Repression of Na,K-ATPase β_1 -Subunit by the Transcription Factor Snail in Carcinoma

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The Na,K-ATPase consists of two essential α - and β -subunits and regulates the intracellular Na⁺ and K⁺ homeostasis. Although the α -subunit contains the catalytic activity, it is not active without functional β -subunit. Here, we report that poorly differentiated carcinoma cell lines derived from colon, breast, kidney, and pancreas show reduced expression of the Na,K-ATPase β_1 -subunit. Decreased expression of β_1 -subunit in poorly differentiated carcinoma cell lines correlated with increased expression of the transcription factor Snail known to down-regulate E-cadherin. Ectopic expression of Snail in well-differentiated epithelial cell lines reduced the protein levels of E-cadherin and β_1 -subunit and induced a mesenchymal phenotype. Reduction of Snail expression in a poorly differentiated carcinoma cell line by RNA interference increased the levels of Na,K-ATPase β_1 -subunit. Furthermore, Snail binds to a noncanonical E-box in the Na,K-ATPase β_1 -subunit promoter and suppresses its promoter activity. These results suggest that down-regulation of Na,K-ATPase β_1 -subunit and E-cadherin by Snail are associated with events leading to epithelial to mesenchymal transition.

INTRODUCTION

Epithelia form a barrier between two biological compartments and regulate the molecular composition of and exchange between the compartments they separate. The plasma membrane of epithelial cells is divided into two functionally and biochemically distinct domains, the apical and basolateral (basal and lateral) plasma membranes, by tight junctions (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). This unique structural organization of epithelial cells, referred to as polarized epithelial phenotype or well-differentiated phenotype, is lost in carcinoma (cancer derived from epithelial cells). In general, carcinoma cells show a more fibroblastic or mesenchymal phenotype. Events associated with the phenotypic conversion of epithelial cells to mesenchymal cells in cancer are referred to as epithelial to mesenchymal transition (EMT). Molecular mechanisms involved in EMT are beginning to be understood.

Recent studies indicate that the Snail family members that encode transcription factors of the zinc-finger type play a crucial role during EMT (Batlle *et al.*, 2000; Comijn *et al.*, 2001; Savagner, 2001; Nieto, 2002). The consensus binding site for Snail-related proteins contains a core of six bases,

CAGGTG, also referred to as the E-box (Mauhin *et al.*, 1993; Fuse *et al.*, 1994; Inukai *et al.*, 1999; Batlle *et al.*, 2000; Cano *et al.*, 2000; Kataoka *et al.*, 2000). On binding to the E-box, Snail family members act as transcriptional suppressors (Batlle *et al.*, 2000; Cano *et al.*, 2000; LaBonne and Bronner-Fraser, 2000; Mayor *et al.*, 2000). A direct correlation has been observed between Snail induction and the acquisition of metastatic properties in human tumor cell lines of different epithelial origin, including breast, pancreas, colon, bladder, oral squamous carcinomas, and melanomas (Batlle *et al.*, 2000; Cano *et al.*, 2000; Yokoyama *et al.*, 2001; Jiao *et al.*, 2002).

E-cadherin is a calcium-dependent cell adhesion molecule implicated in the maintenance of the polarized phenotype of epithelial cells (Takeichi, 1990, 1991; Gumbiner, 1996). Expression of E-cadherin is highly reduced during EMT (Hay, 1995; Hay and Zuk, 1995; Sun *et al.*, 1998; Lilien *et al.*, 2002; Masszi *et al.*, 2003). Snail binds to the E-boxes present in the E-cadherin promoter and represses E-cadherin transcription (Batlle *et al.*, 2000; Comijn *et al.*, 2001; Savagner, 2001; Nieto, 2002). Thus, Snail seems to be a key regulator involved in the suppression of E-cadherin in carcinoma.

Na,K-ATPase is an abundantly expressed protein in epithelial cells. Localized to the basolateral plasma membrane, the oligomeric Na,K-ATPase catalyzes an ATP-dependent transport of three sodium ions out and two potassium ions into the cell per pump cycle to maintain the membrane potential and sodium and potassium gradients across the plasma membrane. This sodium and potassium homeostasis is necessary to regulate the functions of the various ion and solute transporters in epithelial cells. Na,K-ATPase is composed of two essential polypeptide subunits, the α -subunit (~112 kDa) (Shull *et al.*, 1985) and the β -subunit (~55 kDa) (Shull *et al.*, 1986) and an optional regulatory γ -subunit (~6.5 kDa) (Beguín *et al.*, 1997). Although the α -subunit contains the catalytic activity of the enzyme, it is not functional

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Abbreviations used: EMSA, electrophoretic mobility shift assay; EMT, epithelial to mesenchymal transition; MDCK, Madin-Darby Canine kidney; MSV-MDCK, Moloney Sarcoma Virus-transformed MDCK; MRE/GREs, mineralocorticoid/glucocorticoid responsive elements; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction.

without the β -subunit. However, the precise function of the β -subunit in Na,K-ATPase enzyme activity remains unclear. It is known that the β -subunit is essential for the transport of the α -subunit to the plasma membrane (Geering, 1990; McDonough *et al.*, 1990; Chow and Forte, 1995). Four α -isoforms have been described in mammals (α_1 , α_2 , α_3 , and α_4); the α_1 isoform is expressed in most of the tissue types (Shamraj and Lingrel, 1994; Blanco *et al.*, 1999; Woo *et al.*, 1999). Of the three isoforms described (β_1 , β_2 , and β_3), the β_1 isoform is expressed in most of the tissues (Lingrel *et al.*, 1994; Mobasheri *et al.*, 2000).

We have shown that the Na,K-ATPase function is necessary for the formation (Rajasekaran *et al.*, 2001a) and maintenance (Rajasekaran *et al.*, 2003) of tight junctions in epithelial cells. Recent studies have shown that in *Drosophila* both Na,K-ATPase α - (*ATP α*) and β - (*Nervana*) subunits are localized to septate junctions and are essential for maintaining the permeability of the septate junctions (Genova and Fehon, 2003; Paul *et al.*, 2003). These studies demonstrate that Na,K-ATPase function plays a crucial role in the maintenance of the polarized phenotype of epithelial cells (Rajasekaran and Rajasekaran, 2003).

The importance of Na,K-ATPase in the regulation of the polarized phenotype of epithelial cells is further exemplified by its altered subunit levels and reduced enzyme activity in carcinoma. Na,K-ATPase β -subunit levels and Na,K-ATPase enzyme activity are highly reduced in an invasive and prevalent form of human renal clear cell carcinoma (Rajasekaran *et al.*, 1999). Moloney sarcoma virus transformation of Madin-Darby canine kidney cells (MSV-MDCK) resulted in the loss of its polarized epithelial phenotype and was associated with a drastic reduction in the levels of the β -subunit of Na,K-ATPase, indicating that oncogenic transformation of epithelial cells is associated with reduced Na,K-ATPase β_1 -subunit expression (Rajasekaran *et al.*, 2001b). The reduced expression of E-cadherin has been correlated to the loss of polarized phenotype and increased invasiveness of MSV-MDCK cells (Behrens *et al.*, 1989). However, repletion of E-cadherin alone did not induce tight junction formation and a well-differentiated phenotype in MSV-MDCK cells (Rajasekaran *et al.*, 1996). Repletion of both E-cadherin and Na,K-ATPase β_1 -subunit induced epithelial polarization, including the formation of tight junctions, and reduced invasiveness and cell motility in MSV-MDCK cells (Rajasekaran *et al.*, 2001b). Based on these results, we proposed that a functional synergism between E-cadherin and Na,K-ATPase β -subunit is involved in the regulation of the well-differentiated phenotype of epithelial cells (Rajasekaran *et al.*, 2001b).

Consistent with the idea of functional synergism between E-cadherin and Na,K-ATPase, the β -subunit promoter also revealed five potential E-boxes (Derfoul *et al.*, 1998). Because Snail is known to bind to E-boxes and suppress E-cadherin expression, we hypothesized that Snail might be involved in the suppression of Na,K-ATPase β_1 -subunit in carcinoma. In this study, we validate this hypothesis by demonstrating an inverse correlation between the levels of Na,K-ATPase β_1 -subunit and Snail in carcinoma cell lines derived from breast, colon, pancreas, and kidney. We also show that Snail binds to the Na,K-ATPase β_1 -subunit promoter and is involved in the down-regulation of the β_1 -subunit in carcinoma cells. These studies strongly indicate that the normal levels of Na,K-ATPase β_1 -subunit and E-cadherin expressed in epithelial cells are important for the maintenance of the well-differentiated phenotype of epithelial cells.

MATERIALS AND METHODS

Cell Lines

MDCK (Clone II, Philadelphia) was provided by Dr. Enrique Rodriguez-Boulan (Cornell University, Ithaca, NY). MCF7, MDA435, Caco2, SW480, HPAF-II, MiaPaCa-2, and MSV-MDCK cells were obtained from American Type Culture Collection (Manassas, VA). MCF7, MDA435, Caco2, SW480, MiaPaCa-2, MDCK, and MSV-MDCK cells were grown in DMEM with 10% fetal bovine serum, nonessential amino acids, 1% penicillin-streptomycin, and glutamine at 37°C in a humidified atmosphere containing 5% CO₂. HPAF-II cells were grown in RPMI with 10% fetal bovine serum, nonessential amino acids, penicillin-streptomycin, and glutamine.

Plasmid Constructs

Construction of the plasmids containing the human Na, K-ATPase β_1 -subunit promoter (H β_1 -1141-Luc) or a truncated piece of the 5' flanking region (H β_1 -456-Luc) fused to the luciferase reporter gene has been described previously (Feng *et al.*, 1993). For transfection efficiency control, *Renilla* plasmid was purchased from Promega (Madison, WI).

A 795-base pair cDNA containing the coding region of human Snail (Snail) was generated by reverse transcription-polymerase chain reaction (RT-PCR) via the Titan RT-PCR kit (Roche Diagnostics, Indianapolis, IN) by using a set of primers containing *Xba*I and *Bam*HI restriction sites. We used total RNA from MiaPaCa-2 cells, which have been shown to overexpress Snail (Battle *et al.*, 2000). The cDNA was subcloned into the expression vector pCDNA3 containing the cytomegalovirus promoter (Invitrogen, Carlsbad, CA). The construct, designated pCDNA3-Snail, was confirmed by sequencing (Laragen, Los Angeles, CA).

pPGS-Snail was constructed as described previously (Hajra *et al.*, 2002). Full-length Snail cDNA was amplified by RT-PCR. Constructs were then subcloned into the pPGS-CMV-CITE-neo retroviral expression vector. Amphotropic retroviral packaging vector was a gift from J. Colicelli (University of California, Los Angeles, Los Angeles, CA).

Antibodies

Mouse monoclonal antibodies raised against Na,K-ATPase α_1 - (M7-PB-E9) and β_1 -subunit (M17-P5-F11) recognize epitopes that are common in human, sheep, and dog and have been characterized and described previously (Abbott and Ball, 1993; Sun and Ball, 1994). Mouse monoclonal antibody against canine E-cadherin (DECMA) was from Sigma-Aldrich (St. Louis, MO) and monoclonal anti-human E-cadherin antibody was from Zymed Laboratories (South San Francisco, CA). Anti-SNAI-1 antibody, a goat polyclonal antibody, was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-linked anti-mouse antibody was purchased from BD Transduction Laboratories (Lexington, KY). Horseradish peroxidase-linked anti-rabbit antibody was from Cell Signaling (Beverly, MA).

Immunoblot Analysis

Monolayers were lysed in a lysis buffer (95 mM NaCl, 25 mM Tris, pH 7.4, 0.5 mM EDTA, 2% SDS, 1 mM phenylmethylsulfonyl fluoride, and 5 μ g/ml each of antipain, leupeptin, and pepstatin). The lysates were briefly sonicated and centrifuged at 14,000 rpm in a microcentrifuge for 10 min. The supernatants were used for further analysis. Protein concentrations of the cell lysates were determined with the use of Bio-Rad DC reagent (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Equal amounts of protein (200 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were blocked with 10% nonfat dry milk in phosphate-buffered saline (PBS) and then incubated for 2 h at room temperature with primary antibody diluted in 10% milk/PBS. After incubation, the blots were washed three times with PBS/0.3% Tween 20 and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:4000 in 10% milk). Bound antibody was detected by peroxidase-catalyzed enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA).

Rubidium Transport Assay

Ouabain-sensitive ion transport was determined via $^{86}\text{Rb}^+$ uptake as described previously (Lambrecht *et al.*, 1998; Rajasekaran *et al.*, 2001b). Cells were washed once with ice-cold wash buffer (144 mM NaCl, 10 mM HEPES, pH 7.4, 0.5 mM CaCl₂), incubated for 10 min at 37°C with uptake buffer (144 mM NaCl, 10 mM HEPES, pH 7.4, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mM RbCl, 1 mg/ml glucose, 1 μ Ci $^{86}\text{Rb}^+$, PerkinElmer Life Sciences), and then washed three times with wash buffer. To determine the ouabain-sensitive $^{86}\text{Rb}^+$ transport, the cells were treated with 50 μ M ouabain (Sigma-Aldrich) for 30 min at 37°C before the first wash. Cells were lysed with 500 μ l of 0.5 N NaOH for 1 h at room temperature and $^{86}\text{Rb}^+$ by using a scintillation counter. Counts were normalized to protein content, and the ouabain-sensitive $^{86}\text{Rb}^+$ flux was determined. The data are presented as mean $\pm$ SE.

Northern Blot Analysis

Total RNA was prepared using TRIzol reagent according to the manufacturer's instructions (Ambion, Austin, TX), transferred to nitrocellulose membranes (Schleicher & Schuell), and probed for Na,K-ATPase α_1 - and β_1 -subunits, E-cadherin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using the PrimeIt labeling kit (Stratagene, San Diego, CA).

RT-PCR Analysis

RT-PCR was performed using the Titan RT-PCR system (Roche Diagnostics). Two micrograms of total RNA were used for each reaction. The sequences for the primers (Invitrogen) are the following: human Snail (60°C annealing): forward, 5' AATCGGAAGCCTAACTACAG 3'; reverse, 5' GGAAGAGGCTGAAGTAGAG 3'; canine Snail (60°C annealing): forward, 5' CCCAAGCCAGCCGATGAG 3'; reverse, 5' CTTGGCCACGGAGAGCCC 3'; GAPDH (60°C annealing): forward, 5' GTGAAGGTCGGAGTCAACGG 3'; reverse, 5' TGATGACAAGCTTCCCGTTCTC 3'; human E-cadherin (60°C annealing): forward, 5' TTCCTCCCAATACATCTCCCTTACAGCAG 3'; reverse, 5' CGAAGAAACAGCAAGAGCAGCAGAAATCAGA 3'; human Na,K-ATPase β_1 -subunit (60°C annealing): forward, 5' ACTGAAATTCCTTCGTCCTAA 3'; reverse, 5' ATCACTGGGTAAGTCTCCA 3'; human Na,K-ATPase α_1 -subunit (57°C annealing): forward, 5' GACGTGATAAGTATGAGCCTG 3'; reverse, 5' AATCCCGGCTCAAGTCTGT 3'. RT-PCR products were analyzed by electrophoresis in 1.0% agarose.

Retroviral Transduction

Snail-expressing retroviruses were generated by transfecting plasmids (15 μ g of pPGS-Snail or pPGS) and 15 μ g of amphotropic retroviral packaging vector into human embryonic kidney 293T packaging cells by using the calcium phosphate method. After overnight transfection (14 h) into the packaging line, the medium was changed and virus containing supernatant was harvested 24, 36, 43, and 48 h later. The supernatant was filtered and then supplemented with 8 μ g/ml Polybrene (Sigma-Aldrich) and used to infect MDCK and MCF7 cells. After infection (48 h), selection was initiated in 0.5 mg/ml G418 (Invitrogen). The G418-resistant clones were pooled and used for experiments.

Small Interfering RNA (siRNA)-mediated RNA Interference

The Snail siRNA duplex was obtained from Dharmacon (Lafayette, CO). The target sequences used are the following: Snail (5'-GCGAGCUGCAGGACUCUAA-3') and green fluorescent protein (GFP) (5'-GGCTACGTCAGGAGCGCACC-3'). Snail siRNA was transfected into poorly differentiated SW480 cells by using Oligofectamine following manufacturer's instructions (Invitrogen). Cell density at 60% confluence was used at the time of transfection. At 96 h posttransfection, total RNA was prepared using TRIzol reagent according to the manufacturer's instructions (Ambion).

Reporter Gene Assays

Transient transfections were performed in six-well plates by using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. The following constructs were used: 10 μ g of luciferase reporter plasmid and 0.1 μ g of *Renilla* control reporter plasmid. Transfections were performed in triplicate. Lysates were made 72 h posttransfection by using 1 $\times$ Passive Lysis buffer (Promega) according to manufacturer's instructions. Lysates were then used for luciferase assays. A luminometer was utilized to measure both β_1 -subunit promoter-luciferase reporter units and *Renilla* luciferase units. Luciferase values were normalized to *Renilla* reporter activity. Statistical significance was determined by Student's *t* test or one-way analysis of variance (Prism; GraphPad Software, San Diego, CA).

Electrophoretic Mobility Shift Assay (EMSA)

Preparation of nuclear extracts and EMSA experiments were performed as described previously (Batlle *et al.*, 2000) with minor modifications. Cells were lysed in 0.5% NP-40 and centrifuged to pellet the nuclei. Nuclear proteins were then extracted in 20 mM HEPES, pH 7.6, 25% glycerol, 840 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and a standard protease inhibitor cocktail. The double-stranded oligonucleotide used as a probe for EMSA experiments corresponds to the following sequence in the Na,K-ATPase β_1 -subunit promoter: E-box1wt (position -81 to -52), 5' GGCGAT-TGGC - CACCGG - GCCGCTAGAGGGCG 3'. 5' CACCGG 3' nucleotides were changed in the mutated version (5' AAATTT 3') used in the competition experiments. Probes were labeled with [γ -³²P]ATP and polynucleotide kinase and then purified using a spin column (Bio-Rad). Reactions with equal amounts of nuclear extracts (10 μ g/reaction) were performed in a 20- μ l final volume containing binding buffer (20 mM HEPES, pH 7.6, 150 mM KCl, 3 mM MgCl₂, 10% glycerol, 0.2 mM ZnSO₄, 0.3 mg/ml bovine serum albumin), 50,000 cpm probe, and 1 μ g of poly(dI:dC) for 30 min at 4°C. For competition assays, 200-fold unlabeled oligonucleotides or 8 μ g of anti-SNAI-1 antibody (Santa Cruz Biotechnology), respectively, was preincubated with cell extracts at 4°C for 30 min before addition of probe. Complexes were resolved on 4% nondenaturing polyacrylamide gels in Tris borate-EDTA buffer.

RESULTS

Decreased Na,K-ATPase β_1 -Subunit Levels in Poorly Differentiated Carcinoma Cell Lines

We have shown previously that the protein levels of the Na,K-ATPase β_1 -subunit were highly reduced in an invasive form of renal carcinoma (Rajasekaran *et al.*, 1999). Subsequently, we showed that the β_1 -subunit protein levels were also reduced in MSV-MDCK cells (Rajasekaran *et al.*, 2001b). To test whether reduced β_1 -subunit expression is also observed in other carcinomas, we determined the levels of Na,K-ATPase β_1 -subunit in various well-differentiated and poorly differentiated cell lines derived from tissues such as breast (MCF7 and MDA435), colon (Caco2 and SW480), pancreas (HPAF-II and MiaPaCa-2), and kidney (MDCK and MSV-MDCK). Well-differentiated cell lines maintain an epithelial morphology and in most cases have tight junctions and show a polarized phenotype. Poorly differentiated cell lines show a fibroblastic phenotype, lack tight junctions and epithelial polarity, and in general are more motile than the well-differentiated cell lines. We compared the protein levels of E-cadherin, Na,K-ATPase β_1 - and α_1 -subunits in well-differentiated cell lines (MCF7, Caco2, HPAF-II, and MDCK) and poorly differentiated cell lines (MDA435, SW480, MiaPaCa-2, and MSV-MDCK) (Figure 1A). Poorly differentiated carcinoma cell lines showed reduced expression of E-cadherin compared with well-differentiated cell lines. Like E-cadherin, the Na,K-ATPase β_1 -subunit protein levels were drastically reduced in all poorly differentiated cell lines compared with well-differentiated cell lines. The reduced β_1 -subunit levels detected in the immunoblot is not due to the failure of the antibody to detect differentially glycosylated β_1 -subunit expressed in these cell lines because cell lysates subjected to *N*-glycosidase treatment also revealed a reduction in β_1 -subunit levels (our unpublished data). The Na,K-ATPase α_1 -subunit levels were similar in poorly differentiated breast and colon carcinoma cell lines and showed some decrease in poorly differentiated pancreatic and kidney carcinoma cell lines. We then tested using ⁸⁶Rb⁺ flux whether reduced β -subunit levels in poorly differentiated cell lines was accompanied by reduced Na,K-ATPase activity. The ouabain sensitive ⁸⁶Rb⁺ flux was 70.6 $\pm$ 5.3, 67.2 $\pm$ 1.2, 89.4 $\pm$ 10, and 60.7 $\pm$ 4.7% for MDA435, SW480, MiaPaCa-2, and MSV-MDCK cells, respectively, compared with their well-differentiated counterparts (Figure 1B). Interestingly, the MiaPaCa-2 cell line, which showed reduced levels of α_1 -subunit, did not show a significant decrease in the Na,K-ATPase activity. Whether this cell line express another isoform of α -subunit is currently not known. These results indicate that a drastic reduction in β_1 -subunit levels is not accompanied by a substantial decrease in the activity of the enzyme.

We then determined whether the decreased protein levels of the Na,K-ATPase β_1 -subunit are associated with decreased mRNA levels by a Northern blot analysis (Figure 2A). β_1 -subunit mRNA levels were drastically reduced in poorly differentiated carcinoma cell lines compared with well-differentiated cell lines, suggesting that the expression of the Na,K-ATPase β_1 -subunit is reduced at the transcriptional level in poorly differentiated carcinoma cell lines. The α_1 -subunit mRNA levels were similar in breast and colon carcinoma cell lines, whereas the levels were reduced in poorly differentiated pancreas and kidney carcinoma cell lines. Reduced E-cadherin expression in poorly differentiated carcinoma cell lines was previously found to be inversely correlated to the level of expression of the transcription factor Snail (Batlle *et al.*, 2000; Poser *et al.*, 2001;

Figure 1. Correlation between E-cadherin, Na,K-ATPase α_1 -subunit, β_1 -subunit protein levels, and Na,K-ATPase enzyme activity. (A) E-cadherin, Na,K-ATPase β_1 -subunit, and α_1 -subunit protein levels were analyzed by an immunoblot in a panel of epithelial cell lines: breast, MCF7, and MDA435; colon, Caco2, and SW480; pancreas, HPAF-II and MiaPaCa-2; and canine kidney, MDCK, and MSV-transformed MDCK. Actin immunoblot analysis confirmed equal loading of whole cell lysates. MCF7, Caco2, HPAF-II, and MDCK are well-differentiated whereas MDA435, SW480, MiaPaCa-2, and MSV-MDCK are poorly differentiated cell lines. (B) Ouabain-sensitive $^{86}\text{Rb}^+$ flux in well- and poorly differentiated cell lines. The $^{86}\text{Rb}^+$ uptake assay was performed as described in MATERIALS AND METHODS. Bars show SE of two independent determinations performed in triplicates.

Yokoyama *et al.*, 2001; Jiao *et al.*, 2002). We therefore determined the levels of Snail mRNA in well-differentiated and poorly differentiated carcinoma cell lines by RT-PCR analysis. Snail expression was elevated in all poorly differentiated carcinoma cell lines compared with the well-differentiated cell lines (Figure 2B). These results demonstrate that Na,K-ATPase β_1 -subunit expression is inversely correlated to the expression of Snail, suggesting that Snail might be involved in the down-regulation of Na,K-ATPase β_1 -subunit in carcinoma cell lines.

Snail Expression Modulates Na,K-ATPase β_1 -Subunit Levels

To test whether increased Snail expression leads to reduced Na,K-ATPase β_1 -subunit levels, we exogenously overexpressed Snail in two well-differentiated cell lines, MCF7 and MDCK. Both cell lines were transduced with an amphotropic retrovirus harboring human Snail cDNA and neomycin-resistant clones were pooled and used for the experiments. RT-PCR analysis confirmed that human Snail is

Figure 2. Reduced Na,K-ATPase β_1 -subunit mRNA levels in poorly differentiated cell lines. (A) Northern blot analysis of E-cadherin, Na,K-ATPase β_1 -subunit, and α_1 -subunit mRNA levels in well- and poorly differentiated cell lines. GAPDH Northern blot analysis confirmed equal loading of total RNA. (B) Levels of Snail mRNA expression in well- and poorly differentiated cell lines were analyzed by RT-PCR. The expression of GAPDH was analyzed in the samples as a control for the amount of cDNA present in each sample.

overexpressed in MCF7-Snail and MDCK-Snail cell lines compared with vector-transduced cell lines (Figure 3D). Vector-transduced MCF7 and MDCK cells showed a typical cobblestone-like appearance (Figure 3A). In contrast, Snail-transduced cells were less adherent to each other and were more elongated and displayed a mesenchymal phenotype (Figure 3A). Immunoblot analysis revealed highly reduced levels of E-cadherin and Na,K-ATPase β_1 -subunit in both Snail-overexpressing MCF7 and MDCK cells. Although some E-cadherin was observed in MCF7-Snail cells, Na,K-ATPase β_1 -subunit expression was barely detected (Figure 3B). Snail overexpression did not affect the α_1 -subunit levels in either cell lines (Figure 3B). Consistent with the α_1 -subunit levels, the $^{86}\text{Rb}^+$ flux in Snail-overexpressing cells did not show a significant difference compared with control cells (Figure 3C). These results strongly suggest that Snail is involved in the down-regulation of Na,K-ATPase β_1 -subunit but not the α_1 -subunit of the enzyme.

RNA interference is a mechanism of gene silencing that is mediated by short strands of duplex RNA (called siRNA) that target the corresponding mRNA for degradation (Zamore, 2001). To further confirm that Snail is involved in the suppression of Na,K-ATPase β_1 -subunit, we used RNA interference (RNAi) to reduce Snail expression in a poorly differentiated cell line (SW480), which expresses high levels of endogenous Snail. Transfection with Snail siRNA resulted

Figure 3. Snail expression modulates Na,K-ATPase β_1 -subunit levels. (A) Phase contrast image of MCF7 and MDCK cells constitutively expressing pPGS empty vector or pPGS-Snail. (B) Immunoblot analysis measured E-cadherin, Na,K-ATPase β_1 - and α_1 -subunit protein levels in MCF7 and MDCK cells constitutively expressing pPGS empty vector or pPGS-Snail. Actin immunoblot shows equal loading of whole cell lysates. (C) Ouabain-sensitive $^{86}\text{Rb}^+$ flux in MCF7 and MDCK cells expressing pPGS vector or pPGS-Snail. The $^{86}\text{Rb}^+$ uptake assay was performed as described in MATERIALS AND METHODS. Bars show SE of two independent determinations performed in triplicate. (D) RT-PCR analysis results show that with the exception of Na,K-ATPase α_1 -subunit, E-cadherin and Na,K-ATPase β_1 -subunit protein levels inversely correlated with Snail expression. GAPDH RT-PCR analysis was performed as a control for the amount of cDNA present in each sample. (E) RNA interference-mediated reduction of Snail increases Na,K-ATPase β_1 -subunit mRNA levels in SW480 cells. RNAi was performed in SW480 cells by using siRNAs for GFP (control) and Snail (refer to MATERIALS AND METHODS). After transfection, total RNA was extracted, and RT-PCR was performed using primer pairs specific for either Snail, β_1 -subunit, α_1 -subunit, E-cadherin, or GAPDH. RT-PCR products were analyzed on a 1% agarose gel.

in a 32.7% reduction of Snail mRNA levels compared with SW480 cells transfected with GFP siRNA control (Figure 3E). Concomitantly, Snail down-regulation also lead to 30.0 and 28.2% increases in the levels of β_1 -subunit and E-cadherin mRNAs, respectively (Figure 3E). The levels of α_1 -subunit mRNA, however, were similar between GFP siRNA-transfected and Snail siRNA-transfected SW480 cells. These results demonstrate that Snail is involved in the modulation of Na,K-ATPase β_1 -subunit levels but not the α_1 -subunit levels in epithelial cell lines.

Reduced β_1 -Subunit Promoter Activity in Cell Lines with Increased Snail Expression

To test whether Snail transcriptionally suppresses Na,K-ATPase β_1 -subunit expression, we transiently transfected well-differentiated (MCF7, Caco2, and MDCK) and poorly differentiated (MDA435, SW480, and MSV-MDCK) carcinoma cell lines with a luciferase reporter construct under the control of the human β_1 -subunit promoter (H β_1 -1141-Luc). This fragment consists of half-sites of three potential mineralocorticoid/glucocorticoid responsive elements (MRE/GREs) (Derfoul *et al.*, 1998), four E-boxes, a nuclear factor-1 binding site (Derfoul *et al.*, 1998), and a noncanonical E-box (5' CACCGG 3') (Figure 4A). The β_1 -subunit promoter activity in MDA435 was reduced to 67.4% compared with MCF7. β_1 -subunit promoter activities for SW480 and MSV-MDCK were 43.5 and 38.5% compared with Caco2 and MDCK cells, respectively (Figure 4B). We then tested H β_1 -456-Luc, which contains one MRE/GRE and the noncanonical E-box element (Figure 4C). This fragment revealed similar results as the H β_1 -1141-Luc. The MDA435 cell line showed a reduction to 58.3% β_1 -subunit promoter activity compared with MCF7 cells. The promoter activities were 24.7 and 37.2% in SW480 and MSV-MDCK cells compared with Caco2 and MDCK cells, respectively (Figure 4D). Because the H β_1 -456-Luc construct yielded similar results as that of the H β_1 -1141-Luc construct, we used the H β_1 -456-Luc construct for further analysis.

Snail Represses the Activity of the β_1 -Subunit Promoter

To directly test whether Snail represses the activity of the β_1 -subunit promoter, we transiently cotransfected pCDNA3-Snail or pCDNA3 vector with the H β_1 -456-Luc promoter construct in MCF7, Caco2, and MDCK cells. Cotransfection with the vector did not affect the promoter activity, whereas pCDNA3-Snail showed a dose-dependent repression of the β_1 -subunit promoter activity (Figure 5A). In MCF7 cells, cotransfection with 1 μ g of pCDNA3-Snail reduced the promoter activity to 83.0%, whereas in Caco2 and MDCK cells, the β_1 -subunit promoter activities were reduced to 34.1 and 21.9%, respectively. Transfection with 2 μ g of pCDNA3-Snail revealed a reduction to 30.2, 15.9, and 4.8% β_1 -subunit promoter activity in MCF7, Caco2, and MDCK cells, respectively. Finally, transfection of varying concentrations of pCDNA3-Snail in COS cells revealed a dose-dependent repression of β_1 -subunit promoter activity (Figure 5B). These data demonstrate that Snail has an inhibitory effect on the Na,K-ATPase β_1 -subunit promoter *in vitro* and that transcription of the β_1 -subunit is directly controlled by Snail regulating the activity of its promoter.

Snail Binds to an E-box in the Na,K-ATPase β_1 -Subunit Proximal Promoter

We then examined whether Snail binds to the Na,K-ATPase β_1 -subunit promoter by using EMSA. Nuclear lysates from Snail-overexpressing well-differentiated cell lines (MDCK-Snail and MCF7-Snail) and two poorly differentiated cell

lines (MiaPaCa-2 and MDA435) were used to test Snail binding to the β_1 -subunit promoter. Because Snail is known to bind E-boxes (Mauhin *et al.*, 1993; Nakayama *et al.*, 1998) and because a putative noncanonical E-box was present at position -71 to -66 of the β_1 -subunit promoter, we generated an oligonucleotide probe (E-box1wt), encompassing the putative noncanonical E-box (position -81 to -52) for the EMSA (Figure 6A). As control, we also generated a probe in which the E-box is mutated into "AAATTT" (E-box1mut) (Figure 6A). Inclusion of nuclear lysates clearly showed a band (arrow, lanes 2, 6, 11, and 15) (Figure 6B) having retarded electrophoretic mobility compared with the labeled oligonucleotide without nuclear lysates (probe alone, lane 1). Furthermore, the shifted band seen when E-box probe was incubated with nuclear lysates was specifically competed out with excess unlabeled probe (lanes 3, 7, 12, and 16). However, a mutant probe where the E-box was altered showed no competition (lanes 4, 8, 13, and 17). The only difference between the cold wild-type and mutant probes is in the E-box element. Finally, inclusion of an antibody against Snail in the EMSA reaction abolished the detection of the shifted band (lanes 5, 9, 14, and 18), whereas the control antibody did not show any effect (lane 10), indicating that the shifted band contains Snail bound to the β_1 -subunit promoter. Together, these results demonstrate that the transcription factor Snail binds to the noncanonical E-box element of the β_1 -subunit promoter and is involved in the down-regulation of the Na,K-ATPase β_1 -subunit.

DISCUSSION

In this study, we demonstrated that Na,K-ATPase β_1 -subunit levels are reduced in a variety of cell lines derived from carcinoma and that the transcription factor Snail is involved in the repression of Na,K-ATPase β_1 -subunit in carcinoma cells. Loss of the well-differentiated phenotype with concomitantly reduced β_1 -subunit and E-cadherin levels in Snail-overexpressing MDCK and MCF7 cells demonstrate that normal levels of E-cadherin and Na,K-ATPase β_1 -subunit are necessary to maintain the well-differentiated phenotype of epithelial cells. Consistent with this observation, we have shown previously that repletion of Na,K-ATPase β_1 -subunit and E-cadherin was sufficient to induce a well-differentiated phenotype in MSV-MDCK cells (Rajasekaran *et al.*, 2001b). These results demonstrate that a functional synergism between E-cadherin and Na,K-ATPase is involved in the maintenance of the well-differentiated phenotype of epithelial cells.

Although the Na,K-ATPase α_1 - and β_1 -subunits are expressed together at the cell surface, our results suggest that the genes encoding these proteins are differentially regulated in carcinoma. Highly reduced β_1 -subunit but not α_1 -subunit levels in Snail-overexpressing MDCK and MCF7 cells and up-regulation of β_1 -subunit levels in SW480 cells transfected with Snail siRNA demonstrate that Snail specifically down-regulates the Na,K-ATPase β_1 -subunit. In addition, interaction of Snail to the 30 nucleotide promoter fragment containing the noncanonical E-box element and competition of Snail binding by the cold wild-type probe but not the cold mutant probe demonstrate that Snail binds to the noncanonical E-box element "CACCGG" located at position -71 to -66 of the β_1 -subunit promoter. Noncanonical E-boxes have been discovered in a number of genes. The basic helix-loop-helix protein sisterlessB in *Drosophila* binds to the noncanonical E-box CA(G/C)CCG (Yang *et al.*, 2001), whereas the upstream stimulatory factor-1 interacts with CACGGG in the insulin-like growth factor binding protein-1

Figure 4. Reduced Na,K-ATPase β_1 -subunit promoter activity in poorly differentiated cells. (A) Schematic representation of Na,K-ATPase β_1 -subunit proximal promoter elements. Luciferase reporter used is under the control of the human β_1 -subunit promoter (H β_1 -1141-Luc). This fragment consists of half-sites of three potential MRE/GREs, four E-boxes, a nuclear factor-1 (NF-1) binding site, and a noncanonical E-box (5' CACCGG 3'). +1 indicates the transcription start site. (B) Comparison of the β_1 -subunit promoter activity in well-differentiated and poorly differentiated cell lines by using the H β_1 -1141-Luc construct. MCF7, MDA435, Caco2, SW480, MDCK, and MSV-MDCK cells were transiently transfected with 10 μ g of H β_1 -1141-Luc (full-length) reporter plasmid. Promoter activity was determined by luciferase reporter assays. Luciferase values were normalized to *Renilla* reporter activity. The results shown correspond to the average of three independent experiments. (C) Schematic representation of H β_1 -456-Luc (truncated) containing one MRE/GRE and the noncanonical E-box. (D) Comparison of the β_1 -subunit promoter activity in well-differentiated and poorly differentiated cell lines by using the H β_1 -456-Luc construct. MCF7, MDA435, Caco2, SW480, MDCK, and MSV-MDCK cells were transiently transfected with 10 μ g of H β_1 -456-Luc reporter plasmid. Promoter activity was determined by luciferase reporter assays, and luciferase values were normalized to *Renilla* reporter activity. Results shown correspond to the average of three independent experiments.

promoter (Matsukawa *et al.*, 2001). The sequence in the β_1 -subunit promoter has similarity to the CANNNG sequence found in insulin-like growth factor binding protein-1 promoter; both contain a "G" at position 5 instead of a "T." Our results strongly suggest that Snail binding to the non-

canonical E-box element is sufficient to reduce activity of the β_1 -subunit promoter. Whether the upstream E-box elements in the β_1 -subunit are also involved in Snail binding and down-regulation of the β_1 -subunit in carcinoma remains to be elucidated.

Figure 5. Increasing Snail expression reduces Na,K-ATPase β_1 -subunit promoter activity in well-differentiated cell lines (MCF7, Caco2, and MDCK) and COS cells. (A) MCF7, Caco2, and MDCK cell lines were transfected with pCDNA3 vector containing human Snail cDNA or with empty pCDNA3. One or two micrograms of pCDNA3-Snail were used in the transient transfection. The H β 1-456-luc reporter plasmid was used in the luciferase reporter assays. Luciferase values were normalized to *Renilla* reporter activity. The results shown correspond to the average of three independent experiments. (B) Increasing Snail expression reduces Na,K-ATPase β_1 -subunit promoter activity in COS cells in a dose-dependent manner. COS cells were transfected with pCDNA3-Snail or with empty pCDNA3 plasmid. Increasing amounts of pCDNA3-Snail were used in the transient transfection. The H β 1-456-luc plasmid was used in the luciferase reporter assays. Luciferase values were normalized to *Renilla* reporter activity. The results shown correspond to the average of three independent experiments.

Although the β_1 -subunit levels were drastically reduced in poorly differentiated and Snail-overexpressing cell lines, we did not observe a substantial decrease in the activity of

the Na,K-ATPase, indicating that high levels of β_1 -subunit are not critical for pump activity. Several studies have shown that the β_1 -subunit plays a role in the stability and the transport of α_1 -subunit of Na,K-ATPase to the plasma membrane (Geering, 1990; McDonough *et al.*, 1990; Noguchi *et al.*, 1990a,b; Chow and Forte, 1995). Our results, showing normal α_1 -subunit levels but highly reduced β_1 -subunit levels in Snail-overexpressing carcinoma cell lines, imply that carcinoma cells have either adapted alternative mechanism/s to stabilize and transport α_1 -subunit to the plasma membrane or a small amount of β_1 -subunit might be sufficient to stabilize and to transport the α -subunit to the plasma membrane. It is tempting to speculate that the β_1 -subunit might have functions that are independent from its role in the regulation of Na,K-ATPase enzyme activity in epithelial cells.

How does Snail act to repress Na,K-ATPase β_1 -subunit expression? Na,K-ATPase β_1 -subunit expression is up-regulated by glucocorticoids (Taormino and Fambrough, 1990; Liu and Glick, 1992; Derfoul *et al.*, 1998; Devarajan and Benz, 2000). Functional GRE and MRE responsive elements in the β_1 -subunit promoter have been characterized (Derfoul *et al.*, 1998). Although glucocorticoids can up-regulate expression of the β_1 -subunit in a tissue-specific manner (Derfoul *et al.*, 1998), whether these factors are necessary to maintain the expression of β_1 -subunit in epithelial cells is not known. However, reduced β_1 -subunit levels and reduced promoter activity in various Snail-overexpressing carcinoma cell lines suggest that Snail is involved in the down-regulation of β_1 -subunit in a variety of carcinoma. Quenching is a form of gene regulation in which repressors and activators cooccupy flanking regions in the promoter with the repressor either preventing the activator from interacting with the transcription factor complex or masking the latter's activation surfaces (Hemavathy *et al.*, 2000). It is possible that Snail could prevent factors necessary for the transcription of β_1 -subunit such as the mineralocorticoid and glucocorticoid receptors and their coactivators from binding to the promoter and hindering transactivation of the β_1 -subunit. Moreover, Snail may reduce Na,K-ATPase β_1 -subunit promoter activity by inhibiting activators that interact with the GC-rich proximal region of the CCAAT box. Future studies are necessary to understand the mechanism of β_1 -subunit transcriptional regulation in normal epithelial cells and its repression in carcinoma. Although our studies demonstrate that the β_1 -subunit expression is suppressed by Snail, whether Snail is also involved in the regulation of the β_2 and β_3 isoforms remains to be investigated.

Reduced Na,K-ATPase β_1 -subunit expression in poorly differentiated carcinoma cell lines and its down-regulation by Snail is consistent with a role for the Na,K-ATPase in the conversion of the epithelial cells to mesenchymal cells that occur during epithelial to mesenchymal transition (EMT). EMT is a physiological process that occurs during embryonic development, and this process has been found to be involved in cancer development as well (Savagner, 2001). During both of these processes, epithelial cells dissociate and migrate to reach different locations due to their increased cell motility. Down-regulation of E-cadherin by Snail facilitates increased motility of cells undergoing EMT. We have shown that β_1 -subunit expression alone in MSV-MDCK cells significantly reduced their motility and when coexpressed with E-cadherin, the motility was even further reduced. Moreover, Na,K-ATPase β_1 -subunit suppresses cell motility in a Rac1-dependent manner in MSV-MDCK cells (Barwe, Anilkumar, Moon, Zheng, Rajasekaran and Rajasekaran, unpublished data). These results demonstrated

Figure 6. Endogenous Snail from multiple cell lines binds to the noncanonical E-box element of the Na,K-ATPase β_1 -subunit promoter. (A) Noncanonical E-box is present at position -71 to -66 of the β_1 -subunit promoter. An oligonucleotide probe, called E-box1wt, was generated, which encompasses the noncanonical E-box (position -81 to -52) for the EMSA. As a control, we also generated a probe in which E-box1 is mutated into "AAATTT" (E-box1 mut). (B) Nuclear extracts from MDCK-Snail, MCF7-Snail, MiaPaCa-2, and MDA435 cells were incubated with double stranded 32 P-labeled oligonucleotides containing the 5' CACCGG 3' sequence corresponding to E-box1 of the Na,K-ATPase β_1 -subunit promoter. For competition assays, a 200-fold molar excess of unlabeled E-box1 wt (wt; lanes 3, 7, 12, and 16), E-box1 mut (mut; lanes 4, 8, 13, and 17) oligonucleotides, 8 μ g of anti-SNAI-1 antibody (Snail; lanes 5, 9, 14, and 18) or a nonspecific antibody (Cont; lane 10) was added before addition of probe to the binding reactions. Snail-containing complexes were detected in all cell lines (arrow). Addition of anti-SNAI-1 antibody or cold wild-type probe competed Snail binding to E-box1.

that the β_1 -subunit of Na,K-ATPase is also involved in the suppression of cell motility in epithelial cells (Rajasekaran *et al.*, 2001b). Coordinate down-regulation of Na,K-ATPase β_1 -subunit and E-cadherin by Snail in carcinoma cells is consistent with the hypothesis that the reduced expression of both these proteins might be involved in increased motility of cells undergoing EMT. Experiments are in progress in our laboratory to further understand the role of Na,K-ATPase in EMT.

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