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Focal adhesion kinase and β1 integrin regulation of Na^+, K^+, $2Cl^-$ cotransporter in osmosensing ion transporting cells of killifish, *Fundulus heteroclitus*

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ABSTRACT

Focal adhesion kinase (FAK), also known as PYK2, is a tyrosine kinase that functions in integrin-mediated signaling in mechanosensitive cells but its role in osmosensing cells is unknown. Antibodies directed against phosphorylated FAK, whose epitopes are conserved among vertebrates, were used to follow phosphorylation patterns in an osmosensing ion secreting epithelium, the killifish (*Fundulus heteroclitus*) opercular membrane. At the electron microscopic level, a unique combination of integrin β1, the phosphorylated form of FAK at tyrosine 407 (pY407) and Na^+, K^+, $2Cl^-$ cotransporter (NKCC1) were all colocalized only on the basolateral membrane in chloride cells. The three proteins were also coimmunoprecipitated with each other in isotonic conditions, suggesting an osmosensing complex involving the three proteins. Only FAK pY407 was sensitive to hypotonic shock and became dephosphorylated with hypotonic shock, while FAK pY576 in the apical membrane and pY861 in cell–cell adhesions were insensitive to hypotonicity. NKCC1 contributes to NaCl secretion in seawater and previous reports showed that hypotonic shock (−60 mOsm/kg) rapidly inhibits Cl^- secretion. These results indicate that chloride cells respond to hypotonic shock using integrin β1 as an osmosensor that is connected to dephosphorylation of FAK pY407 which leads to NKCC1 deactivation in the basolateral membrane and the inhibition of NaCl secretion by these epithelial cells.

1. Introduction

Focal adhesion kinase (FAK), also known as PYK2, is a ubiquitous non-receptor tyrosine kinase with several tyrosine phosphorylation sites located in the focal adhesion (Parsons, 2003). FAK is autophosphorylated at position 397 and self-activated to perform a number of different functions. Mammalian FAK also has phosphorylation sites at Y407, Y576, Y577, Y861 and Y925. Phosphorylated Y397, Y576/577, Y861 were discovered in zebrafish (Henry et al., 2001; Crawford et al., 2003) and Y407, Y576 and Y861 in killifish (Marshall et al., 2005). FAK is well known to be essential in the motility of epithelial cells and is associated with the initiation of invasive malignancies (Ilic et al., 1995; Owens et al., 1995; Cary et al., 1998; Parsons, 2003). Hypertonic cell shrinkage has been found to stimulate FAK in a variety of mammalian cell types (Hoffmann and Pederson, 2006). FAK is also involved in integrin signaling of mechanosensitive cells, such as vascular epithelium. In these systems, integrins are the mechanosensor actively forced to change its conformation by forces transduced by the extracellular matrix. Integrin then activates cSrc as serine threonine kinase which activates FAK. Although FAK in zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*) has high identity compared with human (~70% in amino acid sequence), very little is known of FAK operation outside of mammals, thus the present study examines FAK function in teleost fish, in the regulation of the secretory form of Na^+, K^+, $2Cl^-$ cotransporter, NKCC1. FAK is present in teleosts and has been studied in terms of a role in zebrafish embryonic development (Crawford et al., 2003) but involvement of FAK in ion transport regulation is unknown for any vertebrate.

The common killifish or mummichog (*Fundulus heteroclitus*) is a hardy euryhaline and euryoxic teleost fish that lives in estuaries of Eastern North America. Part of the euryhaline adaptiveness is associated with the response of NaCl secreting epithelial cells of the gills to slight changes in blood osmolality (Zadunaisky et al., 1995; Marshall et al., 2000). As little as 10 mOsm decrease in blood osmolality produces immediate inhibition of NaCl secretion by chloride cells. A 60 mOsm decrease will produce steady state inhibition of NaCl secretion by 80% or more and the effect cannot be elicited by isotonic decreases in NaCl, thus demonstrating that the effect is purely osmotic (Marshall et al., 2000). By immunocytochemistry, previous reports showed that NKCC1 was localized to the extensive infoldings of the basolateral membrane in chloride cells, along with several other regulatory proteins, including ste20-like stress associated proline rich kinase (SPAK) and oxidative stress response protein 1 (OSR1), and the phosphorylated form of FAK at tyrosine 407 (pY407) (Marshall et al., 2005), thus making FAK a likely candidate kinase to be involved in this osmosensing regulation of epithelical ion transport (see Hoffmann et al., 2007 for a preliminary model including these components).

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The present study investigates the possibility that Y407 is an osmotically sensitive phosphorylation site and broadens the survey of different phosphorylation sites to include Y397, the autophosphorylation site in mammals, and Y576, Y577 and Y861 sites with no established function. In each case the epitope for the well conserved phosphorylation site in the human is confirmed to have consensus with teleost genomes (pufferfish and zebrafish). We also detected integrin β1 (a family of heterodimeric transmembrane receptors) localization and functional relationship with FAK and NKCC1. In this way, the colocalization of integrin β1, FAK pY407 and NKCC1 at the light and electron microscope levels supports the idea of a regulatory metabolon involving an osmotic shock sensor, a group of kinases and the target ion transporter, NKCC1. We showed that Y407 dephosphorylates with hypotonic shock and other stimuli known to inhibit NaCl secretion and extend this to include electron microscopy level and coimmunoprecipitation evidence for a novel functional association between integrin β1 and FAK in its regulation of NKCC1. The importance of the result with reference to a model system for studying FAK action is discussed.

2. Materials and methods

2.1. Animals

Adult killifish (*F. heteroclitus* L.) of both sexes were obtained from the Antigonish estuary (Nova Scotia, Canada), transferred to indoor holding facilities and kept in full strength seawater with salinity of 32 g L^{-1} at 17–21 °C and ambient photoperiod under artificial light. Fish were fed marine fish food blend (Nutrafin flakes; R.C. Hagen, Montreal, QC, Canada) twice daily at a rate of 1.0 g 100 g^{-1} body mass day^{-1}, supplemented three times weekly with mealworms (*Tenebrio molitor*). Killifish were netted and killed by decapitation prior to the experiments. The opercular epithelia were dissected and bathed in modified Cortland's saline (composition in mmol L^{-1}: NaCl 160, KCl 2.55, $CaCl_2$ 1.56, $MgSO_4$ 0.93, $NaHCO_3$ 17.85, NaH_2PO_4 2.97 and glucose 5.55, pH 7.8 (353 mOsm kg^{-1}) when equilibrated with a 99% O_2/1% CO_2 gas mixture).

2.2. Antibodies

The primary antibodies used to detect FAK phosphorylated at tyrosine sites were rabbit polyclonal anti-human FAK pY397, pY407, pY576, pY577 and pY861 that are immunopurified against the epitope (Biosource Int., Camarillo, CA, USA). The phosphorylated tyrosine epitope regions of FAK corresponding to these antibodies are known to be highly conserved between human (GenBank accession no. L05186) and pufferfish *T. rubripes* (Blast search on fugu genomics project website, http://www.fugu-sg.org/). We used rabbit polyclonal anti-mouse FAK (Biosource Int.) as a primary antibody to detect unphosphorylated FAK, which we call "general FAK". The antigen was the C-terminal 150 amino acid sequence that is highly (97%) conserved between mammals and pufferfish, and it does not overlap with other phosphorylation site specific antibodies which were used in this study. The primary antibody against NKCC was T4 (Lytle et al., 1992; Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, IA, USA), which has been shown to bind to several isoforms of NKCC across several species (Haas and Forbush, 1998; Wilson et al., 2000) including killifish opercular epithelium (Marshall et al., 2002). The primary antibody used for detection of killifish Cystic Fibrosis Transmembrane conductance Regulator (CFTR) was mouse monoclonal anti-human CFTR (R&D Systems, Minneapolis, MN, USA) with conserved carboxy terminal domain in killifish (Singer et al., 1998), thus the antibody is selective for this protein (Marshall et al., 2002). The primary antibody used to detect integrin β1 was mouse monoclonal 8C8 (Gawantka et al., 1992; Developmental Studies Hybridoma Bank, University of Iowa). The functional features and signaling properties of the integrin β1 subunits are highly conserved in xenopus, zebrafish and human; additionally, β1-1 and β1-2 are both expressed in zebrafish gill tissue (Mould et al., 2006). Anti-Na^+, K^+-ATPase antibody used for immunoprecipitation negative control was α5 from Developmental Studies Hybridoma Bank, University of Iowa. The secondary antibodies used for immunofluorescence microscopy were either goat anti-mouse IgG or goat anti-rabbit IgG polyclonal conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR., USA). Secondary antibodies used for immunogold labeling were anti-mouse IgG conjugated with 15 nm gold and anti-rabbit IgG conjugated with 10 nm gold (EM Science, PA, USA).

2.3. Immunofluorescence microscopy

The opercular epithelia were dissected without the dermal chromatophore layer and pinned to modeler's wax. Control membranes that received isotonic treatment were incubated in Cortland's saline before being fixed. This was done in parallel with the test membranes that received hypotonic shock treatment for 1, 15 and 60 min. Test membranes that received hypotonic shock treatment were flushed with a diluted 80% Cortland's saline (288 mOsm kg^{-1}) and continuously aerated with a 99% O_2/1% CO_2 gas mixture to maintain pH balance of the solutions. For genistein pretreatment, paired membranes were incubated in aerated Cortland's saline with 0.14% v/v dimethylsulfoxide (DMSO) vehicle or 100 μmol L^{-1} genistein in an equivalent volume of DMSO. For clonidine pretreatment, membranes were incubated in aerated Cortland's saline with 10 μmol L^{-1} clonidine. Preparations were rinsed three times in rinsing buffer comprising 0.1% bovine serum albumin (BSA) in 0.05% Tween 20 in phosphate-buffered saline (TPBS) (composition in mmol L^{-1}: NaCl 137, KCl 2.7, Na_2HPO_4 4.3, and KH_2PO_4 1.4 at pH 7.4). The membranes were fixed for 2 h at −20 °C in 80% methanol/20% DMSO, then rinsed and immersed in a blocking solution with 5% normal goat serum (NGS), 0.1% BSA, 0.2% NaN_3 in TPBS, pH 7.4 for 30 min at room temperature in the dark. The membranes were then incubated in the appropriate primary antibody (anti-FAK pY397, pY407, pY576, pY577, pY861 and general FAK (10 μg/mL in blocking solution), T4 (8 μg/mL), anti-CFTR (10 μg/mL), and 8C8 (10 μg/mL) at 4 °C overnight. Control and test membranes were then rinsed three times and exposed to the secondary antibody (8 μg/mL in 0.5% BSA in PBS), singly and in combination for 4 h at room temperature. After three final rinses the membranes were mounted in mounting medium (Geltol; Immunon Thermo Shandon, Pittsburgh, PA, USA). Slides were viewed in single blind fashion and images were collected with a laser scanning confocal microscope (Olympus, Markham, ON, Canada; model FV300). In each opercular membrane, randomly selected Z-stack series were collected using a 40× water objective (N.A. 1.15W), zoom of 3.0 and with optical sections of 1.0 ± 0.05 μm. An average of 35 sections were collected for each image.

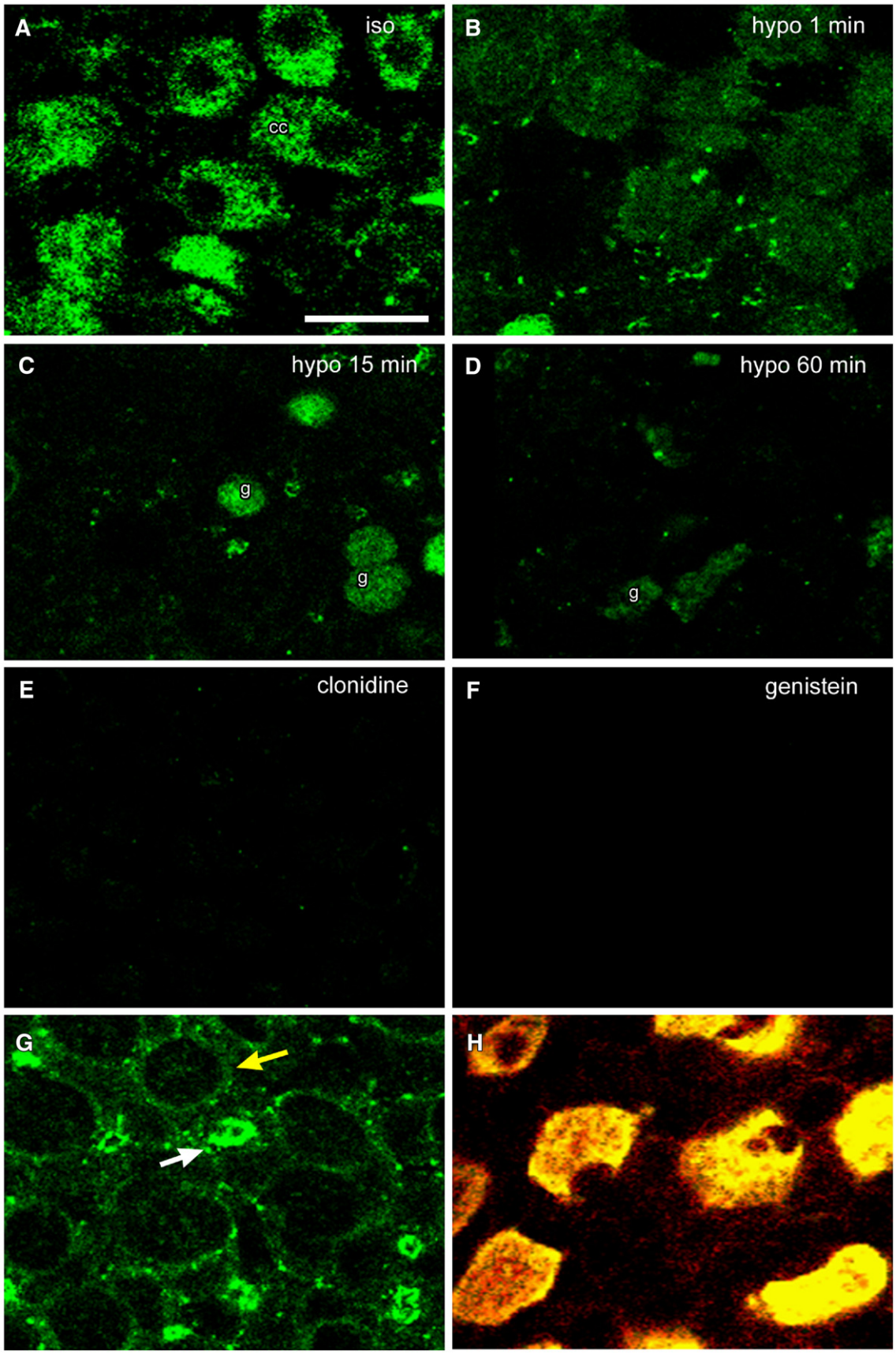
2.4. Oregon Green phalloidin staining

The dissected and pinned opercular membranes, as mentioned above, were fixed in 5% paraformaldehyde (PFA)–0.2% glutaraldehyde (GA) in Cortland's saline for 15 min. Tissues were permeabilized with 0.03% of Triton X-100 for 10 min. Oregon Green phalloidin (Molecular Probes) was added to 132 nmol L^{-1} (final concentration in Cortland's saline) for 90–150 min. Tissues were rinsed three times with fresh Cortland's saline and mounted in Geltol for observation by confocal microscopy (Olympus FV300).

2.5. Immunogold TEM

Opercular membranes from seawater killifish ($N=3$) were fixed in 0.2% GA–2% PFA in 0.1 mol L^{-1} phosphate buffer (PB, pH 7.4) for 3 h.

After dehydration in ethanol, the membrane tissues were embedded in LR white resin (London Resin Company Ltd, UK) and ultrathin sections (80 nm) were cut with a diamond knife. The ultrathin sections on nickel grid were incubated sequentially with 1) blocking solution (2% NGS, 0.1% BSA, 0.05% Triton X, 0.01% NaN_3 in PBS) for 10 min, 2) primary antibody (T4, 6 μg mL^{-1}; anti-FAK pY407, 20 μg



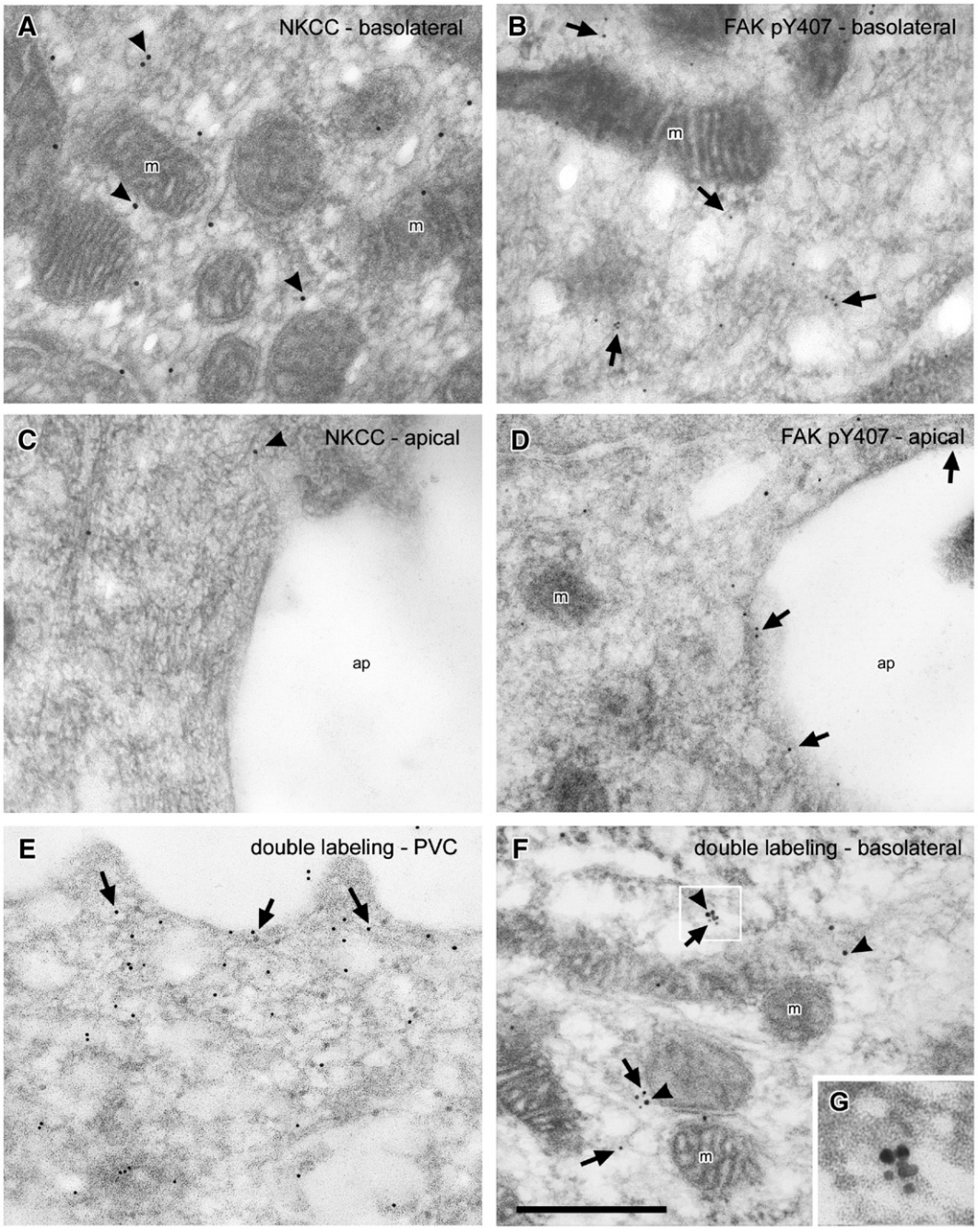
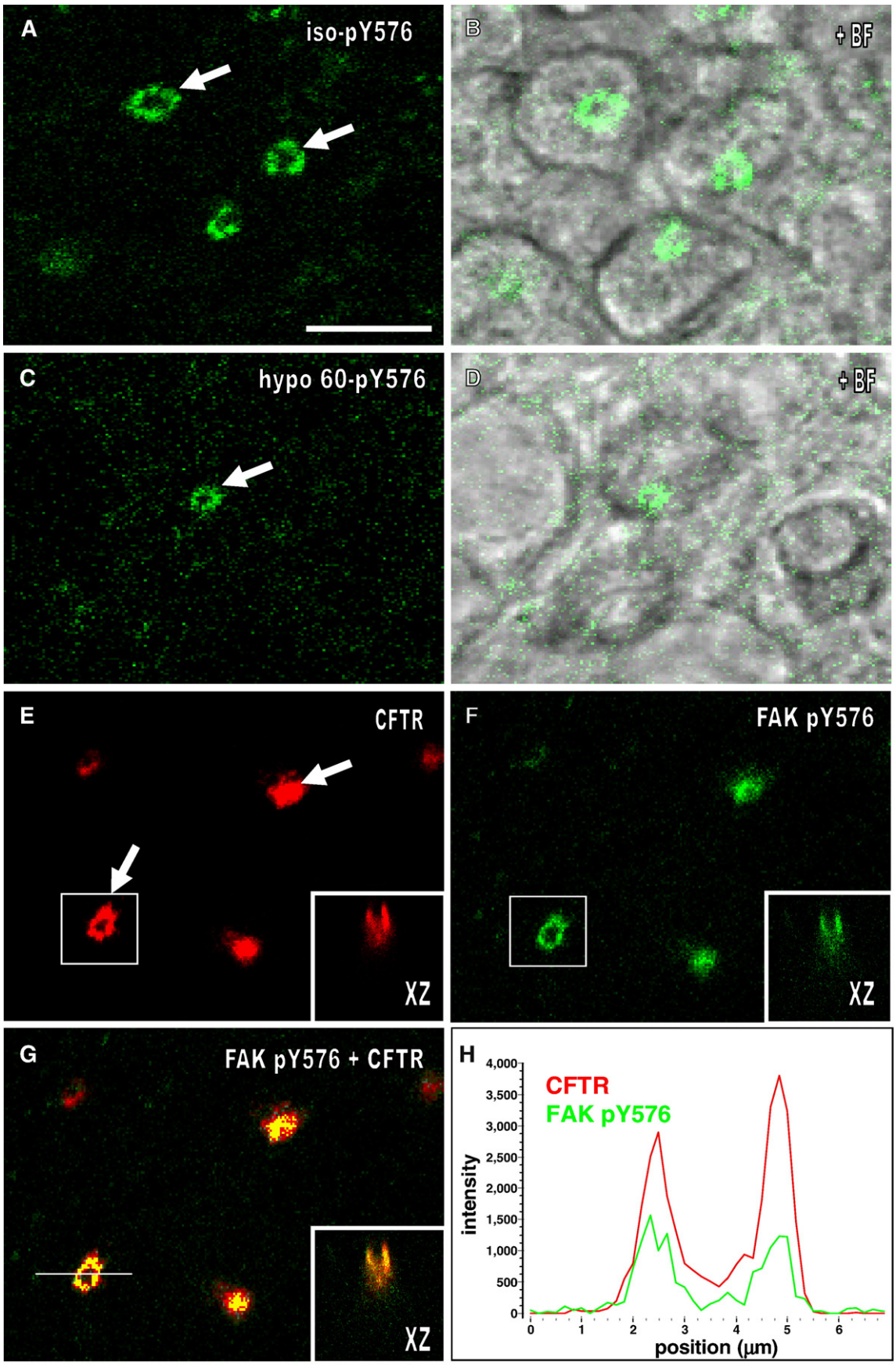


Fig. 2. Transmission electron micrographs of immunogold labeling using anti-NKCC (primary: T4 mouse anti-human NKCC; secondary: goat anti-mouse IgG-15 nm gold) and FAK pY407 (primary: rabbit anti-h FAK pY407; secondary: goat anti-rabbit-10 nm gold). NKCC (A, arrowheads) and FAK pY407 (B, arrows) were detected in the tubular system continuous with the basolateral membrane of chloride cells of seawater killifish opercular membrane. FAK pY407 was also distributed in apical area of the chloride cells (D, arrows), although NKCC was hardly detected (C, arrowhead). In pavement cells of opercular membrane (E), double labeling using both anti-NKCC and FAK pY407 showed that only the FAK pY407 was present. Double labeling in cytoplasmic area of chloride cells shows close association of NKCC (arrowheads) and FAK pY407 (arrows) in the basolateral membrane of the cell (F). (G) Magnified view of the double labeling of NKCC and FAK pY407 in the box of plate F. m, mitochondria; ap, apical crypt. Scale bar, 0.5 μm.

mL^{-1}; or a mixture of both diluted in PBS at 4 °C overnight, 3) immunogold secondary antibodies diluted at 1:50 in PBS for 2 h at room temperature and 4) 5 rinses in PBS for 5 min each. The immunolabeled sections were then washed with filtered water, stained with uranyl acetate for 3 min and lead citrate for 2 min, and viewed using a transmission electron microscope (TEM, Philips EM 410, Netherlands). From each of three separate animal experiments, gold particle density was estimated from three images.

Fig. 1. Immunocytochemistry for FAK phosphorylated at tyrosine 407 (FAK pY407) (primary: rabbit anti-phosphorylated human FAK pY407; secondary: goat anti-rabbit IgG Alexa Fluor 488) and for NKCC (primary: T4 mouse anti-human NKCC; secondary: goat anti-mouse IgG Alexa Fluor 594) of killifish opercular epithelia. (A) FAK pY407 immunofluorescence was present in chloride cells (green) under isotonic conditions ($N=12$). (B) FAK pY407 immunofluorescence was weaker following 1 min hypotonic shock pretreatment ($N=3$) and practically absent following 15 min (C) ($N=6$) and 60 min (D) ($N=6$) hypotonic shock. (E) Clonidine pretreatment (10 μmol L^{-1}) for 15 min ($N=3$) and (F) Genistein pretreatment (100 μmol L^{-1}) for 60 min ($N=3$) eliminated FAK pY407 immunofluorescence. (G) FAK pY407 and NKCC immunofluorescence showed only FAK pY407 immunofluorescence (green) in the pavement cells (yellow arrow) and in the apical crypts (white arrow) of the chloride cells. NKCC immunofluorescence (red) was absent at this level ($N=7$). (H) Same field as G but 10 μm below. At this plane, there was a high degree of colocalization of FAK pY407 and NKCC (yellow) ($N=7$). cc, chloride cell; g, granular cell. Scale bar, 10 μm.



2.6. Protein expression analysis

Dissected opercular membranes from 13 adult killifish were pooled and treated in ~10 mL of either Cortland's saline (isotonic), 80% Cortland's saline (hypotonic), 100 μmol L^{-1} genistein, or 10 μmol L^{-1} clonidine in Cortland's saline, aerated with a 99% O_2/1% CO_2 gas mixture, for 15 or 60 min. The treated membranes were homogenized in 4 mL of ice cold SEI buffer (300 mmol L^{-1} sucrose, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} imidazole) using a Polytron homogenizer. Pellets obtained from centrifuge at 2000 ×g for 5 min at 4 °C were resuspended and rehomogenized in 1 mL of 2.4 mmol L^{-1} deoxycholic acid (DOC) in SEI buffer. The samples were centrifuged at 2000 ×g for 5 min at 4 °C and the supernatant (crude protein) was collected and the protein concentration of each sample was determined using the Bradford method (Bradford, 1976). The above experiment was repeated three times (N=3). 28 μg of extracted crude proteins extracted were denatured in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) supplemented with β-mercaptoethanol to 0.05 mol L^{-1} final concentration.

2.7. Coimmunoprecipitated protein preparation

Coimmunoprecipitations were performed by incubating 250 μg of crude protein sample extracted as mentioned above. The crude protein in DOC–SEI buffer was incubated with 5 μL of each primary antibody, under gentle rotation, overnight at 4 °C, followed by the addition of 50% slurry of 20 μL of protein A-Sepharose (4B Fast Flow, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4 °C. After 5 washes in SEI buffer, the immunoprecipitate-Sepharose bead complexes were resuspended in warm NuPAGE LDS sample buffer (Invitrogen) with 0.05 mol L^{-1} of β-mercaptoethanol and denatured at 65 °C for 15 min. The immunoprecipitates were recovered after centrifugation at 5000 ×g for 3 min at 4 °C. To confirm the specificity of immunoprecipitation, we performed the following negative controls. 1) without any protein sample (only DOC–SEI buffer); 2) with no primary antibodies for precipitation; 3) immunoprecipitation performed using Na^+, K^+-ATPase antibody to make sure the SEI extraction process successfully separated basolateral membrane associated proteins.

2.8. SDS-PAGE and Western blotting

Both of the crude proteins and coimmunoprecipitated proteins in NuPAGE LDS sample buffer were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Precast Ready Gels 10% Tris–HCl, Bio-Rad Laboratories, Mississauga, ON, USA) and electro-transferred to Immobilon-P membrane (Millipore, Bedford, MA, Canada). To determine protein band sizes, Kaleidoscope standards (Bio-Rad Laboratories) were loaded in the same gel. After transfer, the blots were incubated in blocking buffer (Tris-buffered saline plus 0.05% Tween 20 (TTBS; composition in mmol L^{-1}: Tris–HCl 20, NaCl 500, KCl 5, pH 7.5), supplemented with 3% BSA for 4 h at room temperature. All primary antibodies were diluted 1:1000 and applied overnight at 4 °C. The membranes were then washed three times in TTBS for 5 min each. The anti-rabbit, alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) was diluted 1:500 in blocking buffer and applied for 2 h at room temperature. The membranes were then washed and visualized using 5-bromo, 4-chloro, 3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) membrane phosphate substrate (Sigma-Aldrich). Quantification of immunoreactive bands was done using an HP PSC 2400 scanner (Hewlett Packard, Oceanside, CA, USA) and the UN-SCAN-IT gel version 5.1 software for Windows (Silk Scientific Inc., Orem, UT, USA). Background of each image was subtracted by selecting a square of background with no specific bands.

2.9. Statistical analyses

Data are expressed as means±1 S. E. M. The minimum level of significance is $P<0.05$ from a two tailed t-test. Statistical analyses for the immunoblots were determined by single classification analysis of variance (ANOVA) with a Bonferroni post test.

3. Results

3.1. FAK pY407 and NKCC

FAK pY407 was present in seawater killifish opercular epithelium. There was positive staining in all chloride cells under isotonic conditions by immunofluorescence microscopy (Fig. 1A). FAK pY407 immunofluorescence decreased considerably following hypotonic shock pretreatments of 1 and 15 min (Fig. 1B, C) and fluorescence was even weaker following 60 min hypotonic shock (Fig. 1D). This result confirms previous findings (Marshall et al., 2005). Clonidine as well as genistein, as previously shown (Marshall et al., 2005), pretreatments also eliminated FAK pY407 immunofluorescence completely (Fig. 1E, F). In seawater chloride cells, NKCC was absent from the apical crypt membrane but was present in the lower level of chloride cells excluding the nucleus (Marshall et al., 2002). Colocalization of FAK pY407 and the NKCC cotransporter revealed solely the presence of FAK pY407 in the pavement cells and in the apical crypts of the chloride cells (Fig. 1G). Although NKCC was absent at the surface level of the chloride cells, it was highly colocalized with FAK pY407 throughout the basal portion of the cells (Fig. 1H).

Immunoelectron microscopy revealed the ultrastructural localization of proteins; NKCC and FAK pY407 were codistributed throughout the extensive tubular system continuous with the basolateral membrane of chloride cells in seawater killifish opercular membrane (Fig. 2A, B). Although the FAK pY407 immunoreactivity was also detected in apical area of the chloride cells (Fig. 2D), there was no apically located NKCC in the chloride cells (Fig. 2C). In pavement cells, we detected the presence of FAK pY407 but not NKCC in double-labeled sections (Fig. 2E). Double labeling using both anti-FAK pY407 and NKCC revealed that these two proteins are associated on the basolateral membrane of seawater killifish chloride cells (Fig. 2F, G). Gold clusters indicating colocalization of FAK 407 and NKCC appeared in sections from all three animals we examined and the frequency was 6 clusters in 9 images. For FAK 407, 4% to 17% of gold particles appeared in clusters

Fig. 3. Immunocytochemistry for FAK phosphorylated at tyrosine 576 (FAK pY576) (primary: rabbit anti-phosphorylated human FAK pY576; secondary: goat anti-rabbit IgG Alexa Fluor 488) and for CFTR (primary: mouse anti-human CFTR; secondary: goat anti-mouse IgG Alexa Fluor 594) of killifish opercular epithelia. (A) In isotonic treatments, FAK pY576 immunofluorescence was present in apical crypts of chloride cells (white arrows) (N=9). (B) Same frame as for (A) but viewed under brightfield (BF) as well as confocal fluorescence, showing the chloride cells below the apical crypts. (C) FAK pY576 immunofluorescence, following 60 min hypotonic shock pretreatment, was similarly distributed in the apical crypt (white arrow) (N=4). (D) Same frame as C but viewed under brightfield as well. (E) CFTR immunofluorescence was present in apical crypts of chloride cells (white arrows). The insert panel in E shows an XZ view of the CFTR immunofluorescence in the chloride cell apical crypt (white box in E) (N=4). (F) Same frame as E, showing FAK pY576 immunofluorescence in the apical crypts of chloride cells. The insert panel shows an XZ view of FAK pY576 immunofluorescence in the apical crypt (white box in F). (G) There was a high degree of colocalization of FAK pY576 and CFTR in the apical crypts (yellow). The insert panel shows an XZ view of the chloride cell apical crypt (N=4). (H) A line scan of fluorescence intensity (arbitrary units) *versus* distance in μm across the apical crypt of a double labeled chloride cell (indicated by the white line in G) showed good correspondence in the colocalization of FAK pY576 (green line) and CFTR (red line) in the crypt periphery (peaks) and not in the central region. Scale bar, 10 μm.

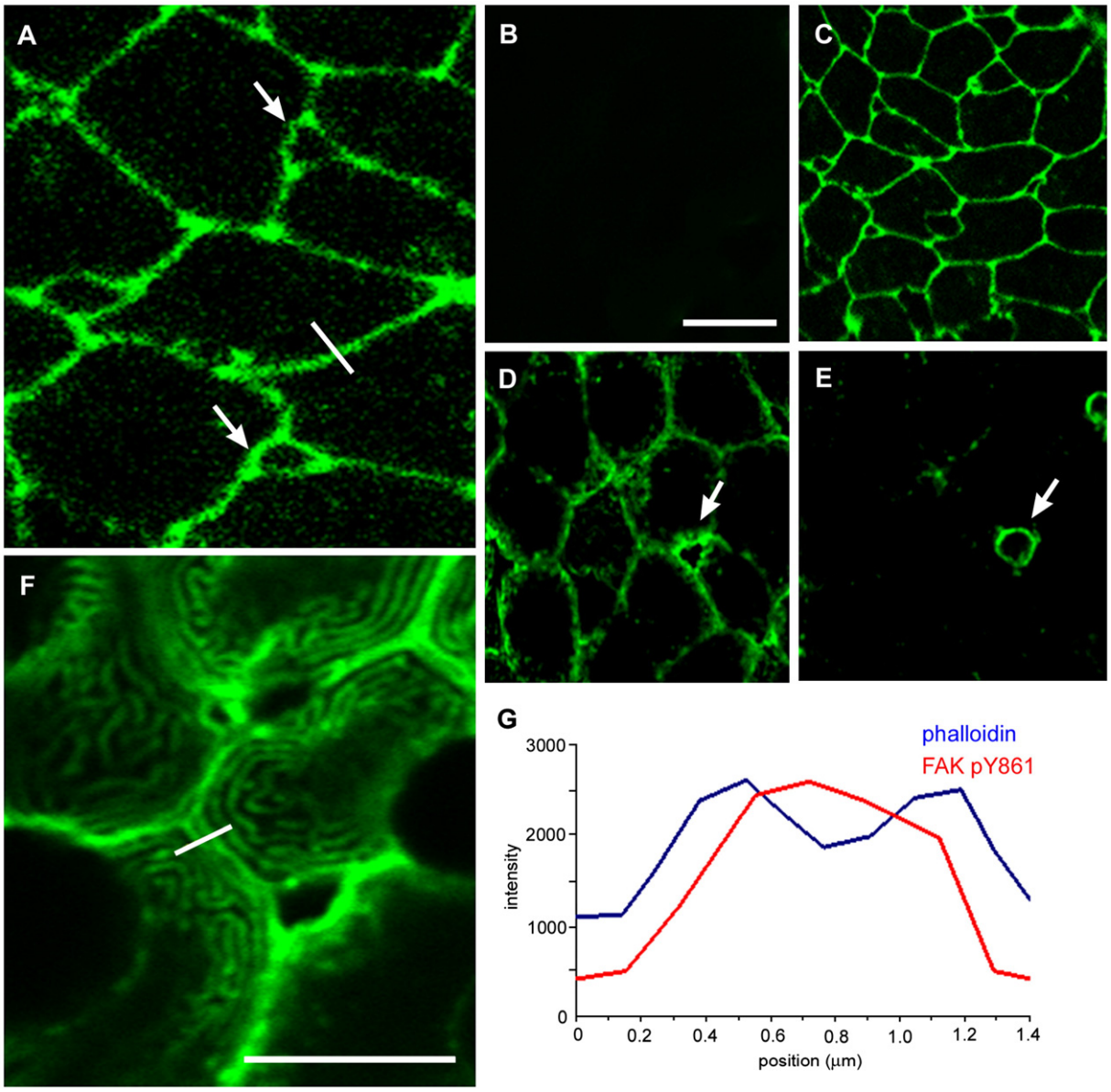


Fig. 4. Immunocytochemistry for FAK phosphorylated at tyrosine 861 (FAK pY861) (primary: rabbit anti-phosphorylated human FAK pY861; secondary: goat anti-rabbit IgG Alexa Fluor 488) and F actin (stained with Oregon Green phalloidin) of killifish opercular epithelia. (A) FAK pY861 immunofluorescence in isotonic condition was present in the pavement cell–cell adhesions and fluorescence appeared as a single line ($N=11$). Angular gaps (white arrows) indicate the presence of an apical crypt below. (B) Same frame as A, 2 μm below. The lack of fluorescence showed an absence of FAK pY861 in the apical crypt of chloride cells. (C) FAK pY861 immunofluorescence, following 60 min hypotonic shock pretreatment, was unchanged ($N=3$). (D) F actin fluorescence in junctions between pavement cells ($N=10$). The angular gap (white arrow) indicates the presence of an apical crypt below. (E) Same frame as D, 2 μm below. Immunofluorescence of apical crypt (white arrow) revealed the presence of a ring of F actin in the apical crypts of chloride cells. (F) Phalloidin fluorescence in pavement cell junctions appeared as two distinct lines of fluorescence ($N=3$). (G) Line scan of the fluorescence from the highlighted areas (white lines) of A and F showed the two peaks of phalloidin fluorescence (blue) and the one peak of FAK pY861 fluorescence (red) in pavement cell junctions. Scale bars, 10 μm, same magnification in B, C, D, E and A, F.

with NKCC and for NKCC, 8% to 12% of gold particles appeared in clusters.

3.2. FAK pY576

FAK pY576 distribution was similar in isotonic and hypotonic treatments. Immunofluorescence was localized in the apical crypt of all CFTR-positive chloride cells in isotonic conditions (Fig. 3A, B) and in hypotonic conditions (Fig. 3C, D). Killifish CFTR distribution in seawater killifish was found exclusively in the apical region of chloride cells (Marshall et al., 2002). At this level, there was a high degree of colocalization between killifish CFTR and FAK pY576 (Fig. 3E–G). The spatial correspondence of the CFTR and FAK pY576 across the apical crypt of doubly labeled cells was very close (Fig. 3H).

3.3. FAK pY861

Immunocytochemistry of FAK pY861 revealed positive staining in intercellular tight junctions between pavement cells. Although there was obvious fluorescence on the surface of the membranes defined by a single fluorescent line and the presence of angular gaps indicating the presence of apical crypts below (Fig. 4A), there was no fluorescence at the apical crypt level (2 μm below the pavement cell surface) (Fig. 4B). Hypotonic shock pretreatment did not affect FAK pY861 distribution (Fig. 4C). Oregon Green phalloidin is a fluorescent dye that binds to filamentous actin (F actin) microfilaments. Opercular epithelium fixed in PFA and stained with Oregon Green phalloidin using methods outlined in Daborn et al. (2001) showed the presence of F actin in junctions between pavement cells (Fig. 4D). Angular gaps

within the pavement cell layer represent points at which pavement cells are connected to chloride cells, rather than to adjacent pavement cells; chloride cell apical crypts, exposed to the external environment through the break in the pavement cell layer, exist immediately below these angular gaps. The gaps themselves were fluorescent in membranes stained to reveal FAK pY861 (Fig. 4A, C) and have also been found to be fluorescent in membranes stained with phalloidin (Fig. 4D). Furthermore, positive staining of the crypts found to be present several μm below these angular gaps, revealed the presence of a ring of F actin surrounding the apical crypts of chloride cells (Fig. 4E). Although both FAK pY861 and phalloidin fluorescence were found in the pavement cell junctions, the former was distributed in a single line (Fig. 4A), while the latter was distributed in two distinct lines (Fig. 4F). Further analysis of fluorescence revealed that the single peak of FAK pY861 fell within the two peaks of phalloidin fluorescence (Fig. 4G). We infer that FAK pY861 is very close to or in the junctional complex while the actin is in the cytosol subjacent to the junction.

3.4. FAK pY397 and pY577

Immunocytochemistry was also done on opercular epithelia to determine FAK pY397 ($N=18$) and FAK pY577 ($N=6$) distribution. In both cases, immunofluorescence was not detectable in pavement and chloride cells of opercular epithelia in control isotonic and isotonic and test hypotonic shock pretreatments (images not shown).

3.5. General FAK

General FAK (polyclonal antibody directed against C-terminal 150 amino acid sequence) was present in seawater killifish opercular epithelium. Immunofluorescence was present in all locations where phosphorylated forms of FAK (pY407, pY576 and pY861) distribute, cytoplasmic area of chloride cells (Fig. 5A, B), pavement cells and intercellular junctions between pavement cells under isotonic conditions. General FAK fluorescence was unchanged following 60 min hypotonic pretreatments ($N=6$) (not shown).

3.6. Integrin β1 distribution

Integrin β1 immunocytochemistry showed the same distribution as did FAK pY407. Both were present at the pavement cell level (Fig. 2E, 6E) and throughout the basal portion of chloride cells in seawater killifish opercular membrane (Fig. 6A, B) with a high degree of colocalization (Fig. 6C). In addition, immunogold labeling at transmission electron microscopic level showed that the basolaterally located integrin β1 in the chloride cells by fluorescent microscopy distributed in the basolateral tubular system of the chloride cell (Fig. 6D) in which FAK pY407 and NKCC1 were localized (Fig. 2A, B). Integrin β1 was not detected in the tight junctions between pavement cells or in the apical membrane of chloride cells (Fig. 6F).

3.7. Protein expression

FAK pY407 activation in pretreated opercular epithelia was measured by Western blot analyses. The antibody specific to FAK pY407 detected a single band at ~135 kDa (Fig. 7A, iso). Immunoreactivity was the strongest in the isotonic treated membranes. Lighter bands were visible for all other treatments (Fig. 7A). Hypotonic shock is known to inhibit chloride secretion in seawater opercular epithelia (Marshall et al., 2000). In this case, FAK pY407 expression was significantly lower than the control group following 15 and 60 min hypotonic shock pretreatment (Fig. 7A, hypo 15 min and hypo 60 min). The α_2-adrenergic agonist clonidine (Fig. 7A, clon 60 min) and the protein tyrosine kinase inhibitor genistein (Fig. 7A, gen 60 min) also elicited similar results. However, significant decrease of FAK pY407 phosphorylation was detected only in genistein treated membranes (Fig. 7B). Although the decrease was not significant (Fig. 7B), the band detected following pretreatment with the Ca^{2+} ionophore ionomycin appeared weaker (Fig. 7A, iono 60 min) compared to isotonic group (iso).

The antibodies specific to FAK pY576 and FAK pY861 detected a visible band at ~135 kDa in both control and hypotonic shock pretreated membranes (Fig. 8A). This was consistent with the immunohistochemistry results mentioned above. Although immunofluorescence was not detected by immunocytochemistry for FAK pY397 and FAK pY577 control and pretreated membranes, very faint bands were present in the Western blots (Fig. 8A). However, Y407 was the only phosphorylation site significantly dephosphorylated after the hypotonic pretreatment (Fig. 7A, iso, hypo 15 min and 60 min; Fig. 8B) and there was no significant difference in immunoreactivity between the control and pretreated membranes for the other phosphorylated FAK proteins (Fig. 8B).

3.8. Coimmunoprecipitation of FAK pY407, NKCC and integrin β1

To examine the relationship between FAK pY407, NKCC and integrin β1, we isolated the proteins from seawater (isotonic) and hypotonic shocked opercular membrane of killifish. We revealed that

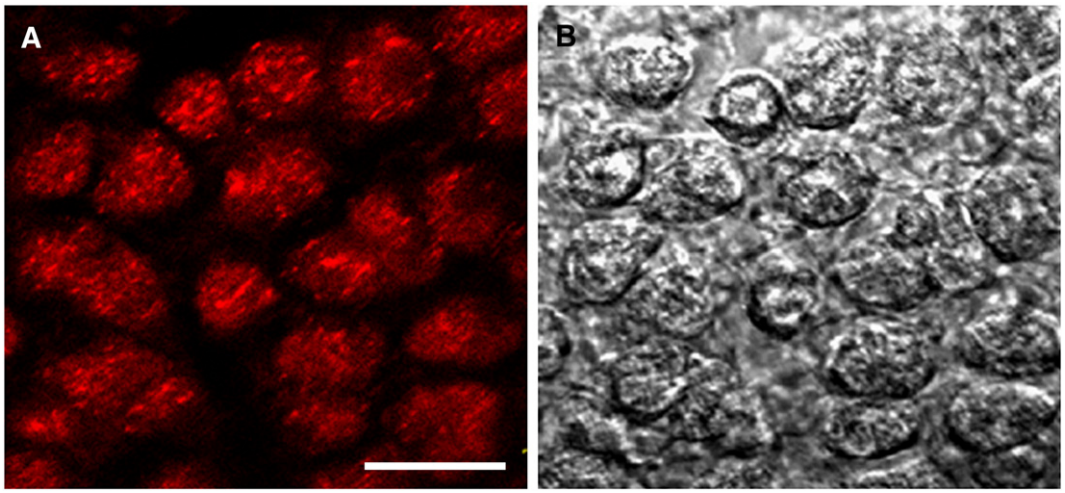


Fig. 5. Immunocytochemistry for general FAK in seawater killifish opercular epithelium (primary: rabbit polyclonal general FAK; secondary: goat anti-rabbit IgG Alexa Fluor 488) ($N=5$). (A) General FAK immunofluorescence was present in chloride cells (red). (B) Same frame as A but viewed under brightfield. General FAK was also present in apical membrane and in intercellular tight junctions between pavement cells (not shown). Scale bar, 20 μm.

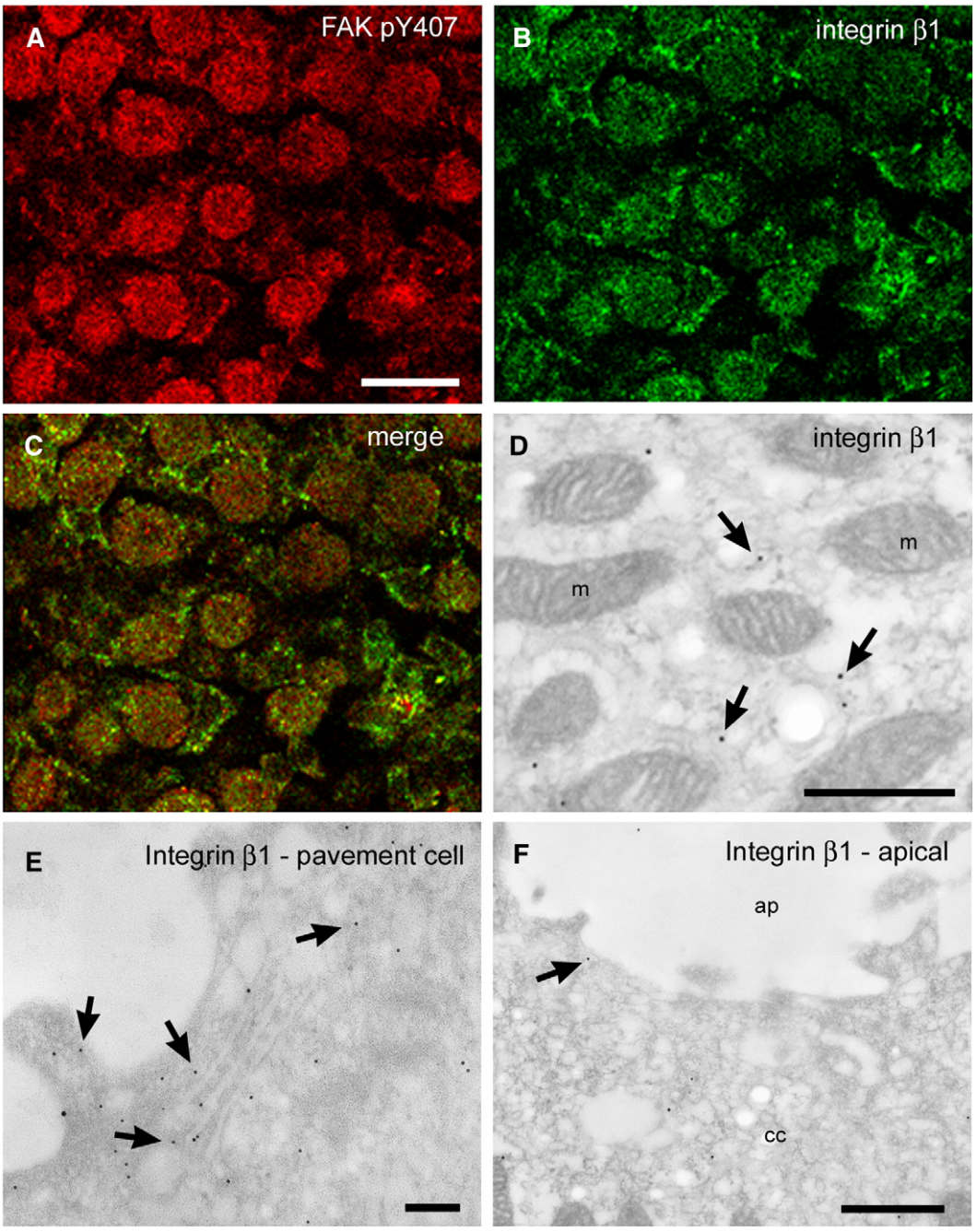


Fig. 6. Immunocytochemistry for FAK pY407 (primary: rabbit anti-phosphorylated human FAK pY407; secondary: goat anti-rabbit IgG Alexa Fluor 488) and integrin β1 (primary: mouse anti-integrin β1; secondary goat anti-mouse IgG Alexa Fluor 594) in seawater killifish opercular epithelium ($N=3$). (A) FAK pY407 immunofluorescence alone (red). (B) Integrin β1 immunofluorescence alone (green). (C) Same field as A and B but with both channels open (red and green). Colocalization of FAK pY407 and integrin β1 was visible in all chloride cells (yellow). (D–F) Immunogold labeled transmission electron micrograph of integrin β1 (primary: 8C8 mouse anti-*Xenopus* integrin β1; secondary: goat anti-mouse IgG 15 nm gold). Immunoreactivity was detected in the basolateral membrane of chloride cells (D) and pavement cells (E) but not in apical area of chloride cell (F) in seawater killifish opercular membrane (arrows). ap, apical crypt; cc, chloride cell; m, mitochondria. Scale bars, 20 μm (A–C); 0.5 μm (D); 1 μm (E, F).

the NKCC coimmunoprecipitated with both FAK pY407 and integrin β1, and FAK pY407 coimmunoprecipitated with integrin β1 in seawater killifish opercular membrane (Fig. 9A, iso samples). Because hypotonic shock dephosphorylates FAK pY407, this treatment should disrupt the coimmunoprecipitation. As expected, with the 60 min hypotonic shock pretreated membrane protein, FAK pY407 and NKCC were not detectable after the immunoprecipitation with anti-FAK pY407 (Fig. 9A, hypo samples). This was consistent with the immunoblotting result (Fig. 7B, hypo 60 min) using crude proteins after hypotonic treatment. When both immunoprecipitation and blotting were done using anti-FAK pY407 as a positive control, one specific band at the size of ~135 kDa was detected (Fig. 9B, lane 1). The specific band pointed by arrow in the lane 5 of Fig. 9B indicates the specific band of NKCC detected following the immunoprecipitation using anti-FAK 407 antibody and immunoblotting using T4 (i.e. coimmunoprecipitation). As negative controls, we carried out the immunoprecipitation 1) without any tissue protein sample and whole process was done by the regular methods (Fig. 9B, lane 2, IP no protein); 2) with no primary antibody for immunoprecipitation step (Fig. 9B, lanes 3 and 6, IP no 1^{o}AB); 3) using anti-Na^{+}, K^{+}-ATPase antibody, the basolateral membrane associated protein in chloride cells (Fig. 9B, lane 4, IP NAK). In these negative control blots, the

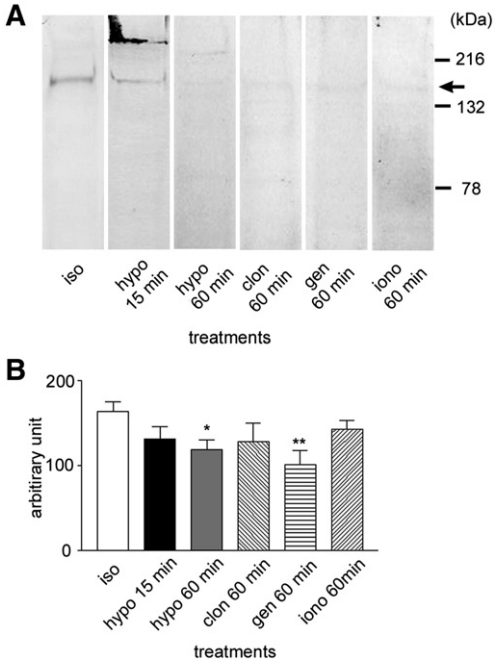


Fig. 7. Immunoblots of FAK pY407 from opercular epithelia pretreated with isotonic solution (iso, $N=6$), hypotonic saline for 15 min and 60 min (hypo, $N=4$), 10 µmol L^{-1} clonidine solution for 60 min (clon, $N=4$), genistein solution 100 µmol L^{-1} for 60 min (gen, $N=4$) and 2 µmol L^{-1} ionomycin solution for 60 min (iono, $N=3$). (A) FAK pY407 was detected at ~135 kDa in isotonic conditions. Weaker bands were visible for all the other treatments. (B) Quantitative Western analysis revealed significantly lower levels of FAK pY407 expression in membranes pretreated 60 min with hypotonic saline ($*P<0.05$) and 60 min with genistein ($**P<0.01$) compared to the expression of the control (iso) membranes.

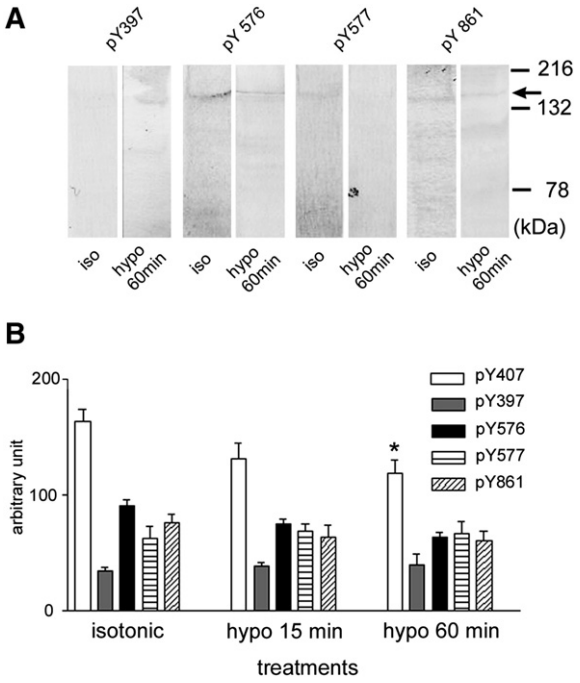


Fig. 8. Immunoblots of FAK pY397, FAK pY576, FAK pY577 and FAK pY861 from opercular epithelia in isotonic solution (iso, $N=3$) and hypotonic saline for 60 min (Hypo, $N=3$). (A) Weak bands were detected at 135 kDa for FAK pY397 and FAK pY577 following isotonic and hypotonic pretreatments. Stronger bands were visible for FAK pY576 and FAK pY861 controls compared to their respective pretreated membranes with hypotonic saline. (B) Quantitative Western analysis of A did not reveal a significant difference in expression levels between FAK pY397, FAK pY576, FAK pY577, FAK pY861 controls (iso) and their respective 15 min or 60 min hypotonic pretreatments. Only the pY407 (e.g. blot in Fig. 7A) showed significant decrease ($*P<0.05$) of phosphorylated protein after 60 min hypotonic shock pretreatment.

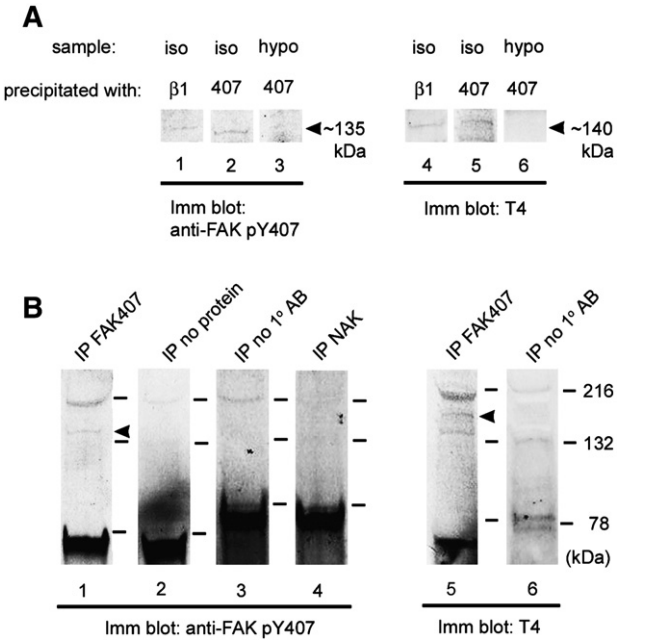


Fig. 9. (A) Coimmunoprecipitation of FAK pY407 and NKCC with integrin β1 and pY407. Immunoblots of FAK pY407 (pY407) and NKCC (T4) immunoprecipitated with integrin β1 (β1) or FAK pY407 (407) showed visible bands at ~135 kDa and ~140 kDa for FAK pY407 and NKCC, respectively under isotonic conditions (iso, $N=3$). There were no detectable bands following 60 min hypotonic shock pretreatment (hypo). (B) Coimmunoprecipitation of NKCC with FAK pY407 (lane 5) shown with controls (lanes 1, 2, 3, 4 and 6) of immunoprecipitation Western blots. Lane 1: immunoprecipitated with anti-FAK pY407 and blotted using anti-FAK pY407 (positive control). Lane 2: immunoprecipitated with anti-FAK pY407 in DOC–SEI buffer with no protein sample and blotted using anti-FAK pY407. Lane 3: immunoprecipitated using no primary antibody and blotted with anti-FAK pY407. Lane 4: immunoprecipitated with anti-Na^+, K^+-ATPase and blotted with anti-FAK pY407. Lane 5 (test): immunoprecipitated with anti-FAK pY407 and blotted using T4. Lane 6: immunoprecipitated using no primary antibody and blotted with T4. Only lanes 1 and 5 show specific bands for FAK pY407 and NKCC which appeared at ~135 and ~140 kDa in lanes 1 and 5, respectively (arrowheads). Molecular size (216, 132 and 78 kDa) of proteins is indicated for each blot using Kaleidoscope standards (Bio-Rad Laboratories).

specific bands which appeared in the first and fifth lanes (Fig. 9B, arrowheads) were not detectable.

4. Discussion

4.1. Hypotonic effect on FAK pY407

The striking dephosphorylation of FAK pY407 in chloride cells with hypotonic shock implicates FAK in the rapid transport responses already discussed for hypotonic shock, even small hypotonic exposures significantly decrease transepithelial NaCl transport (Marshall et al., 2000). This was detected by immunofluorescence and confirmed by Western blotting. As an internal control for the immunocytochemistry, granular leucocytes that are highly motile in the epithelium and are interspersed among the chloride cells (Ostergaard and Lysechko, 2005) were positive for FAK pY407 in control (isotonic) and in hypotonic treatments (Fig. 1A–D). This illustrates the possible distinction between chloride cells that are osmosensitive and the leucocytes that are less sensitive, being able to maintain cellular motility in a broad range of salinities. Presumably in teleost leucocytes, FAK is primarily associated with cell motility rather than osmosensitivity. The fact that none of the other phosphorylation sites responded to hyposmotic stimuli consolidates pY407 as the only identified locus thus far that is responsive to osmotic stimuli. Because cell swelling is an essential response in osmoregulation and in inflammation, it is a core cellular response that has repercussions in osmotic, ionic and antigenic functions.

4.2. FAK pY407 and NKCC

There is an important close association between FAK pY407 as a regulatory kinase and the cotransporter NKCC1 (Fig. 2F). Evidence for this includes the colocalization of FAK pY407 and NKCC1 at the light and electron microscopic levels as well as coimmunoprecipitation of NKCC with FAK in the present study (Fig. 9). The apparently lower signal in immunoelectron microscopy, as compared to immunocytochemistry, is attributed to the strength of TEM fixatives and the thinness of the sections, such that fewer immunoreactive sites are available. NKCC1 is known to be a membrane resident protein (Russell, 2000; Haas and Forbush, 2000), while FAK is cytosolic and can occur in a variety of locations (Tani et al., 1996; Rafiq et al., 2006). In chloride cells the two proteins colocalized to the tubular system that comprises a network of the basolateral membrane. In this same location we previously detected, by immunocytochemistry, SPAK and OSR1 at the light microscopic level (Marshall et al., 2005), and integrin β1, a mechanosensing protein, at the electron microscopic level (Fig. 6D). The former two proteins, SPAK and OSR1, have been shown to coimmunoprecipitate with NKCC1 (Johnston et al., 2000; Piechotta et al., 2003), strongly suggesting that these kinases may be the regulatory proteins that ultimately phosphorylate and activate NKCC1, as well as maintaining NKCC1 activity. Proximal to these must be a phosphatase, as inhibition of NKCC1 involves dephosphorylation of NKCC1. The likely location of FAK in the cascade is upstream of SPAK and OSR1, but distal to the phosphatase, as inhibition of NaCl secretion by hypotonic shock (Fig. 1B–D) or by clonidine (Fig. 1E) both dephosphorylate FAK pY407.

The hypotonic shock effect, logically should parallel integrin signaling in other systems, particularly vascular epithelial cells (Wu, 2005). In mechanosensitive vascular endothelial cells, integrin conformational change is linked to cSrc activation, phosphatase activation and FAK dephosphorylation. We infer these steps in chloride cells and add FAK dephosphorylation at pY407 (specifically), dephosphorylation of SPAK or OSR1 and the ultimate dephosphorylation and deactivation of NKCC1. The rapidity of the response plus the close molecular level association of NKCC1, SPAK, OSR1 and FAK suggests the existence of a metabolon like structure wherein the group of proteins is stably associated and can act as a unit to rapidly regulate NKCC1 transport rate. An interesting implication of this conclusion is that the titer of the regulatory proteins and the cotransporter should be close to stoichiometric equivalence.

NKCC transports Na^+, K^+ and Cl^- into the cytosol down the electrochemical gradient for Na^+, thus driving Cl^- into the cell to levels above its electrochemical equilibrium (Russell, 2000). In this way, the active step in Cl^- secretion is accomplished. The inactivation of NaCl secretion occurs when the chloride cells experience hypotonic shock on the basolateral side (Marshall et al., 2000). The present work links mechanosensing protein integrin through a novel series of regulatory proteins to the cotransporter.

Immunocytochemistry using general FAK antibody was consistent with immunocytochemistry using phosphorylated forms of FAK antibodies (pY407, pY576 and pY861) and showed that the expression of FAK protein is at the same level in isotonic and hyposmotic treated opercular chloride cells, although the FAK was dephosphorylated at Y407 after incubation of membranes in hypotonic saline. This finding suggests that FAK protein is stably located in the basolateral membrane at least after 60 min treatment in hypotonic conditions, and the Y407 site will be dephosphorylated following the hypotonic treatment. Our results suggest a stable association of regulatory proteins with the transporter, not one that is changed after outside stimuli.

4.3. Other phosphorylated forms of FAK

FAK is variously phosphorylated by cSrc, depending on the location of tyrosine phosphorylation (Parsons, 2003). FAK tyrosine phosphorylation was enhanced at least 4 sites (Y397, 407, 861 and 925) in aortas at high pressure and at 1 site (Y861) in pulsatile aortas (Lehoux et al., 2005). Y397, the autophosphorylation site in mammals, is often phosphorylated during activation of FAK (Hirakawa et al., 2004; Parsons, 2003). In this study, however, FAK phosphorylation at Y397 could not be detected in the killifish opercular membrane, and this is possibly due to this form being highly labile. In a previous report by Crawford et al. (2003), FAK pY397 was observed in zebrafish embryos when orthovanadate (10 mM) was used to heighten phosphorylation.

We were also unable to detect immunofluorescence of pY577 in resting or stimulated opercular membranes, but pY576 was strongly positive in chloride cells and only in the apical membrane. This localization and distribution is exactly the same as for the CFTR Cl^- channel (Fig. 3G). This is unique because although general FAK and pY407 are clearly in the basolateral membrane, pY576 is conspicuously absent. We therefore suggest that FAK pY576 could be involved in the regulation of CFTR in the apical membrane of chloride cells; however, this will require independent verification. Currently, antibodies to phosphorylated forms of CFTR are lacking, however. The immunolocalization of a mechanosensor integrin was limited in basolateral membrane of chloride cells where NKCC and FAK pY407 localized (Fig. 6D, F) and the phosphorylation state at Y576 is insensitive to osmotic shock (Fig. 2A, C), suggesting that osmotic regulation of NaCl secretion is limited to the basolateral control of the cotransporter, NKCC1. The osmosensing function in the basolateral side of chloride cells has been observed using electrophysiological analysis by Marshall et al. (2000). We showed that a hypotonic shock applied on the basolateral side of killifish opercular epithelia reduced the I_{sc}. This effect was purely osmotic because the similar reductions in [NaCl], but with added mannitol to maintain osmolality, were without effect (Marshall et al., 2000). At the apical membrane, CFTR is known to be activated instead by cAMP/protein kinase A in teleosts and quickly is deactivated and presumably dephosphorylated and the shrinkage activation of NKCC is independent of this apical Cl^- channel regulation (Hoffmann et al., 2002). In responding to hypotonic shock it is understandable that the main object and pathway is to downregulate the entry of solutes at the basolateral membrane, rather than to limit solute exit at the apical membrane, as the latter response would exacerbate the volume changes, while the former would help ameliorate the shock and tend to restore cell volume. Importantly, integrin is apparently lacking in the apical membrane as well as FAK pY576 in the apical membrane is not dephosphorylated by hypotonic shock. Stimulation of NaCl secretion by cAMP and inhibition by other means has not been attempted thus far, so it is possible that CFTR regulation could involve FAK pY576 and possibly FAK pY407 that is also present.

The surprising distribution of FAK pY861 in cell–cell adhesions near the intercellular junctions between epithelial pavement cells (Fig. 4) implies that phosphorylation at this site is associated with structural integrity of the zona occludentes. Our result confirms findings in zebrafish early embryos, wherein the same primary antibody binds to FAK in cell–cell adhesions subjacent to intercellular tight junctions between the epithelial cells of the chorionic membrane (Crawford et al., 2003). Although tight junctions can respond to changes in transport rates and osmotic stimuli in some epithelia, in the killifish opercular membrane, the phosphorylation at Y861 of FAK is unresponsive to osmotic shock (Fig. 4).

4.4. Osmosensor proteins

In the present study, we found the integrin β1 localization in the basolateral membrane of chloride cells in which NKCC1 and FAK pY407 are localized (Fig. 6). Also the association of these three proteins was detected by coimmunoprecipitation and Western blot analysis (Fig. 9). Integrins are a family of heterodimeric transmembrane receptors consisting of an α and β subunit. There exist 18

different α and 8 different β subunits known in mammals, that yield at least 24 different integrin receptors (Hynes, 2002; Brakebusch and Fässler, 2005). Integrin α5β1 plays a pivotal role in hypotonic stress-induced responses in human umbilical cord vein endothelial cells (Hirakawa et al., 2004, 2006; Flatman, 2002; Hoffmann and Dunham, 1995). When the ligands bind to integrin at the extracellular side, the cytoplasmic integrin tail connects to the actin cytoskeleton and elicits intracellular signaling pathways. Because integrin molecules lack an actin binding domain and any enzymatic activity, all these effects are mediated by integrin associated molecules (Brakebusch and Fässler, 2005). On the other hand, the N-terminal domain of FAK binds to sequences in the cytoplasmic domain of integrin β1 subunit *in vitro*, although a demonstration of a direct interaction between FAK and integrin *in vivo* is still lacking (Parsons, 2003; Krarup et al., 1998; Schaller et al., 1995). FAK coimmunoprecipitates with integrin β1 in human pancreatic cancer cell lines, AsPC-1, BxPC-3 and Capan-2 (Sawai et al., 2005) and our results confirmed this and extended the result to include an epithelial system. Thus in functional terms, involvement of FAK in integrin signaling has been established. Kajimura et al. (1997) demonstrated the necessity of integrin–endothelial cell membrane binding in transducing changes in cell volume and/or shape into changes in permeability, using the integrin–extracellular matrix interaction-blocking protein (RGD peptide). Kahle et al. (2006) also reported that total FAK phosphorylation was reduced (at 150 mm Hg) by treatment with the RGD peptide as well as the Src family kinase inhibitor PP2, again suggesting the involvement of FAK in integrin signaling. These previous studies support our hypothesis that integrin is the osmosensor and FAK protein an intermediary in a pathway regulating NKCC1 ion transport function.

The Ca^{2+} permeable TRP (transient receptor potential) channels are known to be activated upon binding of intracellular and extracellular messengers, chemical compounds, mechanical stimuli and osmotic stress (Nilius and Voets, 2005) and seem to be another candidate for the osmosensor protein. In early indications of osmosensors in epithelial cells, hyposmotic shock to renal epithelial cells apparently directly activated both Ca^{2+} and K^+ channels (Montrose-Rafizadeh and Guggino, 1990, 1991). The TRP superfamily has seven subfamily members and most of them have permeability ratios of P_{Ca}/P_{Na}=0.3–10.0. The TRP super family homologs have been discovered widely from the nematode (*C. elegans*) to human including fish species (O'Niel and Heller, 2005; Nilius and Voets, 2005; Shahsavarani et al., 2006). In vertebrates, one of the TRP channels, TRPV (vanilloid) 4 ion channel, also known as VR-OAC, apparently is an osmo/mechano-sensing channel because the TRPV4 is activated by cell swelling, in a number of cell types, as well as chemical stimulus, thermal stimulus, cellular Ca^{2+} level, etc (Liedtke et al., 2003; Liedtke and Friedman, 2003; Liedtke, 2005; Nilius and Voets, 2005). Liedtke and colleagues reported that *C. elegans* behaviors in response to mechanical, osmotic, or olfactory stimuli require the OSM-9 protein (Colbert et al., 1997), which has structural similarity to vertebrate TRPV1, 2 and 4, using *osm-9* mutant *C. elegans* (Liedtke et al., 2003) and also that *trpv4* null mice possess abnormalities of their osmotic regulation (Liedtke and Friedman, 2003; Mizuno et al., 2003). Interestingly, the Ca^{2+} mediated inhibition may be distal to FAK because FAK pY407 is not strongly dephosphorylated by ionomycin, yet ionomycin strongly inhibits transepithelial NaCl secretion in this system (Marshall et al., 1993). We also saw previously that clonidine (α_2-adrenergic agonist) *via* α_2-adrenergic receptors, acts *via* intracellular Ca^{2+}, mostly derived from intracellular stores and Ca^{2+} ionophore ionomycin mimicked the clonidine action and inhibited the Cl^- transport of the membrane (Marshall et al., 1993). In the present study, pretreatment of membranes in clonidine as well as with genistein (protein tyrosine kinase inhibitor) dephosphorylate FAK at Y407, suggesting that osmotic-mediated inhibitions of NaCl secretion at a point proximal to FAK in the cascade and another pathway regulated by [Ca^{2+}] in the cell, possibly related to TRPV4 channel as an osmosensor.

4.5. Pathway

There must be other enzymes involving in our hypothetical signaling pathway beginning with integrin and regulating NKCC 1 in the chloride cells. Because there is no tyrosine phosphorylation site on NKCC1 and it is reported that NKCC1 would be phosphorylated at threonine 212 and 217 in mammals and both of these tyrosines are conserved in teleosts NKCC1 (tilapia, GenBank accession numbers AY513737 (α) and AY513738 (β)). FAK and NKCC could be associated *via* other proteins in the signaling pathway. Between FAK and the transporter NKCC1, it has been suggested that WNK (with no K (lysine) protein kinase) lies upstream of SPAK and OSR1, which in turn phosphorylate the N-terminal domain of the NKCC1 (Gamba, 2005). Vitari et al. (2005) reported that WNK1 and WNK 4 interact with both SPAK and OSR1 (see above, "FAK pY407 and NKCC1") which are serine/threonine kinase, phosphorylation by WNK1 and WNK4 increased SPAK and OSR1 activity. When SPAK and OSR1 were incubated with active WNK1 in the presence of Mg-ATP, NKCC1 was markedly phosphorylated. They also showed that WNK1 phosphorylated OSR1 at threonine 185, not serine 325, and SPAK at serine 373 and threonine 233. In killifish opercular membrane, protein expression of OSR1 and SPAK were colocalized with NKCC in chloride cells (Marshall et al., 2005). Marshall et al. (2005) also demonstrated that the involvement of serine/threonine protein phosphatase in recovery of ion transport from hypotonic treatment in killifish opercular membrane. SPAK and OSR1 have been coimmunoprecipitated using NKCC antibody indicating their association (Johnston et al., 2000; Piechotta et al., 2003). Piechotta et al. (2002) have demonstrated that SPAK and OSR1 interact with several members of cation-chloride cotransporter superfamily (KCC3b, NKCC1 and NKCC2, but not KCC1 and KCC4) using yeast hybrid assays. Dowd and Forbush (2003) described the regulation of NKCC1 by PASK (SPAK) in transfected HEK cells.

In summary, our results showed that 1) integrin, FAK pY407 and NKCC1 colocalized in basolateral membrane of chloride cells of killifish opercular membrane; 2) The three proteins, integrin, FAK and NKCC1 were coimmunoprecipitated; 3) Y407 is the sole hypotonic shock sensitive phosphorylation site in FAK protein; 4) In the apical membrane, lacking integrin, pY407 is sensitive to hypotonic shock. These results suggest that integrin functions as osmosensor which is sensing the hypotonic shock to the membrane and phosphorylation of FAK at Y407 is involved in the cell signaling pathway regulating NKCC1 ion transporting function in killifish opercular membrane.

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References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brakebusch, C., Fässler, R., 2005. β1 integrin function *in vivo*: adhesion, migration and more. Cancer Metastasis Rev. 24, 403–411.

Cary, A.L., Han, D.C., Polte, T.R., Hanks, S.K., Guan, J.L., 1998. Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. J. Cell. Biol. 140, 211–221.

Colbert, H.A., Smith, T.L., Bargmann, C.I., 1997. OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in *Caenorhabditis elegans*. J. Neurosci. 17, 8259–8269.

Crawford, B.D., Henry, C.A., Clason, T.A., Becker, A.L., Hille, M.B., 2003. Activity and distribution of paxillin, focal adhesion kinase and cadherin indicate cooperative roles during zebrafish morphogenesis. Mol. Biol. Cell. 14, 3065–3081.

Daborn, K., Cozzi, R.R.F., Marshall, W.S., 2001. Dynamics of pavement cell–chloride cell interactions during abrupt salinity change in *Fundulus heteroclitus*. J. Exp. Biol. 204, 1889–1899.

Dowd, B.F.X., Forbush III, B., 2003. PASK (praline–alanine-rich ste20-related kinase), a regulatory kinase of the Na–K–Cl cotransporter (NKCC1). J. Biol. Chem. 278, 27347–27353.

Flatman, P.W., 2002. Regulation of Na–K–2Cl cotransport by phosphorylation and protein–protein interactions. Biochim. Biophys. Acta 1566, 140–151.

Gamba, G., 2005. WNK lies upstream of kinases involved in regulation of ion transporters. Biochem. J. 391, e1–e3.

Gawantka, V., Ellinger-Ziegelbauer, H., Hausen, P., 1992. β1-integrin is a maternal protein that is inserted into all newly formed plasma membranes during early *Xenopus* embryogenesis. Development 115, 595–605.

Haas, M., Forbush III, B., 1998. The Na–K–Cl cotransporters. J. Bioenerget. Biomemb. 30, 161–172.

Hass, M., Forbush III, B., 2000. The Na–K–Cl cotransporters of secretory epithelia. Annu. Rev. Physiol. 62, 515–534.

Henry, C.A., Crawford, B.D., Yian, Y.L., Postlethwait, J., Cooper, M.S., Hille, M.B., 2001. Roles for zebrafish focal adhesion kinase in notochord and somite morphogenesis. Dev. Biol. 240, 474–487.

Hirakawa, M., Oike, M., Karashima, Y., Ito, Y., 2004. Sequential activation of RhoA and FAK/Paxillin leads to ATP release and actin reorganization in human endothelium. J. Physiol. 558, 479–488.

Hirakawa, M., Oike, M., Watanabe, M., Karashima, Y., Ito, Y., 2006. Pivotal role of integrin α5β1 in hypotonic stress-induced responses of human endothelium. FASEB J. 20, 1992–1999.

Hoffmann, E.K., Dunham, P.B., 1995. Membrane mechanisms and intracellular signaling in cell volume regulation. Int. Rev. Cytol. 162, 173–262.

Hoffmann, E.K., Hoffmann, E., Lang, F., Zadunaisky, J.A., 2002. Control of Cl^- transport in the operculum epithelium of *Fundulus heteroclitus*: long- and short-term salinity adaptation. Biochim. Biophys. Acta 1566, 129–139.

Hoffmann, E.K., Pederson, S.F., 2006. Sensors and signal transduction pathways in vertebrate cell volume regulation. Contrib. Nephrol. 152, 54–104.

Hoffmann, E.K., Schettino, T., Marshall, W.S., 2007. The role of volume-sensitive ion transport systems in regulation of epithelial transport. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 48, 29–43.

Hynes, R.O., 2002. Integrins: bidirectional, allosteric signaling machines. Cell 110, 673–687.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., Aizawa, S., 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 377, 539–544.

Johnston, A.M., Naselli, G., Gonez, L., Martin, R.M., Harrison, L.C., DiAizpurua, H.J., 2000. SPAK. a Ste 20/sps 1-related kinase that activates the p38 pathway. Oncogene 19, 4290–4297.

Kahle, K.T., Rinehart, J., Ring, A., Gamba, G., Hebert, S.C., Lifton, R.P., 2006. WNK protein kinases modulate cellular Cl^- flux by altering the phosphorylation state of the Na–K–Cl and K–Cl cotransporters. Physiology 21, 326–335.

Kajimura, M., O'Donnell, M.E., Curry, F.E., 1997. Effect of cell shrinkage on permeability of cultured bovine aortic endothelia and frog mesenteric capillaries. J. Physiol. 503, 413–425.

Krarup, T., Jakobsen, L.D., Jensen, B.S., Hoffmann, E.K., 1998. Na–K–2Cl cotransport in Ehrlich cells: regulation by protein phosphatases and kinases. Am. J. Physiol. 275, C239–C250.

Lehoux, S., Esposito, B., Merval, R., Tedgui, A., 2005. Differential regulation of vascular focal adhesion kinase by steady stretch and pulsatility. Circulation 111, 643–649.

Liedtke, W., Tobin, D.M., Bargmann, C.I., Friedman, J.M., 2003. Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U.S.A. 100, 14531–14536.

Liedtke, W., 2005. TRPV4 as osmosensor: a transgenic approach. Pflügers Arch. 451, 176–180.

Liedtke, W., Friedman, J.M., 2003. Abnormal osmotic regulation in trpv4−/− mice. Proc. Natl. Acad. Sci. U.S.A. 100, 13698–13703.

Lytle, C., Xu, J.C., Biemsderfer, D., Haas, M., Forbush III, B., 1992. The Na–K–Cl cotransport protein of shark rectal gland. I. Development of monoclonal antibodies, immunoaffinity purification, and partial biochemical characterization. J. Biol. Chem. 267, 25428–25437.

Marshall, W.S., Bryson, S.E., Garg, D., 1993. α_2-adrenergic inhibition of Cl− transport across the opercular epithelium is mediated by intracellular Ca^{2+}. Proc. Natl. Acad. Sci. U.S.A. 990, 5504–5508.

Marshall, W.S., Bryson, S.E., Luby, T., 2000. Control of epithelial Cl^- secretion by basolateral osmolality in the euryhaline teleost *Fundulus heteroclitus*. J. Exp. Biol. 203, 1897–1905.

Marshall, W.S., Lynch, E.M., Cozzi, R.R.F., 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. J. Exp. Biol. 205, 1265–1273.

Marshall, W.S., Ossum, C.G., Hoffmann, E.K., 2005. Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. J. Exp. Biol. 208, 1063–1077.

Montrose-Rafizadeh, C., Guggino, W.B., 1990. Cell volume regulation in the nephron. Ann. Rev. Physiol. 52, 761–772.

Montrose-Rafizadeh, C., Guggino, W.B., 1991. Role of intracellular calcium in volume regulation by rabbit medullary thick ascending limb cells. Am. J. Physiol. 260, F402–F409.

Mizuno, A., Matsumoto, N., Imai, M., Suzuki, M., 2003. Impaired osmotic sensation in mice lacking TRPV4. Am. J. Physiol. 285, C95–C101.

Mould, A.P., McLeish, J.A., Huxley-Jones, J., Goonesinghe, A.C., Hurlstone, A.F., Boot-Handford, R.P., Humphries, M.J., 2006. Identification of multiple integrin β1 homologs in zebrafish (*Danio rerio*). BMC Cell Biol. 7, 24–38.

Nilius, B., Voets, T., 2005. TRP channels: a TR(I)P through a world of multifunctional cation channels. Pflugers Arch. 451, 1–10.

O'Niel, R.G., Heller, S., 2005. The mechanosensitive nature of TRPV channels. Pflügers Arch. 451, 193–203.

Ostergaard, H., Lysechko, T.L., 2005. Focal adhesion kinase-related protein tyrosine kinase Pyk2 in T-cell activation and function. Immunol. Res. 31, 267–282.

Owens, L.V., Xu, L., Craven, R.J., Dent, G.A., Weiner, T.M., Kornberg, L., Liu, E.T., Cance, W.G., 1995. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. Cancer Res. 55, 2752–2755.

Parsons, J.T., 2003. Focal adhesion kinase: the first ten years. J. Cell. Sci. 116, 1409–1416.

Piechotta, K., Lu, J., Delpire, E., 2002. Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline–alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). J. Biol. Chem. 277, 50812–50819.

Piechotta, K., Garbarni, N., England, R., Delpire, E., 2003. Characterization of the interaction of stress kinase SPAK with the Na^+–K^+–$2Cl^-$ cotransporter in the nervous system. J. Biol. Chem. 278, 52848–52856.

Rafiq, K., Kolpakov, M.A., Abdelfettah, M., Streblow, D.N., Hassid, A., J Dell'Italia, L., Sabri, A., 2006. Role of protein tyrosine phosphatase SHP2 in focal adhesion kinase downregulation during neutrophil cathepsin G-induced cardiomyocytes anoikis. J. Biol. Chem. 281, 19781–19792.

Russell, J.M., 2000. Sodium–potassium-chloride cotransport. Physiol. Rev. 80, 211–276.

Sawai, H., Okada, Y., Funahashi, H., Matsuo, Y., Takahashi, H., Takeyama, H., Manabe, T., 2005. Activation of focal adhesion kinase enhances the adhesion and invasion of pancreatic cancer cells *via* extracellular signal-regulated kinase-1/2 signaling pathway activation. Mol. Cancer 4. doi:10.1186/1476-4598-4-37.

Schaller, M.D., Otey, C.A., Hildebrand, J.D., Parsons, T., 1995. Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. J. Cell Biol. 130, 1181–1187.

Shahsavarani, A., McNeill, B., Galvez, F., Wood, C.M., Goss, G.G., Hwang, P.P., Perry, S.F., 2006. Characterization of a branchial epithelial calcium channel (ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 209, 1928–1943.

Singer, T.D., Tucker, S.J., Marshall, W.S., Higgins, C.F., 1998. A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F heteroclitus*. Am. J. Physiol. 274, C715–C723.

Tani, T., von Koskull, H., Virtanen, I., 1996. Focal adhesion kinase pp125FAK is associated with both intracellular junctions and matrix adhesion sites in vivo. Histochem. Cell Biol. 105, 17–25.

Vitari, A.C., Deak, M., Morrice, N.A., Alessi, D.R., 2005. The WNK 1 and WNK4 protein kinases that a mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and PSR1 protein kinases. Biochem. J. 391, 17–24.

Wilson, J.M., Randall, D.J., Donowitz, M., Vogl, A.W., Ip, A.K.Y., 2000. Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). J. Exp. Biol. 203, 2297–2310.

Wu, M.H., 2005. Endothelial focal adhesions and barrier function. J. Physiol. 569, 359–366.

Zadunaisky, J.A., Cardona, S., Au, L., Roberts, D.M., Fisher, E., Lowenstein, B., Cragoe Jr., E.J., Spring, K.R., 1995. Chloride transport activation by plasma osmolality during rapid adaptation to high salinity of *Fundulus heteroclitus*. J. Membr. Biol. 143, 207–217.