Osmotically relevant membrane signaling complex: association between HB-EGF, β₁-integrin, and CD9 in mTAL

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Sheikh-Hamad, David, Keith Youker, Luan D. Truong, Soren Nielsen, and Mark L. Entman. Osmotically relevant membrane signaling complex: association between HB-EGF, β₁-integrin, and CD9 in mTAL. Am J Physiol Cell Physiol 279: C136–C146, 2000.—The integral membrane proteins cluster of differentiation-9 (CD9), β₁-integrin, and heparin-binding epidermal growth factor-like (HB-EGF) exist in association in many cell lines and are linked to intracellular signaling mechanisms. Two of the proteins (CD9 and β₁-integrin) are induced by hypertonicity, suggesting that their related signaling processes may be relevant to osmotic stress. The validity of this hypothesis rests upon coexpression and physical association between these molecules in nephron segments that are normally exposed to high and variable ambient osmolality. In this work, we show that CD9 and β₁-integrin are induced in rat kidney medulla after dehydration. Immunohistochemistry and immunoprecipitation studies show that CD9, HB-EGF, and β₁-integrin are coexpressed and physically associated in medullary thick ascending limbs (mTAL), nephron segments that are normally exposed to high and variable extracellular osmolality. Our findings are consistent with the existence of a cluster of integral membrane proteins in mTAL that may initiate or modulate osmotically relevant signaling pathways.

cluster of differentiation-9; heparin-binding epidermal growth factor; β₁-integrin; osmotic stress; thick ascending limb; kidney

MANY ORGANISMS, FROM BACTERIA to mammals, adapt to sustained hyperosmotic stress by the preferential accumulation of protective organic solutes (36). These compounds do not perturb cellular macromolecules and are considered important for the survival of cells in hypertonic environment (36). The accumulation of these substances is facilitated by the induction of specific genes, such as transporters for betaine, inositol, and taurine, as well as the aldose reductase enzyme, which catalyzes the reduction of d-glucose to form the organic solute sorbitol (6, 13, 31, 35). The induction of these genes is thought to correlate with the intracellular ionic strength (the sum of Na and K concentrations; see Ref. 30). However, it remains unclear how changes in ionic strength regulate gene expression.

We recently reported the cloning of a partial cDNA corresponding to the membrane protein CD9 and demonstrated its regulation by hypertonicity in two renal epithelial cell lines of medulla origin, Madin-Darby canine kidney (MDCK) and the rabbit papillary cells, PAP-HT25 (27). Additional studies in MDCK cells demonstrate that the adhesion molecule β₁-integrin is induced by hypertonicity and exists in association with cluster of differentiation-9 (CD9; see Ref. 28). Furthermore, the induction of CD9 and β₁-integrin by hypertonicity is attenuated by supplementation of the culture media with organic osmolytes, suggesting a physiological role for CD9 and β₁-integrin in the adaptation of kidney cells to osmotic stress (27, 28). Previous work by others demonstrated an association between CD9, β₁-integrin, and heparin-binding epidermal growth factor-like (HB-EGF) in monkey kidney cells (20). Increased expression of CD9 upregulates the number of functional cell-surface HB-EGF molecules and their juxtacrine epidermal growth factor (EGF) receptor stimulatory activity (8, 11). Because CD9, HB-EGF, and β₁-integrin are linked to signal transduction pathways, such as extracellular regulated kinase (ERK), Jun NH₂-terminal kinase (JNK), and focal adhesion kinase (125F AK; see Refs. 3, 7, 8, 19, 20, 22, 25, 33), the induction by hypertonicity of CD9 and β₁-integrin may lead to initiation or modulation of CD9-, HB-EGF-, and β₁-integrin-related signaling molecules in an osmolality-dependent manner. The validity of this hypothesis rests upon coexpression and physical association between these proteins in kidney structures that are normally exposed to high and variable osmolality.

In these studies, we report that water deprivation, which leads to increased osmolality in the kidney medulla, upregulates CD9 and β₁-integrin, suggesting that the induction of these molecules by hypertonicity has its in vivo correlate. Furthermore, immunoprecipitation and immunohistochemistry studies demonstrate...
physical association between and coexpression of CD9, HB-EGF, and β1-integrin in cells of the medullary thick ascending limbs (mTAL). The data are consistent with a role for CD9, HB-EGF, and β1-integrin in initiating or modulating osmotically relevant signaling pathways in mTAL cells.

**METHODS**

**Experimental Animals**

Male Sprague-Dawley rats weighing 200 g (Harlan Bioproducts for Science, Indianapolis, IN) were placed on rat chow and either of the following: 1) ad libitum tap water or 2) ad libitum 500 mM sucrose in tap water for water loading. A portion of the sucrose-fed animals was water deprived, whereas the rest served as controls. At predetermined times, the animals were killed by lethal injection of pentobarbital sodium. For urine osmolality determination, a urine sample was collected by needle aspiration directly from the bladder after opening the abdominal cavity. Samples of kidney cortex and medulla were harvested for analysis.

**Northern Blot Analyses**

Total RNA was isolated using RNazol (Tel-Test, Friendswood, TX; see Ref. 4). Poly(A)+ RNA was prepared using oligo(dT) columns (Collaborative Biomedical Products), as previously described (6). Electrophoresis was performed after loading equal amounts of Poly(A)+ RNA per lane in a 1% agarose-2.2 M formaldehyde gel, followed by transfer to Gene

**Fig. 1.** A: characterization of L355 anti-cluster of differentiation-9 (CD9) antibodies. Rat outer medulla protein lysates were immuno-precipitated using L355 anti-CD9 antibodies (lane 3), preimmune serum (lane 1), or L355 that was pretreated with the immunizing peptide (lane 2). Precipitates were run on 12% SDS-PAGE, and immunoblots were reacted with a known anti-CD9 antibody (ALB6). Upper band corresponds to the glycosylated form of CD9, whereas the lower band corresponds to the nonglycosylated form. B: association between CD9 and β1-integrin. Rat outer medulla protein lysates were immunoprecipitated using L355 anti-CD9 antibodies (lane 3), preimmune serum (lane 1), or L355 that was pretreated with the immunizing peptide (lane 2). Precipitates were run on 12% SDS-PAGE and immunoblots reacted with anti-β1-integrin antibodies (AB1997). Ab, antibody.

**Fig. 2.** Urine osmolality after hydration. Male Sprague-Dawley rats were given either tap water or sucrose-supplemented water (500 mM) for 5 days. Urine samples were collected by aspiration directly from the bladder, and osmolality was measured using a vapor-pressure osmometer. Data represent the means and SE of 4 independent determinations.

**Fig. 3.** Decreased kidney medulla CD9 mRNA and protein abundance with water loading. Male Sprague-Dawley rats were given either tap water (3 rats in each group, lanes 1–3) or sucrose-supplemented water for 5 days (3 rats in each group, lanes 4–6). Kidney medulla samples were analyzed for CD9 mRNA and protein abundance. For Western blots, 40 μg protein were loaded per lane; for Northern blots, 3 μg of poly(A)+ RNA were loaded per lane. Top, CD9 protein; middle, CD9 mRNA; bottom, β-actin mRNA.

**Fig. 4.** Increased urine osmolality by water deprivation after water loading. Male Sprague-Dawley rats were placed on sucrose-supplemented water for 5 days. At the end of the 5th day (time 0 point), rats were water deprived for incremental periods up to 48 h. Urine samples were collected by aspiration directly from the bladder, and osmolality was measured using a vapor-pressure osmometer. Data represent the means and SE of 4 independent determinations.
Fig. 5. A: modulation of kidney medulla CD9 mRNA and protein abundance by water balance. Male Sprague-Dawley rats were placed on sucrose-supplemented water. At the end of the 5th day (water [W] loaded), rats were water deprived for incremental time points up to 48 h. Kidney medulla samples were analyzed for CD9 mRNA and protein abundance. For Western blots, 40 µg protein were loaded per lane; for Northern blots, 3 µg of poly(A)⁺ RNA were loaded per lane. Top, CD9 protein; middle, CD9 mRNA; bottom, β-actin mRNA. Experiments were carried out in triplicate (3 rats in each group): lanes 1–3, water loading for 5 days; lanes 4–6, water deprivation for 24 h after water loading; lanes 7–9, water deprivation for 48 h after water loading. B: rat kidney medulla β₁-integrin mRNA abundance after water deprivation. Rats were water deprived as described in A, followed by water deprivation for 10 h. Kidney medulla samples were analyzed for β₁-integrin mRNA abundance. Poly(A)⁺ RNA (3 µg) was loaded per lane. Top, β₁-integrin mRNA; bottom, β-actin mRNA. Experiments were carried out in triplicate (3 rats in each group): lanes 1–3, water loading for 5 days; lanes 4–6, water deprivation for 10 h after water loading.

Screen membrane (New England Nuclear; see Ref. 6). Human full-length (2-kb) β-actin cDNA (Clontech) was labeled with [α-³²P]dCTP (Random Primed DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN) for use as probe. Synthetic antisense CD9-specific oligonucleotide (35 NT), corresponding to base pairs 109–143 of rat CD9 cDNA (accession no. X76489), and β₁-integrin-specific oligonucleotide (46 nt) corresponding to base pairs 522–567 of rat β₁-integrin cDNA (accession no. U12309) were used as probes (12, 15). The CD9 probe sequence (5'-TCTTG CTCGA AGATG CTCTT GGTCT GAGAG CTAAG-3') is unique to CD9 from different species and is not shared with other known members of the tetraspan family (TM4SF), whereas the β₁-integrin probe sequence (5'-TCTTC ATTTG ATTTCA TCAGA TCGCT CCAAC GACTC TTCAC ATTTG C-3') is in the coding region of β₁-integrin and is not shared with other known members of the integrin family of proteins. Sequence specificity of the probes was determined using the BLAST search of the National Center for Biotechnology Information (NCBI) databases. Both oligonucleotide probes were end-labeled with [γ-³²P]ATP (5,000 Ci/mmol; Amersham Life Science, Arlington Heights, IL), as previously described (16). β-Actin probe was hybridized to the blots overnight at 42°C in a solution containing 40% formamide, 5 × SSC (1 × SSC = 150 mM NaCl and 15 mM trisodium citrate, pH 7.0), 5 × Denhardt’s solution (1 × Denhardt’s = 0.02% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) BSA, and 0.2% (wt/vol) Ficoll 400), 0.5% SDS, 250 µg/ml salmon sperm DNA, 10 mTris (pH 7.5), and 10% dextran sulfate. The blots were then washed at 65°C as follows: 1 h in 2 × SSC, 0.5% SDS; 1 h in 0.5 × SSC, 0.5% SDS; 1 h in 0.1 × SSC, 0.5% SDS. The end-labeled oligonucleotide probes were hybridized to the blots overnight at 42°C in a solution as above but containing 100 µg/ml salmon sperm DNA and no formamide. These blots were then washed at 42°C, as described above for β-actin probe. Autoradiographs were prepared using X-Omat AR film (Kodak, Rochester, NY) with an intensifying screen. Bands on Northern blots were scanned using a UMAX Astra 1200S scanner and were quantitated using Adobe Photoshop 4 andUTHSCSA Image Tool software. Band intensities were determined relative to the corresponding β-actin bands.

Antibodies

Because of the inadequacy of commercially available anti-CD9 antibodies for our immunohistochemistry studies, we custom-made polyclonal anti-CD9 antibodies (L355). These were raised in rabbit (Lofstrand, Rockville, MD) against keyhole limpet hemocyanin-conjugated 15-amino acid synthetic oligopeptide (CFYKDYQKLKNKDE; cysteine in the first position was added to facilitate conjugation of the oligopeptide to resin for affinity purification of the antibody), which was derived from the putative major extracellular domain (amino acids 122–135) of rat CD9 protein (accession no. P40241). Sequence analysis of the oligopeptide, using GCG-sequence analysis software (University of Wisconsin Software Package), predicted high antigenicity and no post-translation modification sites. The specificity of the oligopeptide was determined using the BLASTP-BEAUTY/nr protein search protocol of the National Center for Biotechnology Information databases. To determine the specificity of the anti-CD9 antibody, immunoblots carrying L355 immune precipitates of kidney medulla protein lysate were reacted with ALB6 (Immunotech, Westbrook, ME), a known monoclonal anti-CD9 antibody. The antibody detected a 25-kDa protein, the predicted size for CD9. CD9 was not detected by ALB6 in precipitates of preimmune serum or L355 that was pretreated with the immunizing peptide (Fig. 1A). Polyclonal rabbit anti-human (3100) and anti-rat (3096) HB-EGF antibodies (21) were a generous gift from Dr. Michael Klagsbrun (Surgical Research Department, Children’s Hospital, Harvard Medical School, Boston, MA). Anti-BSC1 bumetanide-sensitive cotransporter (BSC1 or Na-K-2Cl transporter) antibody was provided by Dr. Kishore Belamkonda (Division of Nephrology, University of Cincinnati, Cincinnati, OH; see Ref. 5). K20, a mouse anti-β₁-integrin monoclonal antibody, and ALB6, a mouse anti-CD9 monoclonal antibody, were purchased from Immunotech (Westbrook, ME). Polyclonal rabbit anti-human β₁-integrin antibody (AB1937) was purchased from Chemicon International (Temecula, CA). Monoclonal anti-plasma membrane Ca²⁺-ATPase antibody (anti-PMCA, no. MA3–914) was purchased from Affinity Bioreagents (Golden, CO).
Preparation of Kidney Medulla Protein Lysate

Male Sprague-Dawley rats weighing 200 g were killed by lethal injection of pentobarbital sodium, and samples of kidney cortex and inner and outer medulla were obtained. For preparation of total cell protein lysate, tissue samples were homogenized for 60 s in 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer [10 mM HEPES, pH 7.0, 150 mM NaCl, 10 mM CHAPS, 1 μg/ml leupeptin, and 0.4 mM phenylmethylsulfonyl fluoride (PMSF)] using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and were left at 4°C for 30 min. Lysates were cleared from insoluble material by 10 min centrifugation at 3,000 g and 4°C, and the supernatants were collected. For preparation of membrane protein lysate, total cell lysates prepared as above were centrifuged at 16,000 g for 20 min. The pellets were then suspended in Triton lysis buffer (50 mM Tris, pH 7.2, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, and 1 μg/ml leupeptin). Protein was quantitated using the bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL) and was stored at −80°C until used.

In Situ Hybridization

Riboprobe generation and selection. T7 and SP6 RNA polymerases were used (2 U/μl) on linearized template (1 μg/20 μl) to generate FITC-conjugated sense and antisense riboprobes, using an Amersham kit as per the manufacturer’s instructions. After ethanol precipitation and washes (two times in 75% ethanol), the labeled RNA was suspended in 50 μl diethyl pyrocarbonate-treated water. Probe concentration was estimated by comparing serial dilutions of the probe with labeled RNA of known concentration. Probe specificity was verified by Northern blot analysis following the manufacturer’s protocol, as previously described (37). Calorimetric detection of FITC, using alkaline phosphatase-conjugated sec-
ondary antibody, revealed signal with the antisense probe but not with the sense probe.

In situ hybridization. In situ hybridization was performed on 2% paraformaldehyde-embedded sections, as previously described (37). After sectioning and deparaffinization, the tissue was incubated in 2×3 SSC buffer for 5 min and again for 1 h. After 1 h prehybridization at room temperature in 100 μl of hybridization solution (50% formamide, 4× SSC, 1× Denhardt’s reagent, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA, and 10% dextran sulfate), the probe was added (500 ng/ml), and the tissue was hybridized overnight at 42°C followed by serial washes in 2× SSC (1 h at room temperature), 1× SSC (1 h at room temperature), 0.5× SSC (0.5 h at 37°C), and 0.5× SSC (0.5 h at room temperature). Detection was carried out using anti-FITC antibody and nitro blue tetrazolium staining for alkaline phosphatase reaction, as previously described (37). No signal was detected using “sense” riboprobe (data not shown).

Immunohistochemistry

Male Sprague-Dawley rats weighing 200 g were killed by lethal injection of pentobarbital sodium. Coronal sections of the kidney (2–3 mm thick) were fixed in 2% paraformaldehyde. Tissue was embedded in paraffin, sectioned, and subjected to dual immunostaining using FITC-tagged anti-β1-integrin plus indocarbocyanine (Cy3)-tagged anti-CD9 (L355) or FITC-tagged anti-β1-integrin plus Cy3-tagged anti-HB-EGF (3096) antibodies. All antibodies were diluted 1:300 in blocking solution (Vector Laboratories, Burlingame, CA). For BSC1 and anti-CD9 antibody (L355) dual staining, a peroxidase-based detection system was used (Vector Laboratories). First detection (with BSC1) employed 3-amino-9-ethyl carbazol as substrate, followed by ethanol washes (to erase the first staining), sequential immunolabeling with anti-CD9 antibody, and detection using diaminobenzidine as substrate. Preimmune serum or pretreatment of anti-CD9

Fig. 8. CD9 is expressed in bumetanide-sensitive cotransporter (BSC1)-positive tubules. Dual immunostaining with anti-BSC1 (B, D, and F) and L355 anti-CD9 (A, C, and E). A and B: image captured from the border between the IM and ISOM showing identical staining pattern in medullary TAL with both antibodies. At higher magnification, BSC1 staining in the TAL has apical predominance (F) while CD9 staining is mostly basolateral (E). In C and D, the image was captured from the region of interlobular vessels in the cortex. Identical staining pattern in TAL (arrow) is seen with both antibodies. GM, glomerulus. In G, control staining with preimmune serum (L355) shows no labeling.
(L355) with the immunizing peptide served as controls for immunostaining with L355, whereas omission of the primary antibodies served as the negative controls for HB-EGF, β₁-integrin, and BSC1 antibodies.

**SDS-PAGE**

This method is based on Laemmli (14), with slight modifications based on Rubinstein et al. (24) and Seehafer and Shaw (26). Briefly, equal amounts of protein were run on 12% nonreducing SDS-PAGE. Proteins were transferred overnight at 4°C, 40 volts, on Hybond-enhanced chemiluminescence (ECL) membrane (Amersham) in Laemmli buffer (25 mM Tris and 52 mM glycine, pH 8.3) containing 20% methanol. Blots were blocked for 1 h with 5% dried milk in 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20 (TBST), washed quickly in TBST, and incubated overnight at 4°C with primary antibody (1:20 dilution for ALB6; 1:50 dilution for K20; and 1:500 dilution for anti-HB-EGF and anti-β₁-integrin) in TBST containing 1% BSA. After a 30-min wash in TBST, blots were incubated with 1:1,000 dilution of peroxidase-conjugated secondary antibody in TBST containing 1% BSA. Protein bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, UK) as per the manufacturer’s instructions. Blots designated for reaction with anti-PMCA were transferred in Laemmli buffer lacking methanol. Primary antibody dilution was 1:500, and all incubations and washes were as above, except for substitution of PBS (150 mM NaCl and 50 NaPO₄, pH 7.5) for TBST.

**Immunoprecipitation and Immunoblotting**

Protein lysate (100 µg) was incubated with 5 µg of one of the following antibodies: anti-CD9 (L355), anti-HB-EGF (3100), or anti-β₁-integrin. The samples were rocked for 2 h at 4°C in the presence of 30 µl of protein A and G-agarose (Santa Cruz) followed by 6 min centrifugation at 4°C and 6,000 g. The immunoprecipitates were then washed three times in Triton lysis buffer (see above), and the recovered material was analyzed on 12% SDS-PAGE under nonreducing conditions. Blots were reacted with one of the following antibodies: anti-CD9 (ALB6), anti-β₁-integrin (AB1937), or anti-HB-EGF (3100). For control experiments, total cell lysates of outer medulla prepared as above were immunoprecipitated with either anti-β₁-integrin or anti-PMCA. Precipitates were resolved on 12% SDS-PAGE, and immunoblots were reacted with anti-PMCA antibodies.

**RESULTS**

**Regulation of Rat Medulla CD9 and β₁-Integrin by Water Balance**

Because of the urinary concentrating mechanism, cells of the renal medulla are exposed to hyperosmotic milieu. Cells of the renal cortex on the other hand are not. The expression of osmotically regulated genes in the kidney is typically higher in the medulla than in the cortex and correlates with the
hydration state (6, 13, 18, 27, 32, 35). Thus changes in the expression of CD9 and \( \beta_1 \)-integrin with variations in medulla osmolality would suggest a physiological role for these proteins in osmoregulation. Therefore, we examined the abundance of CD9 and \( \beta_1 \)-integrin in rat kidney under conditions of water loading or deprivation. Rats were placed on either tap water or tap water containing 500 mM sucrose for 5 days (for hydration). A portion of the rats on sucrose-supplemented water was then water deprived for incremental intervals up to 2 days. Urine samples were collected for osmolality measurement, and kidney cortex and medulla tissues were analyzed for CD9 and \( \beta_1 \)-integrin abundance. Results were compared with those obtained from water-loaded (sucrose in water) rats or rats maintained on ad libitum tap water. Water loading for 5 days decreased urine osmolality from 2,150 ± 100 to 600 ± 50 mosmol/kgH\(_2\)O (Fig. 2) and was associated with a two- to threefold decrease in the abundance of medulla CD9 mRNA and protein (Fig. 3). Water deprivation for 24 h after water loading increased urine osmolality from 600 ± 50 to 3,100 ± 125 mosmol/kgH\(_2\)O (Fig. 4) and was associated with a two- to threefold increase in medulla CD9 mRNA and protein (Fig. 5A). Similarly, \( \beta_1 \)-integrin mRNA decreased twofold after water loading (data not shown). However, maximal induction (3-fold) of \( \beta_1 \)-integrin mRNA in the medulla after water deprivation occurred after 10 h (Fig. 5B), concomitant with increased urine osmolality from 600 ± 50 to 2,100 ± 150 mosmol/kgH\(_2\)O (Fig. 4). These data are consistent with our previously published findings in cultured MDCK cells in which peak induction of \( \beta_1 \)-integrin mRNA occurred between 6 and 16 h after osmotic stress, whereas that of CD9 occurred after 16–24 h (27, 28). CD9 and \( \beta_1 \)-integrin mRNAs were detectable at lower levels in the cortex and showed no variations with water loading or deprivation (data not shown). There was no equivalent difference in the expression of \( \beta \)-actin mRNA in the medulla under the various experimental conditions. In conclusion, the expression of CD9 and \( \beta_1 \)-integrin in the kidney medulla correlates with extracellular osmolality and suggests a role for these proteins in the cellular adaptation to osmotic stress.

**Coexpression of CD9, \( \beta_1 \)-Integrin, and HB-EGF in mTAL**

Different nephron segments have unique cellular elements that are identified with specific physiological function(s). Identification of the cellular elements that express CD9, \( \beta_1 \)-integrin, and HB-EGF may suggest a function with which the proteins are associated. Therefore, we subjected rat kidney sections to dual immunostaining using a combination of FITC-tagged anti-\( \beta_1 \)-integrin plus Cy3-tagged anti-CD9 or FITC-tagged anti-\( \beta_1 \)-integrin plus Cy3-tagged anti-HB-EGF antibodies. Regional colocalization of all three proteins was found in thick ascending limb (mTAL) cells in the inner stripe of the outer medulla (Fig. 6). Figure 7 demonstrates that this staining, as shown for HB-EGF, occurs exclusively in mTAL and has a predominant basolateral membrane localization. To confirm the mTAL expression of HB-EGF, CD9, and \( \beta_1 \)-integrin, we carried out sequential “dual” immunostaining with anti-BSC1 (Na-K-2Cl cotransporter, a marker for TAL and macula densa) followed by anti-CD9 (L355). BSC1 and CD9 are coexpressed in the same tubular structures (Fig. 8), confirming the localization of HB-EGF, CD9, and \( \beta_1 \)-integrin to mTAL. We note with particular interest, the expression of all three proteins in the cortex, in few tubular elements that are in immediate
proximity to interlobular arteries and veins (Fig. 9). This expression stands in stark contrast with the negative labeling in the majority of tubular elements in the cortex. Representative staining is shown for CD9 (Fig. 10). However, an identical pattern is seen for HB-EGF and β₁-integrin (data not shown). Staining with BSC1 antibodies confirmed the identity of these structures as thick ascending limbs (Fig. 8). To rule out the possibility of nonspecific labeling in these structures secondary to what is known as the “edge effect,” we carried out in situ hybridization using CD9 cDNA as a probe (27). CD9 mRNA expression in these tubular structures is identical to that obtained using immunolabeling (Fig. 11), thus ruling out nonspecific edge effects. In addition to colocalization of all three proteins in mTAL, isolated expression of one or co-expression of only two of the proteins was observed in other nephron segments (see Table 1 for details). Last, consistent with the in vivo quantitative studies for CD9 and β₁-integrin described earlier and in agreement with the in situ hybridization studies previously reported by Homma et al. (10), the highest level of labeling observed for each of these proteins was in the medulla (outer). We conclude that HB-EGF, CD9, and β₁-integrin are coexpressed in mTAL, a nephron segment that is normally exposed to high and variable osmolality.

Physical Association Between CD9, β₁-Integrin, and HB-EGF

To determine whether HB-EGF, CD9, and β₁-integrin are physically associated, we performed immunoprecipitation studies on rat kidney outer medulla protein lysates using anti-CD9 antibody (L355). Precipitates were resolved on SDS-PAGE, and immunoblots were reacted consecutively with a known anti-CD9 antibody (ALB6) and anti-β₁-integrin (AB1937). CD9 (Fig. 1A) and β₁-integrin (Fig. 1B) were detected in anti-CD9 (L355) immunoprecipitates but not in precipitates of preimmune serum or L355 that was pretreated with the immunizing peptide, suggesting physical association between CD9 and β₁-integrin in vivo. In a separate experiment, outer medulla protein lysates were immunoprecipitated with anti-β₁-integrin antibody (AB1937) or anti-HB-EGF (3100). Precipitates were resolved on SDS-PAGE, and immunoblots were reacted with ALB6 (a monoclonal anti-CD9). CD9 is detected in immunoprecipitates of both antibodies (Fig. 12), suggesting an in vivo association between CD9 and β₁-integrin as well as CD9 and HB-EGF. Last, outer medulla protein lysates were immunoprecipitated with anti-β₁-integrin antibody (AB1937) or anti-HB-EGF (3100). Precipitates were resolved on SDS-PAGE, and immunoblots were reacted consecutively with anti-HB-EGF (3100) and anti-β₁-integrin antibody (AB1937). HB-EGF was detected in β₁-integrin precipitates, whereas β₁-integrin was detected in HB-EGF precipitates (Fig. 13), suggesting an in vivo association between HB-EGF and β₁-integrin. As a control experiment, similarly prepared protein lysates were immunoprecipitated with an antibody directed against the PMCA, which is not known to associate with β₁-integrin or HB-EGF. HB-EGF and β₁-integrin were not detected in anti-PMCA immunoprecipitates (Fig. 13). It is concluded that CD9, HB-EGF, and β₁-integrin are physically associated in vivo in outer medulla structures. Based on coexpression of these three proteins in mTAL as discussed above, it is also concluded that
HB-EGF, CD9, and β₁-integrin are physically associated in mTAL.

**DISCUSSION**

Studies in other in vitro systems have suggested that CD9, HB-EGF, and β₁-integrin exist as a complex at the cell membrane (20). Our findings suggest physical association between and coexpression of CD9, HB-EGF, and β₁-integrin in mTAL, lending support for their existence in vivo as a functional unit.

In addition, the current data extend our previously published tissue culture findings of CD9 and β₁-integrin induction by hypertonicity in MDCK cells (27, 28) to the kidney medulla. Like CD9, HB-EGF and β₁-integrin are linked to signal transduction pathways and are physically associated; the induction by hypertonicity of CD9 and β₁-integrin suggests direct relevance of CD9, HB-EGF, and β₁-integrin-related signaling cascades to osmotic stress. HB-EGF is expressed as a membrane-bound molecule that can activate EGF receptor in a juxtacrine manner (8). Increased expression of CD9 upregulates the number of functional cell-surface HB-EGF molecules and their juxtacrine EGF receptor stimulatory activity (8, 11). This in turn leads to activation of ERK and JNK pathways. In parallel, integrin-mediated cell attachment to extracellular matrix activates mitogen-activated protein kinase and pp125FAK (3, 19, 33). Osmotic stress causes immediate shrinkage of cells and raises intracellular ionic strength. Such changes are likely to lead to physicochemical alteration(s) in integrin-matrix or integrin-cytoskeletal interaction(s). A change in the abundance of CD9 and/or β₁-integrin, or altered integrin-matrix-cytoskeletal interaction, may in turn initiate HB-EGF-, CD9-, and/or integrin-mediated signaling. This is conceivably a mechanism by which the cell can sense osmotic stress and initiate adaptive responses to it. These adaptive processes may be important for cell survival or function in a hypertonic environment. The basolateral expression of β₁-integrin, HB-EGF, and CD9 in mTAL, coupled with the

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<td>TAL negative</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>Negative</td>
<td>CD positive</td>
<td>Weak CD</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Ab, antibody; HB-EGF, heparin-binding epidermal growth factor-like; CD9, cluster of differentiation-9; S₃, straight proximal tubules or segment 3; TAL, thick ascending limbs; CD, collecting duct; DCT, distal convoluted tubules.

**Table 1. Distribution of CD9, HB-EGF, and β₁-integrin in rat kidney**

**Fig. 12. CD9-HB-EGF and CD9-β₁-integrin association.** Rat outer medulla lysates were immunoprecipitated using either anti-β₁-integrin (lane 1) or anti-HB-EGF (lane 2). Precipitates were run on 12% SDS-PAGE and blots reacted with anti-CD9 (ALB6). Band on top corresponds to the glycosylated form of CD9, whereas the band on bottom corresponds to the nonglycosylated form.

**Fig. 13. HB-EGF and β₁-integrin association.** Rat outer medulla lysates were immunoprecipitated using one of the following: anti-β₁-integrin (lane 1), anti-HB-EGF (lane 2), or an irrelevant Ab, anti-plasma membrane Ca-ATPase (PMCA, lane 3). Precipitates were run on SDS-PAGE and blots reacted consecutively with anti-β₁-integrin (AB1937) or anti-HB-EGF (3100).
basolateral membrane localization of EGF receptors (2, 9) and the suggestion that MDCK cells respond more efficiently to basolaterally directed hypertonicity, lend further credence to this hypothesis (34). Indeed, such a scenario has been suggested by Rosette and Karin (23) in an in vitro system where clustering of EGF, tumor necrosis factor (TNF), and interleukin (IL)-1 receptors is induced in osmotically stressed HeLa cells and provides an incremental activation of the JNK pathway (23).

Osmotic stress activates ERK, JNK, 125FAK, and p38 kinase (1, 17, 29, 33). Activation of three of these kinases in osmotically stressed cells may be initiated from the HB-EGF/CD9/β₁-integrin protein complex. Does this complex stand alone in this context, and what is the significance of its localization to TAL? Considering the findings of Rosette and Karin, it is conceivable that mammalian cells utilize multiple cell surface molecules to initiate and/or modulate signaling pathways in osmotically stressed cells. In this setting, TNF/EGF/IL-1 receptors may work in synergy with HB-EGF/CD9/β₁-integrin to initiate or modulate osmotically relevant signaling from the cell membrane. Of note, cells of the TAL are metabolically more active than other cells in the medulla. As a result, these cells may be more susceptible to toxic and metabolic injury. Hence, they may have adapted unique protective mechanisms to enhance their survival and/or function in the hypertonic and potentially injurious environment of the kidney medulla.

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