A-kinase anchoring protein 2 is required for calcitonin-mediated invasion of cancer cells

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Abstract

Expression of neuropeptide calcitonin (CT) and its receptor (CTR) is frequently elevated in prostate cancers (PCs) and activation of CT–CTR axis in non-invasive PC cells induces an invasive phenotype. Specific, cell-permeable inhibitors of protein kinase A abolish CTR-stimulated invasion of PC cells. Since PKA is ubiquitously distributed in cells, the present study examined the mechanism(s) by which CTR-stimulated PKA activity is regulated in time and space. CT reduced cell adhesion but increased invasion of PC cells. Both these actions were abolished by st-Ht31 inhibitory peptide suggesting the involvement of an A-kinase anchoring protein (AKAP) in CT action. Next, we identified the AKAP associated with CT action by the subtraction of potential AKAP candidates using siRNAs. Knock-down of membrane-associated AKAP2, but not other AKAPs, abolished CT-stimulated invasion. Stable knock-down of AKAP2 in PC3-CTR cells remarkably decreased their cell proliferation, invasion, clonogenicity and ability to form orthotopic tumors and distant metastases in nude mice. Re-expression of AKAP2-wt restored these characteristics. Primary PC specimens displayed remarkable upregulation of CTR/AKAP2 expression as compared to benign prostates. Metastatic cancers displayed significantly higher CTR/AKAP2 expression than localized cancers. These results for the first time demonstrate that AKAP2 is expressed in human prostates, its expression is elevated in metastatic prostate cancer, and the knock-down of its expression remarkably decreased tumorigenicity and metastatic ability of prostate cancer cells. AKAP2 may serve as a critical component of CTR-mediated oncogenic actions.

Key Words

- calcitonin receptor-AKAP2-prostate cancer-invasion and metastasis

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Introduction

Expression of calcitonin (CT) and its receptor (CTR) is frequently elevated in prostate cancers (PCs). Human prostate CTR belongs to the class B family of G protein-coupled receptors (GPCRs), and is dually coupled to G_q and G_s types of heterotrimeric G proteins (Force *et al.* 1992, Shah *et al.* 1994, Offermanns *et al.* 1996, Parthier *et al.* 2009). In addition to its well-known role in the

regulation of serum calcium levels, CTR is crucially involved in the processes associated with development, growth and differentiation (Wookey 2009). Our recent studies suggest that CTR plays a key role in promotion of prostate cancer (PC) metastasis by destabilizing cell-cell junctions, inducing epithelial-to-mesenchymal transition (EMT) and increasing invasion of PC cells

(Sabbisetti *et al.* 2005, Thomas *et al.* 2006). While the mechanism associated with CTR-mediated junction destabilization remains to be investigated, our results show that CTR-induced invasion can be completely abolished by mPKI, a specific cell-permeable inhibitor of cAMP-dependent protein kinase (PKA; Sabbisetti *et al.* 2005). These results are consistent with the current evidence that PKA, along with Rho-family GTPases, plays a key role in the regulation of cell–cell adhesion and actin cytoskeleton organization of epithelial cancer cells (Leve *et al.* 2008).

PKA, a serine threonine kinase, is universally distributed in a cell (Beebe 1994). However, the fidelity of its action is determined by its location and the duration of its activation within a cell. The time and space of PKA action is usually determined by a molecular framework of adapter, anchoring and scaffold proteins that maintain kinases and phosphatases in defined subcellular compartments (Colledge & Scott 1999). For example, A-kinase anchoring proteins (AKAPs) provide spatial specificity to PKA by tethering the PKA holoenzyme in discrete intracellular environments (Colledge & Scott 1999, Stratakis & Cho-Chung 2002, Gold *et al.* 2006). They may also temporally regulate PKA action by assembling a scaffold of PKA along with its signal terminators such as phosphoprotein phosphatases and cAMP-specific phosphodiesterases. More than 50 AKAPs have been identified. They are highly divergent proteins, but all have a signature PKA regulatory subunit-docking motif (Theurkauf & Vallee 1982, Wong & Scott 2004, McConnachie *et al.* 2006).

To further elucidate the role of PKA in the actions of CTR that decrease cell adhesion and increase invasion of PC cells, we tested the hypothesis that CTR increases invasiveness and metastatic capacity of PC cells by selectively activating AKAP-anchored PKA, and that the AKAP that anchors CTR-activated PKA is localized in the proximity of CTR as well as cell–cell junctions. Our results suggest that CTR-induced actions on cell adhesion and invasion are mediated by PKA that is anchored to AKAP2, a protein localized in the plasma membrane (Gold *et al.* 2012). Knock-down of AKAP2 or the deletion of its PKA-binding site abolishes CTR-stimulated PC cell invasion as well as orthotopic tumor growth and metastasis of PC xenografts in nude mice.

Material and methods

Animals

Male balb/c nu/nu mice (6–8 weeks old) were purchased from Harlan (Madison, WI, USA), and housed two per cage

in microisolator units under 70% humidity and temperature-controlled conditions. The animals were fed *ad lib* on a standard sterilizable laboratory diet (Teklad Lab chow, Harlan Teklad) and quarantined for 1 week prior to their use in the study.

Surgical orthotopic implantation

All animal procedures were conducted in accordance with the principles and procedures outlined by the NIH and Institutional Animal Care and Use Committee at University of Louisiana at Monroe. The Surgical orthotopic implantation was performed under Ketamine/Xylazine anesthesia as previously described (Chien *et al.* 1999, Shah *et al.* 2008, Shah *et al.* 2009a). In brief, tumor cell suspensions (1×10^6 cells/20 μ l) were injected into the dorsal prostate, the mice were maintained on the laboratory diet *ad libitum* for up to 60 days, and were regularly monitored for tumor growth/metastasis with fluorography using Kodak 4000 MM imaging station (Eastman Kodak Company, Rochester, NY, USA) (Thomas *et al.* 2006).

Cell culture

PC cell lines LNCaP, PC-3 and DU-154 were obtained from ATCC (Manassas, VA, USA), and maintained as recommended by ATCC. All cell lines were cultured under previously described culture conditions (Shah *et al.* 2009b). PC-3CTR cell line was generated by stable transfection of pcDNA3.1 containing full-length CTR cDNA cloned from LNCaP cells as described previously (Shah *et al.* 2009b). The cell lines and modified sublines were used only up to passage 12. Primary cell lines were used within 6 months of their purchase. PC-3 and PC-CTR cells were used to generate AKAP2^{KD}, AKAP2-reexp and PKAP2 Δ PKA sublines. Therefore, PC-3 and PC-3CTR cell lines were authenticated by STR profiling at the John Hopkins University Medical Center. The results demonstrating their authenticity are provided as a Shah Supp Fig. 1, see section on supplementary data given at the end of this article. The sublines were used immediately after the stable transfection.

Generation of AKAP2^{KD} cell lines

PC-3CTR cells were used for this study because they are PC-3-derived, but co-express CT and CTR and are suitable to study modulation of CTR action in response to changes/mutations in AKAP2 molecule (Chien *et al.* 2001). Endogenous AKAP2 expression of PC-3CTR cells was silenced by stable expression of constitutive AKAP2

shRNA expression vector (pSuper.neo system; shRNA duplex- 5'- GCCAUAUAAUGAAUGGCXGUTT-3' corresponding 3279–3295 bp of human AKAP2 transcript). Since the constitutive presence of AKAP-2 shRNA in PC-3CTR cells could interfere with the re-expression of AKAP2-wt or its Δ PKA mutant (where the Δ PKA-binding domain of AKAP2 (aa 566–579) was deleted), we generated the cDNA expression vectors refractory to AKAP2 RNAi by introducing a silent mutation in RNAi target sequence in the AKAP2-wt or AKAP2- Δ PKA transgenes. The cells were then transfected with either AKAP2-wt-zeocin or AKAP2- Δ PKA-zeocin transgenes. All AKAP2 plasmids were HA-tagged. The colonies of stable transfectants were screened for the knock-down of AKAP2 as well as its re-expression with AKAP2-wt and AKAP2- Δ PKA constructs by western blotting. The stable clones expressing each vector were pooled and maintained in a medium containing $G418 \pm$ zeocin.

Cell adhesion assay

Twenty-four-well plates were coated with PBS, Laminin-1 or Fibronectin at 4 °C overnight. The plates were then chilled and seeded with 10^5 cells/well. The cells were then incubated for 60 min in the presence/absence of agents. After washing, the cells were fixed in 4% paraformaldehyde and stained with Crystal Violet for 10 min. The plates were then dried, and adherent cells were solubilized in 2% SDS and read for optical density at 550 nm.

In vitro invasion assay

Invasion experiments were conducted in 24-well, two compartmented, Matrigel invasion chambers (Becton Dickinson, Bedford, MA, USA) as previously described (Sabbisetti *et al.* 2005).

Growth Correction Since CT also induces proliferation of PC cell lines, we determined the factor to correct for possible proliferation of cells that may have migrated in early phase of 24-h incubation period. Then, 25×10^4 cells were plated at hourly intervals in six-well dishes and cultured for 1–24 h. Mean percent increase in cell number in each well was determined. This correction was applied to the results of invasion assays.

Orthotopic tumor growth and metastasis

Stable expression of red fluorescence protein To detect micrometastases of implanted tumor cells in mice,

we stably transfected the cell lines with DsRed-MCherry-Hyg-N1, a mammalian expression vector that encodes DsRed-MCherry, a derivative of red fluorescent protein (Clontech, Palo Alto, CA, USA). Hygromycin-resistant colonies were selected, and observed over a period of 4 weeks. All cell lines expressed strong red fluorescence at a steady level over the duration of the observation period. The cells were then used for orthotopic implantation in nu/nu mice. The mice were observed for tumor growth over a period of 6–8 weeks.

Histology At necropsy, primary tumors and several organs were harvested and weighed. Wet sections of organs were examined for the presence of RFP. Fluorescent images of tumor cells were acquired with a charge-coupled device Retiga 2000 RT digital camera connected to a Nikon Optiphot-2 (Nikon, Tokyo, Japan). The remaining tissues were fixed in neutral buffered formalin and embedded in paraffin. The 5- μ m thick sections were processed for H&E staining.

AKAP, ZO-1 and E-cadherin immunocytochemistry

Fixed PC cells were immunolabeled with primary antibodies (mouse Anti-AKAP-KL, BD Transduction Laboratories; rabbit Anti-ZO-1, Invitrogen; goat anti-E-Cadherin, R&D systems) for overnight at 4 °C. The appropriate secondary antibodies conjugated with fluorophores FITC, TRITC or Alexa Fluor 350 was applied. After washes, the slides were observed under Nikon Optiphot-2 microscope equipped for epifluorescence. The images were captured with Retiga 1300 camera connected to an iMac computer loaded with the iVision image analysis program (Biovision Technologies, Exton, PA, USA). All antibodies were characterized for specificity and cross-reactivity as described in Figs S1-S3, see section on supplementary data given at the end of this article.

CTR, AKAP2 IHC of PC specimens

Monoclonal CTR antibody was purchased from Acris (San Diego, CA, USA). Characterization of CTR and AKAP-KL antibodies is presented in Supplementary Figs S1-S3, see section on supplementary data given at the end of this article. Specimens of benign prostatic hyperplasia (BPH), localized and metastatic PCs were purchased from commercial sources (PR955; US Biomax, Rockville, MD, USA and TRP-1 of NCI). The cohorts included BPH ($n=16$); localized PC ($n=30$) and metastatic PC ($n=37$). The slides were deparaffinized, and following antigen retrieval, immunohistochemistry was performed as described

previously (Shah *et al.* 2008). Incubations with anti-CTR (Acris) or AKAP-KL monoclonal antibody were followed by TRITC-conjugated secondary antiserum. The slides were then counterstained with DAPI and assessed for immunostaining. Six randomly selected images of every specimen were captured with a Retiga 1300 camera connected to a Nikon Optiphot-2 microscope equipped for epifluorescence. Immunostaining was scored by two individuals independently using the established methods (Jackel *et al.* 2001), and the mean reading was taken. Number of AKAP2-positive cells (red) and total cells (stained blue) per 400 $\times$ field were counted; and staining intensity was assigned an arbitrary value, on a scale of 0–3 as follows: (–)=0, ($\pm$)=0.5, (+)=1, (++)=2, (+++)=3.

An IHC index for each sample was calculated by multiplying staining intensity with the percentage of positive cells. The results were graded from 0 (negative) to 300 (all cells display strong staining intensity). Reproducibility of the analysis was verified by rescoring of randomly chosen slides. Duplicate readings gave similar results.

Co-immunoprecipitation

Serum-starved PC-3-CTR cells (2×10^6 /100 mm dish) were washed, and lysed in immunoprecipitation buffer as previously described (von Zastrow & Kobilka 1994). In brief, CTR-FLAG was immunoprecipitated from equal lysate proteins per sample (~ 2.5 –4 mg protein per lysate) with anti-FLAG EZ-view beads (Sigma). The CTR-FLAG immunoprecipitates were eluted with FLAG peptide, fractionated on a polyacryl amide gel by electrophoresis and transferred on to PVDF blot. The blot was probed for ZO-1, AKAP2 and E-cadherin by immunoblotting. Preimmune lysate was used as input control.

Statistical analysis

The results were statistically analyzed by one-way ANOVA or as described in the results section. The significance was derived from Newman-Keuls test. The difference was considered statistically significant when $P < 0.05$.

Results

CT reduces cell adhesion and increases invasion of prostate cancer cells: attenuation of its action by steared Ht-31 peptide

Earlier studies from this laboratory have shown that CT significantly increased invasion of PC cells, and mPKI,

a cell-permeable specific inhibitor of PKA, almost abolished this effect (Sabbisetti *et al.* 2005). To investigate whether PKA that mediates the action of CT on cell adhesion and invasion is anchored by an AKAP, we examined the effects of CT in the presence/absence of Ht-31, an anchoring inhibitor peptide that occupies PKA-binding site on AKAP molecules (Jarnaess *et al.* 2008). The peptide does not interfere with the PKA activity, but uncouples the bound PKA from an AKAP molecule (Vijayaraghavan *et al.* 1997, Gold *et al.* 2006).

Figure 1 shows that CT (50 nM) decreased the adhesion of PC-3CTR cells on plastic (Fig. 1A1 and A2), laminin (Fig. 1B1 and B2) and fibronectin (Fig. 1C1 and C2). However, the effect of CT on cell adhesion on laminin was almost half of that on plastic or fibronectin. These results are consistent with our earlier observations and suggest that CT interacts with laminin-activated integrins (Thomas *et al.* 2007). When treated with Ht-31Pro (the control peptide for Ht-31, 50 μ M), the peptide did not affect cell adhesion of either control cells or CT-treated cells (Fig. 1A3 and A4) on plastic; laminin (Fig. 1B3 and B4) as well as fibronectin (Fig. 1C3 and C4). Ht-31 (50 μ M) did not alter the adhesion of control cells on all surfaces, but remarkably reversed CT-induced decline of cell adhesion (Fig. 1A5 and A6 on plastic; Fig. 1B5 and B6 on laminin; and Fig. 1C5 and C6 for fibronectin). Figure 1C shows quantitative analysis of cell adhesion. The results demonstrate that CT significantly decreased the adherence of cells on plastic and fibronectin, and this action was almost abolished or markedly reduced by Ht-31.

In contrast, CT significantly increased the invasion of PC3-CTR cells (Fig. 1D). Ht-31Pro did not affect the invasion of either vehicle-treated or CT-treated cells. Ht-31 did not alter the invasion of vehicle-treated PC-3CTR cells, but significantly decreased CT-stimulated invasion (Fig. 1E). These results suggest that the action of CT on cell adhesion and invasion is mediated by the PKA that is anchored to an AKAP molecule. AKAP plays an important role in CT action by providing spatio-temporal specificity.

Identification of an AKAP associated with CTR actions

Since more than 50 AKAPs have been reported, we attempted to identify an AKAP associated with CTR-mediated invasion. Because CTR is a membrane receptor, we first examined AKAPs that have been localized to the plasma membrane such as type 2, 5, 8, 11, and 12.

Knock-down of membrane AKAPs and CTR-stimulated invasion

To identify the AKAP associated with

Figure 1

Role of AKAP in the actions of CT on cell adhesion and invasion of prostate cancer cells. Representative photomicrographs of PC-3CTR cell adhesion to plastic (A and B) laminin (10 μ g/ml) or (C) fibronectin (10 mg/ml); and its modulation by CT (50 nM) (in the presence/absence of 50 μ M st-Ht-31Pro and 50 μ M stHt-31). Cells were allowed to adhere for 30 min as indicated. (D) Colorimetric analysis of adherent cell lysates of four independent experiments similar to that depicted in Fig. 1A. The results are expressed as mean $\pm$ S.E.M. $P < 0.001$ (**CT vs. st+Ht31; *Ht31Pro vs Ht31+CT; ^CT vs

Ht-31+CT-f; one way ANOVA and Newman-Keuls test). (E) Effect of CT on invasion of prostate cancer cells. PC-3CTR cells were added to the upper insert of invasion chambers along with/without 50 nM CT (in the presence/absence of 50 μ M st-Ht-31Pro and 50 μ M st-Ht-31), and cells that passed through the Matrigel barrier were counted after 48 h. The results are expressed as mean number of invaded cells per 400 $\times$ field $\pm$ S.E.M. for $n=6$. * $P < 0.05$ (PC-3CTR vs PC-3CTR+CT; PC-3CTR+Ht-31Pro vs PC-3CTR+Ht-31Pro+CT; one way ANOVA and Newman-Keuls test).

CT actions, we knocked-down each individual membrane-associated AKAP subtype with specific siRNAs, and tested its impact on CT-induced invasion. Controls received an equivalent amount of scrambled RNAs. The knock-down of each AKAP was verified by western blotting (Fig. 2A).

CT induced a threefold increase in the invasion of scrambled RNA-transfected PC-3CTR cells (Fig. 2B). Knock-down of AKAP8 did not affect either the basal or CT-stimulated invasion. However, the knock-down of either of AKAP 2, 5, 11, and 12 reduced the basal invasion

of PC-3CTR cells to a variable extent. CT could still increase the invasion of cells receiving siRNAs for AKAP 5, 8, 11, and 12 (Fig. 2B). But, AKAP2 siRNA caused a maximal decrease in basal invasion of PC-3CTR cells; and more importantly, CT could not stimulate the invasion of AKAP2 k/d cells, suggesting that AKAP2 may be associated with the stimulatory action of CT on invasion of PC cells (Fig. 2C) (Dong et al. 1998).

Because Ht-31 acts on all AKAPs whereas AKAP2 siRNA should be specific for AKAP2 alone, we compared the effect

Figure 2

Identification of AKAP associated with the action of CT on PC-3CTR cell invasion. (A) Immunoblotting of PC-3CTR lysates for the appropriate AKAP. The cells were lysed 72 h after transfection with appropriate scrambled or siRNA. (B) Invasion of PC-3CTR cells 72 h after transfection with either scrambled or AKAP siRNA as indicated. Each cell well was treated with/without 50 nM CT. The results are expressed as mean number of invaded cells per 400× field $\pm$ S.E.M. for $n=6$. * $P<0.01$ (control vs CT-treated) (one-way ANOVA and Newman-Keuls test). (C) Representative photomicrographs of invaded cells at the end of the treatment as indicated. (D) PC-3CTR cells transfected with either scrambled or siRNA or untreated PC-3CTR cells were added to the upper insert of invasion chambers and treated with/without 50 nM CT (and with st-Ht-31Pro or st-Ht-31 as indicated), and cells that passed through the Matrigel barrier were counted after 48 h. The results are expressed as mean number of invaded cells per 400× field $\pm$ S.E.M. for $n=6$. $P<0.01$ (^a vs. C; ^b vs Ht-31Pro;

one way ANOVA and Newman-Keuls test) (E) PC -3CTR cells were plated in 96-well plate and transfected with either scrambled or siRNA or treated with Ht-31 (or control peptide). After 48 h of incubation, the viability of attached cells was determined. The results are expressed as mean $\pm$ S.E.M. of OD₅₆₀. (F) Presence of AKAP2 immunoreactivity in cell lysates of LNCaP, PC-3 and PC-3M cells. (G) Localization of AKAP2 in PC-3CTR cells. PC-3CTR cells were plated on coverslip and subjected to AKAP2, ZO-1, E-cadherin ICC as described in the methods sections. 400× micrographs presenting AKAP2 immunostaining (TRITC-red), ZO-1 immunostaining (Alexa Fluor 350-Blue) and E-cadherin (FITC-green). (H) Double immunostaining of ZO-1 (Alexa Fluor 350-Blue) and E-cadherin (FITC-Green). No colocalization was observed. (I) A western blot showing the co-precipitation of AKAP2 and ZO-1, but not E-cadherin, with CTR. The column on the right shows input control (PC3-CTR cell lysate prior to immunoprecipitation), which shows all four proteins but in much smaller amounts.

of AKAP2 siRNA with Ht-31 on CT action. The results of Fig. 2D demonstrate that AKAP2 siRNA was at least as potent as Ht-31 in attenuating CT-stimulated invasion, raising a possibility that AKAP2 alone may be associated CTR action that stimulates the invasion of PC cells.

To ascertain that the inhibitory invasion of Ht-31 and AKAP2 siRNA is not due to their cytotoxic effects, we treated the cells with the agents under the experimental conditions of Fig. 2D, and examined their cell viability by MTT assay. The results of Fig. 2E suggest that neither our

Figure 3

Role of AKAP2 in tumor growth and metastasis. (A) Generation of AKAP2-deficient PC-3CTR cell lines. Since AKAP2 is expressed in all tested PC cell lines, we generated AKAP2-deficient cell lines by stable knock-down of AKAP2 as described in the methods section. The AKAP2^{KD} cell line was then used to re-express either AKAP2-wt or AKAP2-ΔPKA. The cell lysates of each of these cell lines were then probed by western blotting. (B) Effect of CT on cell proliferation of AKAP2 sublines. PC3, PC-3CTR or AKAP2 sublines were plated on 96-well plates and were treated with/without 50 nM CT for 24 h as described in the methods section. The number of cells/well at the end of the experiment was determined by the MTT method. The results are expressed as absorbance at 595 nm $\pm$ S.E.M. ($n=6$). * $P<0.01$ (without CT vs CT in appropriate cell line); § $P<0.01$ (PC-3CTR+CT vs AKAP2Reexp+CT). (C) Effect of CT on invasion of AKAP2 sublines. PC3, PC-3CTR or AKAP2 sublines (as indicated) were added to the upper insert of invasion chambers along with/without 50 nM CT, and cells that passed through the Matrigel barrier were counted after 48 h. The results are expressed as mean number of invaded cells per 400 $\times$ field $\pm$ S.E.M. for $n=6$. * $P<0.05$ (with CT vs

without CT for each cell line); ^ $P<0.05$ (each cell line vs PC-3) (one-way ANOVA and Newman-Keuls test). (D) Role of AKAP2 in clonogenic growth of PC cell lines. Representative photomicrographs of colonies formed by various sublines as indicated. PC3, PC-3CTR or AKAP2 sublines (as indicated) were grown on soft agar for 2 weeks as described in the methods section. The cells grown on soft agar were then stained with crystal violet and photographed. Each group had at least four replicates. (E) Role of AKAP2 in prostate cancer xenografts growth in nude mice. PC-3, PC-3CTR and AKAP2 sublines (as indicated) were stably transfected with DsRed-MCherry-Hyg-N1 (Clontech). Hygromycin resistant colonies that expressed strong red fluorescence at a steady level over the observation period were selected, and used for orthotopic implantation. Animals were sacrificed 8 weeks after implantation, and prostate tumors were weighed. * $P<0.01$ (compared to PC-3CTR); one-way ANOVA and Newman-Keuls test. (F) The indicated organs from the mice described in Fig. 3E were removed and observed directly under fluorescent microscope. The images were captured at 100 $\times$ magnification.

experimental procedures nor the AKAP2 knock-down affected the viability of PC3-CTR cells.

Expression of AKAP2 in PC cell lines

We next examined the presence of AKAP2 immunoreactivity in LNCaP, PC-3M and PC-3 cell lines by western blotting. The results of Fig. 2F demonstrate the presence of AKAP2 in all three PC cell lines.

AKAP2 is localized on plasma membrane of PC cells

Available evidence indicates that AKAP2 is localized to the plasma membranes of mouse kidney and lung cells (Dong et al. 1998). However, its presence or the subcellular localization in prostate cells has not been reported. We localized AKAP2 to the cell-cell junctions of PC3-CTR cells by triple immunocytochemistry using zonula occludens-1 (ZO-1), a tight junction marker; and E-cadherin, an adherent junctions marker (Fig. 2G) (Cereijido et al. 1993, Lewis et al. 1994, Rajasekaran et al. 2004). AKAP2-ZO-1 co-localization was observed in 98% of cells. In contrast, AKAP2-E-Cadherin co-localization was much less frequent (~26%). Moreover, the cells that showed co-localization of AKAP2 with E-cadherin also showed co-localization of ZO-1 with E-cadherin. Since ZO-1 and E-cadherin are distinctly localized in tight and adherens junctions respectively (Ebnet 2008), it is very likely that AKAP2-E-cadherin co-localization seen in small number of cells in Fig. 2G may have been due to inability of our microscope to separate tight junctions from adherens junctions in those specific areas (Fig. 2H). Nevertheless, the results demonstrate that AKAP2 is localized in the plasma membrane of PC3-CTR cells in close proximity of cell-cell junctions.

To verify the localization of AKAP2 in tight junctions and examine its relationship with CTR, we examined the presence of AKAP2 and/or ZO-1 in CTR immunoprecipitates. The results of Fig. 2I demonstrate that AKAP2 and ZO-1 co-precipitated with CTR. However, E-cadherin was undetectable. These results support our ICC data and suggest a close association between CTR, ZO-1 and AKAP2.

Characterization of AKAP2-modified PC-3CTR cell lines

To examine the role of AKAP2 in prostate tumor growth and metastasis, we generated multiple PC-3CTR sublines with modulated AKAP2 expression (Fig. 3A) such as silencing of AKAP2 (AKAP2_{KD}), and its re-expression

with either AKAP2-wt or AKAP2-ΔPKA. The presence of AKAP2 protein after these modifications was confirmed by western blot analysis of their cell lysates: PC-3 and PC-3CTR cells displayed AKAP2 immunoreactivity (Lanes 1, 2 of Fig. 3A). AKAP_{KD} cells lacked AKAP2 immunoreactivity (Lane 3 of Fig. 3A), but it reappeared in AKAP2-Re-exp (re-expression) and AKAP2-ΔPKA cells (Lanes 4, 5 of Fig. 3A).

Cell proliferation of AKAP2-modulated PC cell lines

As expected, CT did not alter the proliferation of PC-3V cells (lack endogenous CTR) (Chien et al. 2001). However, PC3-CTR cells displayed higher rate of proliferation; and CT significantly increased their rate of proliferation (Fig. 3B). The knock-down of AKAP2 by itself remarkably reduced the rate of cell proliferation of PC-3CTR cells, and CT-stimulated increase was also remarkably attenuated. Re-expression of AKAP2 restored the baseline rate of cell proliferation, and partially restored CT response. However, the re-expression of AKAP2ΔPKA did not restore the baseline proliferation rate and only partially restored CT-stimulated proliferation. These results suggest that AKAP2-PKA complex may mediate proliferative actions of CT on PC-3CTR cells only partially, and AKAP2-independent mechanisms may also be involved.

Invasion

CT significantly increased invasion of PC-3CTR cells (Fig. 3C). AKAP2_{KD} cells were remarkably less invasive; and CT could not increase the invasion further. Invasion of AKAP2-reexpressed cells was comparable to PC3-CTR cells; and CT increased it further. However, the baseline as

Table 1 Organ weights

Organs	PC-3-CTR	AKAP2 _{KD}	AKAP2 Re-exp.	AKAP2Δ PKA
Seminal vesicles	0.34±0.12	0.03±0.02	0.23±0.11	0.06±0.07
Testes	0.36±0.13	0.04±0.02	0.25±0.12	0.06±0.06
Lymph nodes				0.14±0.10
Mesentery	0.20±0.12	0.05±0.04	0.31±0.14	0.03±0.03
Femur	0.50±0.16	0.06±0.05	0.38±0.17	0.07±0.05
Stomach			0.45±0.11	
Kidneys	0.28±0.066	0.05±0.04	0.33±0.13	0.07±0.10
Lungs	0.48±0.16	0.07±0.05	0.45±0.17	0.06±0.05
Heart	0.41±0.15	0.06±0.06	0.34±0.14	0.05±0.06
Brain	0.30±0.14	0.06±0.06	0.24±0.10	0.05±0.05
Liver	0.37±0.15	0.04±0.03	0.37±0.12	0.06±0.07

Table 2 Frequency of micrometastases in host organs

Organs	PC-3CTR	AKAP _{KD}	AKAP2- ΔPKA	AKAP2 _{REEXP}	Number of animals (n)
Seminal vesicles	6	0	0	6	6
Lymph nodes	6	1	1	6	6
Femur	4	0	0	3	6
Lungs	3	0	0	3	6
Liver	3	0	0	3	6
Kidneys	3	0	0	3	6
Brain	1	0	0	1	6

well as CT-stimulated invasion of AKAP2ΔPKA cells was comparable to that of AKAP2^{KD} cells. These results demonstrate that AKAP2 plays an important role in the invasion of PC-3CTR cells by tethering the PKA activated by CTR; and CTR-stimulated PKA-induced phosphorylation may play a key role in CT-stimulated invasion of these cells.

Clonogenic growth

Cancer cells, but not normal cells, can grow in the absence of substratum or display anchorage-independent growth. Growth in soft agar is reflective of their tumorigenicity and invasiveness. We examined the role of CTR and AKAP2 in tumorigenicity/invasiveness of PC-3 cells by their ability to form colonies on soft agar. The results of Fig. 3D demonstrate that the expression of CTR alone in PC-3 cells caused a dramatic increase in their ability to form colonies. These results are consistent with our earlier report (Thomas *et al.* 2006). AKAP2^{KD} cells displayed greatly reduced ability to grow or form colonies in soft agar. AKAP2-wt re-expression restored this ability, but AKAP2ΔPKA re-expression did not. These results confirm that AKAP2-PKA complex plays a key role in tumorigenicity and invasiveness of PC cells.

Role of AKAP2 in tumorigenicity and metastatic capacity of PC-3CTR cells

We next examined the role of AKAP2 on tumor growth and metastasis of PC-3 cells in orthotopic nu/nu mouse model. We have already shown that the enforced CTR expression significantly increases the ability of PC-3 cells to form orthotopic tumors as well as distant metastases (Thomas *et al.* 2006, Shah *et al.* 2008). In this study, we examined the ability of AKAP2 to modulate tumor growth

and metastatic capacity of PC-3CTR cells. AKAP2^{KD} cells displayed significantly lesser ability to form orthotopic tumors as assessed by the wet weight of the prostate at necropsy (Fig. 3E). However, the re-expression of AKAP2-wt completely restored this ability, while the re-expression of AKAP2ΔPKA did not (Fig. 3E).

We also examined the ability of these cells to form distant metastases in select organs. PC-3-CTRwt cells frequently formed micrometastases in multiple organs, including lymph nodes, femur and lungs (Fig. 3F, Tables 1, 2, and 3). In contrast, AKAP2^{KD} cells did not form micrometastases in all tested organs except one out of six mice showed minor lymph node involvement. AKAP2ΔPKA re-expressed cells also displayed a similar behavior. However, AKAP2-wt re-expressed cells formed metastases in several organs. H&E sections of these tumors are presented in Figs S1-S2, see section on supplementary data given at the end of this article.

Elevation of CTR and AKAP2 expression in primary prostate tumors

Since the present results have identified an important role for AKAP2 in CTR-induced tumor growth and metastasis, we examined CTR and AKAP2 expression in paraffin-embedded specimens of primary human prostate cancer. We selected a cohort of 83 prostate specimens consisting of BPH, localized PC and metastatic PC. As shown in Fig. 4A, all BPH specimens displayed almost undetectable levels of AKAP2 (*n*=16). In contrast, all PC specimens displayed visible-to-intense expression of AKAP2 (*n*=67). When stratified the PC specimens into metastatic vs localized, the AKAP2 IHC index of metastatic PC specimens was significantly higher than localized PCs (Fig. 4B). CTR expression also followed a similar pattern (Fig. 4C and D). These results suggest that increased AKAP2 levels may facilitate the action of increased CTRs to promote PKA-mediated phosphorylation of key proteins, which are important for metastasis of PC.

Table 3 Micrometastases in host organs

Organs	PC-3CTR	AKAP _{KD}	AKAPΔPKA	AKAP _{REEXP}
Seminal vesicles	+++	—	—	+++
Lymph nodes	+++	—	—	++
Femur	+++	—	—	++
Lungs	++	—	—	+
Liver	++	—	—	+
Kidneys	++	—	—	+
Brain	±	—	—	±

Figure 4

AKAP2 expression in human prostate tissues: immunofluorescence. (A) Representative micrographs of AKAP2 immunofluorescence in BPH, localized PC and metastatic PC. (B) AKAP2-positive cells/400 $\times$ field were counted as described in the methods section. The graph presents quantitative data (mean $\pm$ s.e.m. for $n=37$ for localized PC and $n=30$ for

metastatic PC; $*P<0.001$, paired t-test). (C) Representative micrographs of CTR immunofluorescence in BPH, localized PC and metastatic PC. (D) CTR-positive cells/400 $\times$ field were counted as described in the methods section. The graph presents quantitative data (mean $\pm$ s.e.m. for $n=37$ for localized PC and $n=30$ for metastatic PC; $*P<0.001$, paired t-test).

Discussion

Earlier studies from this laboratory have reported that CT is a potent stimulator of prostate cancer cell invasion (Thomas *et al.* 2006). However, specific, cell-permeable inhibitors of PKA almost abolish CT-induced invasion, suggesting that the pro-invasive actions of CT involve PKA-mediated mechanisms (Sabbisetti *et al.* 2005). Considering that PKA catalyzes several cellular processes such

as metabolism, gene transcription, ion channel conductivity, cell growth, cell division and rearrangement of the actin cytoskeleton (Carr *et al.* 1991, Francis & Corbin 1994, Carr *et al.* 2001), it is now accepted that the specificity of PKA action to a stimulus is provided by spatial mechanisms involving adaptor proteins such as AKAPs that target PKA towards particular substrates (Carnegie & Scott 2003, Wong & Scott 2004, Lynch *et al.* 2005). AKAPs also recruit

other signaling molecules to form localized signaling hubs (Wong & Scott 2004, Lynch *et al.* 2005). Present results have used multiple experimental approaches to demonstrate that AKAP2 is expressed in human prostate, and it plays a key role in mediating the actions of CTR, and possibly other receptors, in stimulating tumor growth and metastasis. Specifically, the results that AKAP2 silencing remarkably reduced tumorigenicity and metastatic capacity of PC cells; and the re-expression of AKAP2-wt, but not AKAP2-ΔPKA, restored their tumorigenic and metastatic capacity demonstrate that AKAP2-tethered PKA is crucial for CTR-stimulated prostate cancer growth and metastasis (Fig. 5). Interestingly, this is the first known demonstration of the presence of AKAP2 in human prostate, and its role in PC progression.

The results of immunofluorescence revealed that AKAP2 was co-localized predominantly with ZO-1 but not as much with E-cadherin. Moreover, ZO-1 as well as AKAP2 co-precipitated with CTR, suggesting that the interaction among these three proteins may be important for CTR-induced invasion. The results raise several interesting possibilities. For example, ZO-1, besides being the marker of tight junctions, is an important scaffolding protein with multiple interacting domains including three PDZs, a SH3, a guanylate kinase (GK), actin binding region, and a ZU5 (ZO-1 and Unc5-like netrin receptor domain) (Fanning *et al.* 2002). ZO-1 acts as a key organizer of tight junctions (TJs) at the plasma membrane (Li *et al.*

2005). Based on co-localization and co-precipitation evidence, it appears that ZO-1 brings CTR into close proximity of AKAP2, where CTR can quickly and selectively activate this pool of PKA. Thus, AKAP2 may ensure that the PKA will phosphorylate proteins of junctional complexes. These results can explain multiple actions of CTR such as destabilization of cell-cell junctions, loss of cell adhesion, increase in invasion and the induction of EMT (Shah *et al.* 2009b). The cascade that follows this event may include loss of epithelial integrity, loss of cell polarity, increase in invasion, proliferation and activation of survival pathways that attenuate apoptosis and anoikis (Fig. 6). Further identification and functional characterization of AKAP2 – ZO-1 scaffold and its interaction with CTR will be critically important in revealing CTR-activated events associated with PC progression.

If AKAP2 plays an important role in prostate tumor growth/metastasis, one would expect its upregulation in aggressive prostate cancer. Although its expression in PC was not individually studied, available transcriptome profiling of human prostate cancer specimens suggested an upregulation of AKAP2 gene transcription in metastatic prostate cancer (Lapointe *et al.* 2004). However, there is no evidence for its physiological function or its role in prostate cancer pathogenesis. Therefore, we examined AKAP2 protein expression along with that of CTR in a cohort of human prostate cancer specimens.

Figure 5

Schematic diagram depicting the AKAP2, its PKA-binding domain and the impact of deletion of PKA-binding domain or st-Ht-31 action on the functionality of AKAP2.

Figure 6

Schematic diagram depicting mechanism of CTR action that involves AKAP2 and leads to EMT, increased invasion and tumor growth.

Present results – that both CTR and AKAP2 were very low in benign prostates but were very strong in metastatic prostate cancers – reinforce the association between CTR, AKAP2 and aggressive form of PC. Indeed, a detailed study with a larger cohort will be necessary to investigate the usefulness of AKAP2 as a prognostic indicator for metastatic form of PC.

In conclusion, present results have identified new molecular mechanisms associated with CTR-activated PC progression and metastasis. First, CT-activated pro-invasive actions require AKAP2, a novel finding. Co-localization of AKAP2 with ZO-1 suggests that the PKA tethered to AKAP2 is activated by CTR, and may phosphorylate ZO-1 or its scaffold proteins. This may lead to destabilization of cell-cell junctions, and lead to the loss of epithelial integrity and loss of cell polarity, eventually resulting in EMT and increased invasion (Fig. 6). Upregulation of

AKAP2 in metastatic prostate cancer and its importance in tumor growth/metastasis suggest that AKAP2 may serve as a prognostic indicator of cancer progression and as a new target for molecular therapy of advanced prostate cancer.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0425>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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