoclasts [Hunter and Peters, unpublished observations], we do not know its detailed structure and with which integral membrane proteins it associates. Ultrastructural co-localization of membrane skeleton components with various pumps and transporters (e.g., Na^+K^+ATPase, anion exchange protein) will be especially revealing. Investigation of the cell center staining observed with confocal microscopy will involve co-localization of spectrin with Golgi markers and the use of brefeldin A and nocadazole, agents with known Golgi-perturbing effects. In addition, the effects of hormones with known osteoclast effects will be investigated in regard to their effects on distribution and/or membrane skeleton interactions.

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