Carbonic Anhydrase III Promotes Transformation and Invasion Capability in Hepatoma Cells Through FAK Signaling Pathway

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Carbonic anhydrase III (CAIII) is distinguished from the other members of the CA family by low carbon dioxide hydratase activity, resistance to the CA inhibitor acetazolamide, and a predominant expression in the liver of males. In this report the effects of CAIII expression on liver cancer cells invasiveness were explored. Overexpression of CAIII in the HCC cell line SK-Hep1 resulted in increased anchorage-independent growth and invasiveness. And siRNA-mediated silencing of CAIII expression decreased the invasive ability of SK-Hep1 cells. Furthermore, CAIII transfectants showed elevated focal adhesion kinase (FAK) and Src activity. Silencing of FAK expression in CAIII transfectants led to suppression of HCC cell invasion. More importantly, the CAIII transfectants acidified the culture medium at an accelerated speed than the control cells did. Taken together, these data suggest that the CAIII-promoted invasive ability of HCC cells may probably be mediated through, at least in part, the FAK signaling pathway via intracellular and/or extracellular acidification.

Key words: carbonic anhydrase; anchorage-independent growth; AIG; invasion; metastasis

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancies in the world, particularly in Asia and Africa where the population shows a high prevalence of hepatitis B and C infection [1,2]. HCC has a poor prognosis mainly because of its high propensity for intrahepatic metastasis and recurrence. Several risk factors have been correlated with the development of HCC in humans such as hepatitis B and C infection, alcoholism, and prolonged exposure to aflatoxin B1 [2,3]. Numerous genetic alterations are accumulated during the process of hepatocarcinogenesis. However, the molecular mechanisms of the development and progression of HCC remain largely unclear.

Carbonic anhydrase (CA) is a class of zinc metalloenzymes that catalyze the hydration of CO_2 to generate proton and bicarbonate ion for cellular ion transport and pH homeostasis [4–6]. At least 15 distinct isoforms have been identified in mammals, 12 of which are catalytically active. The CA isozymes differ in their catalytic activity, sensitivity to inhibitors, tissue distribution, and cellular and subcellular localization, and perform a variety of biological functions. Carbonic anhydrase III (CAIII) is an abundant cytoplasmic protein found in skeletal muscle, liver, and adipocytes [7,8]. It is distinguished from other members of the CA family by characteristics such as very low carbon dioxide hydratase activity, resistance to sulfonamide inhibitors, and resistance to oxidant-mediated S-glutathiolation [9–12]. Intriguingly, recently published studies on $Car3^{-/-}$ knockout mice showed no apparent morphological or functional abnormalities [13]. In addition, CAIII has been shown to play an antioxidant role in skeleton muscle injury [14] and to protect cells from hydrogen peroxide-induced apoptosis [15]. Meanwhile, recent observations indicate that inhibition of apoptosis is intrinsically associated

This article contains supplementary material, which may be viewed at the Molecular Carcinogenesis website at http://www.interscience.wiley.com/jpages/0899-1987/suppmat/index.html.

Abbreviations: HCC, hepatocellular carcinoma; CA, carbonic anhydrase; CAIII, carbonic anhydrase III; FAK, focal adhesion kinase; DMEM, Dulbecco's modified Eagles's medium; AIG, anchorage-independent growth; pHe, extracellular pH; EMT, epithelial-mesenchymal transition; pHi, intracellular pH.

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Received 18 January 2008; Revised 13 March 2008; Accepted 24 March 2008

DOI 10.1002/mc.20448

Published online 28 April 2008 in Wiley InterScience (www.interscience.wiley.com)

with cancer metastasis [16]. Several known apoptotic and anti-apoptotic proteins have been shown to influence both anoikis/amorphosis and metastasis. Resistance to anoikis and amorphosis is a crucial event during metastatic spread. Moreover, proteins involved in integrin-mediated signaling, like focal adhesion kinase (FAK), are involved in metastasis. Activation of FAK is sufficient for epithelial cell survival in the absence of contact with the extracellular matrix (ECM) [17]. A large number of reports have described that many primary and metastastic human tumors have an increased FAK expression and activity [18–20]. In HCC, FAK has previously been shown to promote tumor progression, especially vascular invasion [21,22]. In addition, FAK is crucial for regulation of endothelial cell morphology, for example, organization of actin cytoskeleton [23].

Recently it has been demonstrated that expression of CAIII is significantly reduced in HCC and lung adenocarcinoma [24–26], but the role of CAIII in cancers has not been well characterized. In this study, we used ectopic overexpression and siRNA knockdown strategy to investigate whether CAIII would promote invasion capability of HCC cells and the possible molecular mechanisms involved.

MATERIALS AND METHODS

Cell Lines and Constructs

The HCC cell lines HA22T, Hep3B, Huh7, PLC/PRC5, Tong/HCC, Mahlavu, and SK-Hep1, and the hepatoblastoma cell line HepG2, were generously provided by Dr. Yuh-Shan Jou of the Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan. The cell lines were cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% FBS (Gibco BRL, Rockville, MD) in a 37°C humidified incubator containing 5% CO_2. SK-Hep1 cells overexpressing CAIII were obtained by stable transfection with the expression vector using calcium phosphate precipitation (Invitrogen, Carlsbad, CA). After 48 h of incubation, the cells were selected with G418 (0.8 mg/mL; Calbiochem, San Diego, CA). Independent transfectants were cloned and maintained in medium containing 0.5 mg/mL G418 and screened for the expression of CAIII protein by Western blot analysis. SK-Hep1 cells were also transfected with pcDNA3.1-TOPO vector and similarly selected for G418 resistance to serve as a control.

Gene Knockdown

Cells were treated with either 50 nM CAIII SMARTpool siRNA, FAK SMARTpool siRNA or control siRNA (Dharmacon, Lafeyette, CO). Invasion assay was performed using BioCoat Matrigel invasion chambers 48 h after transfection. All experiments were repeated at least three times.

Antibodies and Reagents

The recombinant CAIII protein (RA-4010) was purchased from Spectral Diagnostics (Toronto, Ontario, Canada). Antibodies used in this study include those to CAIII (PA-4010, polyclonal, Spectral Diagnostics), β-actin and α-tubulin (Sigma–Aldrich, St. Louis, MO), Cdc42, FAK, Src and vimentin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Rac1 (Chemicon International, Inc., Temecula, CA), pTyr-397 motif of FAK (Biosource International, Inc., Camarillo, CA), pTyr416 of Src (Upstate Biotechnology, Lake Placid, NY), and pSer-71 of Rac1 (Santa Cruz Biotechnology, Inc.). Western blot analysis was performed according to the protocol provided by the manufacturer.

Anchorage-Independent Growth (AIG) Assay

Low-melting-point agarose (0.5%) in DMEM (supplemented with 10% FBS) was poured into 60 mm culture dishes (3 mL/dish) and allowed to solidify at 4°C to form a bottom layer. Low-melting-point agarose (0.3%; 3 mL/dish) in DMEM (supplemented with 10% FBS), pre-warmed to 37°C, was mixed with 10^4 cells then poured onto the top of the pre-made bottom layer. The agarose was allowed to solidify at 4°C for 30 min, and then 2 mL of complete DMEM was added. The top medium (2 mL/dish) was replaced by fresh complete medium every week during growth at 37°C. After 3 wk, the AIG cultures were dried down and colonies were photographed directly or visualized by staining with 1% crystal violet followed by counting of the colonies. All experiments were repeated at least three times.

In Vitro Migration and Invasion Assays

The in vitro migration and invasion assays were carried out in BD migration and BioCoat Matrigel invasion chambers with upper chamber polycarbonate filters of 8 μm pore size (BD Biosciences, San Diego, CA). For migration assay, 2×10^4 cells in serum free medium with 0.1% BSA were plated on top of the insert. The lower wells contained DMEM with 10% FBS. After 6 h of incubation, the cells on the upper surface of the membrane were wiped away with a cotton swab, the cells on the lower surface were fixed in methanol, stained with 10% Giemsa and counted. Each assay was done in triplicate and repeated at least twice. Data are expressed as mean ± s.d. Tumor cell invasion was assayed by the same procedure, except that the polycarbonate membrane was precoated with Matrigel (a reconstituted basement membrane gel), the number of cells seeded was 5×10^4, and the incubation time was 24 h.

Intracellular pH Measurement

Cell suspension was washed rapidly once with HBSS buffer (Gibco BRL) and resuspended in the

same solution. BCECF-AM (Molecular probes, Interchim, Montlucon, France) was then added to a final concentration of 10 μM and the suspension was incubated for 30 min at 37°C to allow BCECF-AM to be hydrolyzed by intracellular esterase into pH-dependent fluorescent BCECF. The calibration curve was established from labeled cells resuspended in the high potassium buffer of various known pH containing 10 μM nigericin (a proton ionophore used to obtain equilibration between intracellular and extracellular pH, pHe) (Sigma–Aldrich) for 5 min before flow cytometry analysis. A set of high potassium buffers with pH of 6.0–8.0 in 0.2 pH unit steps was prepared by mixing appropriate proportions of buffer A (135 mM KH_2PO_4, 20 mM NaCl) and buffer B (110 mM K_2HPO_4, 20 mM NaCl) at room temperature.

RESULTS

Overexpression of CAIII Enhanced Anchorage-Independent Growth of HCC Cells

To investigate the effects of CAIII on various aspects of HCC cells, we established a cell culture model in which full-length human CAIII cDNA was cloned in the pcDNA3.1-TOPO vector and introduced into SK-Hep1 cells that lack endogenous CAIII expression. Because of the presence of His-tag, the protein size of the ectopically expressed CAIII is larger than the endogenous CAIII by about 5 kDa. The pcDNA3.1-TOPO vector alone was used as a control. After G418 selection, expression of CAIII in the stable CAIII transfectants was confirmed by Western blot. The CAIII-expressing clones C2 and C3 exhibited the highest level of CAIII protein, C15 next, and clone C11 the lowest. The pooled population was also used to avoid clonal bias (Figure 1a). The effect of CAIII on tumorigenicity of the cells in vitro was measured by colony formation in the soft-agar assay. The propensity for AIG in soft agar is an important indicator of malignant transformation. In comparison of the AIG ability, we observed that the CAIII transfectants produced four- to sevenfold more colonies than the control cells did (Figure 1b, $P < 0.01$).

CAIII Enhanced Cell Motility and Invasiveness of HCC Cells

We next examined whether CAIII affects the motility of HCC cells. Expression of CAIII significantly increased cell motility two- to threefold (Figure 2a). Next, we investigated whether CAIII affects the invasiveness of HCC cells. The ability of CAIII transfectants to traverse a Matrigel-covered membrane was found to be dramatically increased 7- to 12-fold for the two clones and the population of pooled CAIII transfectants (Figure 2b). To demonstrate the causal influence of CAIII on invasion, CAIII expression of the pooled CAIII transfectants was further knocked down with siRNA oligonucleotides. The maximum knockdown effects were observed after 48 h of siRNA treatment, resulting in an 80% reduction of CAIII protein levels (Figure 2c). As a result, treatment with 50 nM CAIII-siRNA resulted in a significant reduction of 75% in invasive ability (Figure 2d, $P < 0.01$).

Possible Mechanism of the CAIII-Enhanced Invasiveness of HCC Cells

We next investigated the possible mechanisms of the CAIII-mediated HCC cell migration/invasion process. FAK is an intracellular soluble protein-tyrosine kinase (PTK) that regulates the assembly and disassembly cycles of focal contacts required for efficient cell motility. Increased FAK expression and phosphorylation levels at Y397 have been correlated with progression to an invasive phenotype of cancer cells. Here, we used Western blotting to examine the phospho-FAK (at Y397) and several FAK-interacting proteins in the CAIII transfected cells. Phosphorylation levels of FAK, Src, and Rac1 were significantly increased in CAIII-overexpressing transfectants than the control cells (Figure 3a). In addition, vimentin, a maker of the invasiveness-associated epithelial-mesenchymal transition (EMT), was increased in the CAIII transfectants (Figure 3b). Furthermore, the phosphorylation level of FAK was reduced when CAIII expression was knocked down by siRNA (Figure 3c). To determine whether CAIII enhances invasion capability through FAK pathway, we first successfully knocked down the expression of FAK with 50 nM FAK-siRNA (Figure 3d). For both pooled CAIII transfectants and CAIII clone C2, knockdown of FAK expression with FAK-siRNA gave rise to a significant reduction of about 50% in invasive ability (Figure 3e). The number of invasive cells averaged 3673 ± 327 versus 1737 ± 161 for the pooled CAIII transfectants ($P < 0.01$) and 5114 ± 582 versus 2861 ± 43 for CAIII clone C2 ($P < 0.05$). These results may at least partly explain the increased invasive ability through FAK signaling pathway.

We noticed that, during culture maintenance, the medium was acidified more rapidly by CAIII-transfectants than the vector-only control cells. We measured the pHe and intracellular pH (pHi) during culture of cells using an electrode pH meter and the pHi probe BCECF-AM with flow cytometry, respectively. When cells were grown in medium of pHe 7.4, the CAIII over-expressing cells not only reduced pHe to a greater extent (on day 5) (Figure 4a) but also had a drop in pHi more quickly (on day 3) (Figure 4b), which was associated with a greater invasiveness and motility. Furthermore, to substantiate the effect of pHe on cancer cell motility, we compared the effects of pHe 6.8 with pHe 7.4 on invasive ability. As a result, cells grown in acidic pHe indeed showed a significantly increased motility (supplementary Figure 1a). Moreover, the levels of phospho-FAK,

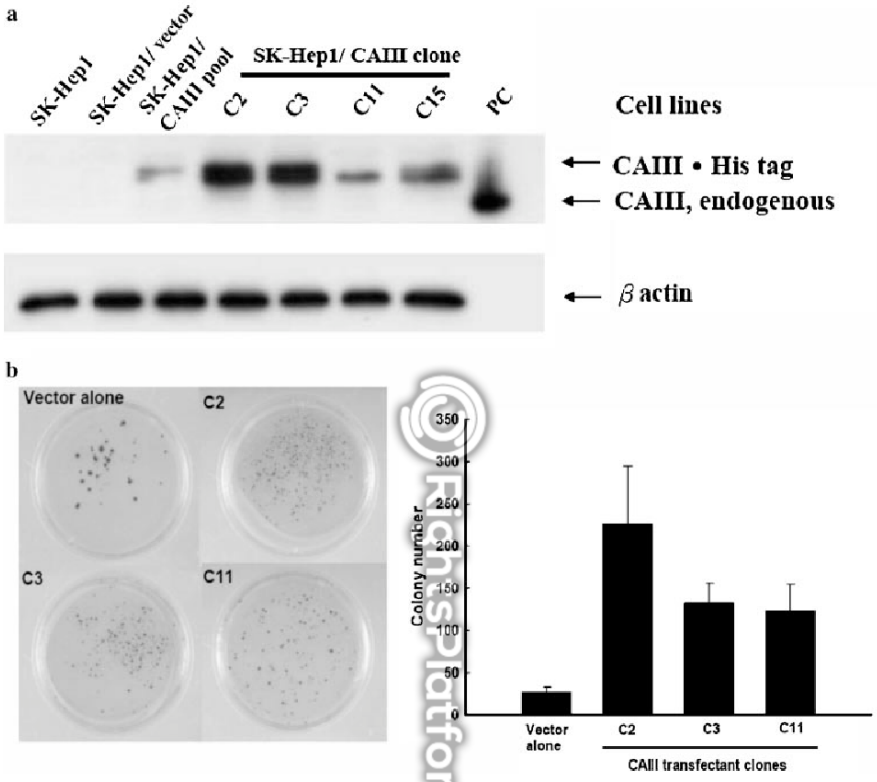

Figure 1. CAIII expression enhances anchorage-independent growth (AIG) of SK-Hep1 cells in soft agar. (a) Western blot analysis of ectopic CAIII protein expression in SK-Hep1 cells. Vector alone transfectants, pooled CAIII transfectants, as well as four CAIII clones are shown. The ectopic CAIII protein is His-tagged. PC, the purified human CAIII protein serves as a positive control. (b) AIG of various CAIII transfectants of SK-Hep1 cells. Cells (1×10^4) were seeded in soft agar and colonies of >20 cells were counted 3 wk later. Each value is the mean of triplicate experiments; bar, SD. All three CAIII transfectant clones were significantly higher than the vector alone control ($P < 0.01$, t-test).

phospho-Rac-1/cdc42 and vimentin were increased in cells grown at pHe 6.8 when compared to cells grown at pHe 7.4 (supplementary Figure 1b and c). When the pHe and pHi of cells grown at an initial culture pH of 6.8 were measured, after 2 d of growth, both pHe and pHi were found to have a reduction of about 0.2 units in pH (7.02 vs. 7.23 and 7.01 vs. 7.18, respectively), in comparison with cells grown at an initial pH 7.4. (supplementary table).

DISCUSSION

Cancer metastasis is a multi-step process that involves cell detachment from the primary tumor, entry into the vascular or lymphatic system, dispersal through the circulation, and proliferation after extravasation and migration into new target organs [27–30]. Many studies have demonstrated the importance of invasion played in the early stages of metastasis. When tumor cells become detached from the substratum or from each other and disperse through the circulation, they lose the signals for growth and survival generated by the cell-substratum interaction. Some cancer cells may survive the loss of these signals, which is a phenomenon related to AIG. In other words, AIG may contribute to metastasis formation. In this report we demonstrate that, for the first time, CAIII expression may increase AIG, motility, and invasiveness, and thereby suggest the possible involvement of CAIII in metastasis of liver cancer.

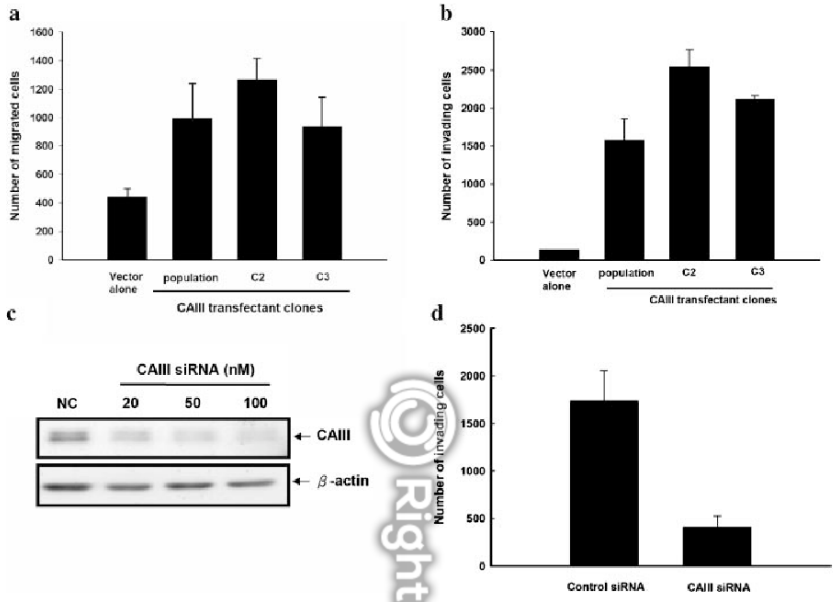

Figure 2. CAIII expression enhances migration and invasion of SK-Hep1 cells. (a) Cell migration was assayed in Boyden chambers fitted with 8-μm pore size filters. All the CAIII transfectants exhibited significantly higher motility than the vector alone control cells ($P < 0.05$, *t*-test). (b) Invasiveness of the HCC cells was assayed using extracellular matrix (ECM)-coated invasion chambers. All the CAIII transfectants showed significantly higher invasiveness than the vector alone control cells ($P < 0.01$, *t*-test). (c) Successful knockdown of CAIII expression by siRNA. Cells were treated with different concentrations of CAIII siRNA for 48 h and assayed by Western blot. NC: scrambled control siRNA. (d) Knockdown of CAIII expression attenuated invasive ability. Cells treated with 50 nM CAIII-siRNA or scrambled control siRNA for 48 h were assayed for their invasiveness. Invading cells were stained, photographed, and counted by Image-Pro Plus 5.0 software. Each value is the mean of triplicate experiments; bar, SD. (Control vs. CAIII siRNA, $P < 0.01$, *t*-test.)

Over the past decades, limited efforts have been made to elucidate the role of CA in tumor progression, either as a biomarker or a tumor-associated protein [31]. However, only the recently characterized *trans*-membrane CAIX and CAXII have so far been associated with tumorigenesis [31–34]. These enzymes may facilitate cancer cell growth and metastasis by maintaining acidic pHe in the tumor microenvironment [35]. The pHe of malignant tumors is lower than that of normal tissues [36,37]. Moreover, Martinez-Zaguilan et al. [38] have recently shown that acidic pH treatment increases the invasiveness of two human melanoma cell lines. The acidity in most solid tumor microenvironments may help the new vessels to grow and survive [39]. In this study, the pHe was significantly lowered in the growth media of transfected SK-Hep1 cells over-expressing CAIII than the growth medium of the vector-alone control cells. This may explain, at least in part, the higher invasive ability of C2 and C3 clones in vitro. We also found that cells grown in acidic pHe indeed had a significantly increased migration capacity.

Recently Kuo et al. [26] demonstrated a reduction of CAI, CAII, and CAIII protein expression in human HCC when compared to the adjacent normal tissues using immunoblotting and immunohistochemistry. Although CAIII expression was shown to be down-regulated in HCC, it should be noted that direct comparison of the CAIII expression levels between primary HCC and the metastases has never been done. The possibility remains that re-expression of CAIII confers invasive ability of cancer cells during later stages of metastatic progression of HCC.

FAK is a nonreceptor tyrosine kinase that plays an important role in cell cycle progression, cell spreading, migration, and survival. FAK is activated by integrin-ECM engagement and transmits signals to a range of targets in promoting cell motility. In addition to adhesion receptor, a variety of stimuli, acting either on specific surface receptors or on intracellular molecules, such as PKC or Rho, can also

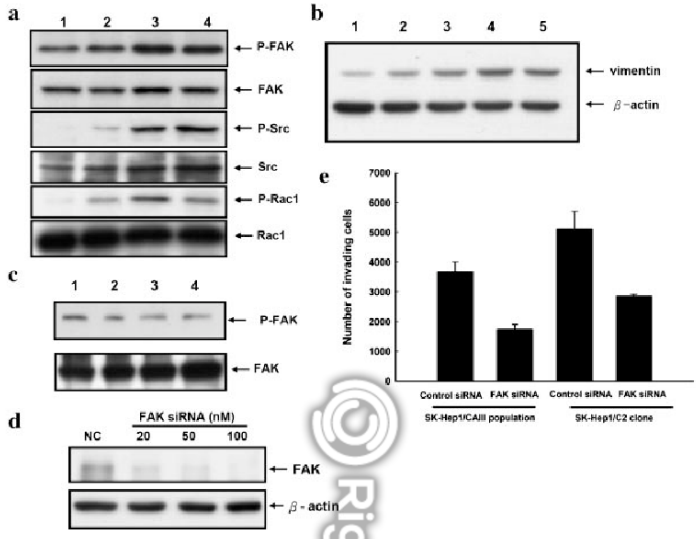

Figure 3. Effects of CAIII expression on protein phosphorylation status. (a) Western blot analysis of phospho-FAK, FAK, phospho-Src, Src, phospho-Rac1, and Rac1. Lanes 1, vector alone control cells; 2, CAIII transfectant population; 3, CAIII clone C2; 4, CAIII clone C3. (b) Western blot analysis of vimentin. Lanes 1, vector alone control cells; 2, CAIII transfectant population; 3, CAIII clone C2; 4, CAIII clone C3; 5, CAIII clone 11. (c) Knockdown of CAIII expression resulted in a reduced level of phospho-FAK. Cells were treated with different concentrations of CAIII siRNA for 48 h then assayed by Western blot for phospho-FAK and FAK. Lanes 1, Scrambled control siRNA; 2, 20 nM CAIII siRNA; 3, 50 nM CAIII siRNA; 4, 100 nM CAIII siRNA. (d) Western blot showing successful knockdown of FAK expression by siRNA treatment. The pooled CAIII-transfectants were treated with scrambled control siRNA (NC, lane 1) or various concentrations of FAK-siRNA for 48 h as indicated (lanes 2–4). (e) Knockdown of FAK expression resulted in reduced invasiveness as measured by ECM-coated invasion chambers. Cells were trypsined 48 h after siRNA treatment and reseeded into the invasion chamber. Invading cells were stained, photographed, and counted by Image-Pro Plus 5.0 software. Each value is the mean of triplicate experiments.

induce tyrosine phosphorylation of FAK [40]. Increased FAK expression and tyrosine phosphorylation of FAK have been correlated with increased malignancy and invasiveness [21,22,41]. In addition, activated FAK has also been shown to enhance the AIG of MDCK cells [42]. Suppression of FAK expression may lead to anoikis and prevent metastasis [43]. In this study we found that, when

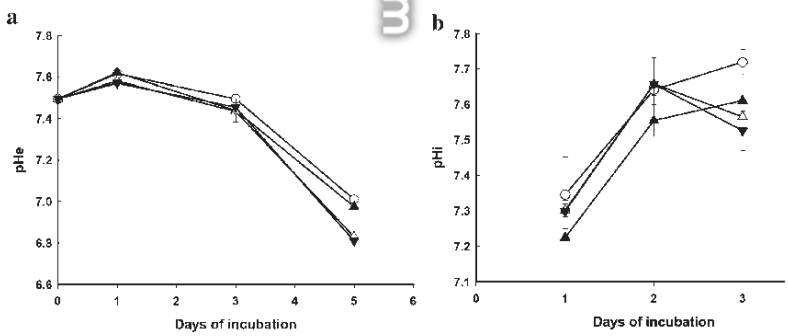

Figure 4. CAIII expression acidifies both intracellular and extracellular pH. (a) Cells (6×10^5) were grown in 60-mm Petri dishes for 5 d, and extracellular pH (pHe) was determined by direct measurement of the pH of the culture medium by a pH meter at intervals. (Day 5, control vs. C2 and C3, $P < 0.05$, t-test) (b) Cells (6×10^5) were grown in 60-mm Petri dish for 3 d, and the intracellular pH (pHi) was determined at intervals by using the pH-sensitive, fluorescent dye BCECF-AM and detected by flow cytometry as described in Materials and Methods Section. (Day 3, control vs. CAIII population, C2 and C3, $P < 0.05$, t-test) (○) SK-Hep1 vector alone; (▲) SK-Hep1/CAIII population; (△) SK-Hep1/C2; (▼) SK-Hep1/C3.

compared with controls, cells over-expressing CAIII had elevated levels of phospho-FAK, phospho-Src and phospho-Rac1, and were more motile and invasive. Down-regulation of FAK in CAIII transfectant cells reduced their invasive capability. These results suggest that CAIII expression confers an increased motility and invasiveness and this effect may be mediated through an increase in the levels of phospho-FAK. What mechanisms are involved in the activation of FAK signaling by CAIII overexpression in HCC cells? In Figure 4, we showed that overexpression of CAIII accelerated the reduction of both pHe and pHi, and in the supplementary figure, we further demonstrated that lowered pH could activate FAK signaling. These phenomena are consistent with the observation of a greater invasiveness and motility. Therefore, we propose that overexpression of CAIII may promote invasive ability of HCC cells through FAK signaling by changing the pHe and/or pHi.

The occurrence of EMT during tumor progression allows the benign tumor cells to acquire the capacity of invading the surrounding tissue and to ultimately metastasize to distant sites [44–47]. Commonly used molecular makers for EMT include increased expression of N-cadherin and vimentin and decreased expression of E-cadherin. Activation of mesenchymal maker enhances the migration ability of cancer cells. In this study, we found that the CAIII tranfectants indeed had a higher vimentin expression than the control cells. But the E-cadherin protein expression was not detectable among these cells (data not shown).

In summary, in the present study we show that overexpression of CAIII promotes AIG, motility, and invasiveness of SK-Hep1 cells. There was an increase in the levels of phospho-FAK and the downstream signaling phospho-Src and phospho-Rac1 in the CAIII transfectants. Moreover, a consistent increase in expression of the EMT maker vimentin in the CAIII transfectants may also contribute to the increased invasiveness. Knockdown of CAIII and FAK, respectively, in the CAIII transfectant cells resulted in a reduction in invasiveness. Thus, these results further support that CAIII might have an important influence in the development of invasion/metastasis in liver cancer. Clinical studies investigating the relationship between CAIII expression and metastatic potential of HCC (e.g., comparison of CAIII expression in primary vs. metastatic cells) and the relationship between CAIII expression and clinical outcomes are highly warranted.

ACKNOWLEDGMENTS

We thank Dr. Chong-Kuei Lii, Department of Nutrition, Chung-Shan Medical University, Taichung, Taiwan, for the gift of CAIII isoform-specific antiserum. This study was supported by grants from the National Health Research Institutes, Zhunan, Taiwan.

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