Identification of Ran-binding protein M as a stanniocalcin 2 interacting protein and implications for androgen receptor activity

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Stanniocalcin (STC), a glycoprotein hormone originally discovered in fish, has been implicated in calcium and phosphate homeostasis. While fishes and mammals possess two STC homologs (STC1 and STC2), the physiological roles of STC2 are largely unknown compared with those of STC1. In this study, we identified Ran-binding protein M (RanBPM) as a novel binding partner of STC2 using yeast two-hybrid screening. The interaction between STC2 and RanBPM was confirmed in mammalian cells by immunoprecipitation. STC2 enhanced the RanBPM-mediated transactivation of liganded androgen receptor (AR), but not thyroid receptor β, glucocorticoid receptor, or estrogen receptor β. We also found that AR interacted with RanBPM in both the absence and presence of testosterone (T). Furthermore, we discovered that STC2 recruits RanBPM/AR complex in T-dependent manner. Together, our findings suggest that STC2 is a novel RanBPM-interacting protein that promotes AR transactivation.

INTRODUCTION

Stanniocalcin 1 (STC1) was first identified in the corpuscles of Stannius in bony fishes as a glycoprotein hormone that inhibited calcium uptake in the gills and intestine, and stimulated phosphate reabsorption in the kidney (1). Different from its role in fish, mammalian STC1 seems to play various roles in developmental (2), physiological (3), and pathological processes (4, 5). A paralog of STC1, STC2, has been identified in mammals and fishes (6, 7). STC2 is also a glycoprotein hormone that is widely expressed in fish (7) and mammals (6, 8). Human STC2 is similar to human STC1 in terms of its genomic structure and the conservation of cysteine residues and glycosylation sites in the mature proteins (9). However, the amino acid sequence of human STC2 shows low sequence identity (~30%) with human STC1 and includes a characteristic C-terminal histidine-rich region (10).

STC2 inhibits phosphate uptake in a renal proximal tubular cell line and is regulated by the calcium- and phosphate-regulating compounds 1,25-dihydroxyvitamin D3 and parathyroid hormone (11). Additionally, STC2 is induced in estrogen receptor-positive breast cancer cells by 17β-estradiol (E2) (12) and increases the resistance to apoptotic cell death via an unfolded protein response, which is induced by the accumulation of misfolded proteins in the endoplasmic reticulum (13). During ovarian development in rats, STC2 suppresses gonadotropin-induced progesterone biosynthesis by ovarian granulosa cells, similar to STC1 (14). However, the physiological roles of STC2, compared with STC1, are still largely unknown in both mammals and fishes.

To reveal the molecular partners for STC2 in male reproductive organs and to understand the biological significance of their molecular partnership, we first performed yeast two-hybrid screening of a human testis cDNA library using human STC2 as a bait and found Ran-binding protein M (RanBPM) as a STC2-interacting protein. RanBPM is a multifunctional protein that interacts with a broad spectrum of proteins involved in microtubule formation (15), apoptosis (16), and gonad development (17). Recent studies have reported that RanBPM can interact with several nuclear receptors (NRs) and influence their transactivation (18, 19). Regarding the regulation of NR transactivation by RanBPM, we found that the overexpression of STC2 enhanced the function of RanBPM as a co-activator in androgen receptor (AR) transactivation. Moreover, the interaction between STC2 and RanBPM was enhanced by AR in the presence of testosterone. In conclusion, we suggest that STC2 regulates the transactivation of AR through RanBPM.

Received 8 May 2014, Revised 21 May 2014, Accepted 11 August 2014

Keywords: Androgen receptor, RanBPM, STC2, Testosterone, Yeast two-hybrid
RESULTS

Identification of RanBPM as a STC2-interacting protein
From several rounds of yeast two-hybrid screening (4.7 × 10^8 cells) using a bait encoding the mature peptide region of human STC2 (STC2M; amino acid residues 24-302; Fig. 1A), eight His^+ colonies were identified in a human testis cDNA library. Among them, one colony was a strongly positive clone by a β-galactosidase assay (Fig. 1B). The nucleotides of the insert were in-frame with a RanBPM cDNA fragment encoding amino acid residues 148-729 (GenBank Accession No. NM005484) (Fig. 1C and Supplementary Fig. 1). RanBPM has multiple domains, including a polyglutaminated region, splA and Ryr domain (SPRY), a lissencephaly type-I-like homology motif (LisH), and a C-terminal to LisH motif (CTLH), which are likely associated with protein-protein interactions.

In vivo interaction between STC2 and RanBPM
The potential interaction of RanBPM with STC2 in mammalian cells was investigated by immunoprecipitation. 293T cells were co-transfected with STC2 mature peptides-histidine (STC2M-HIS) expression vectors, or their maternal ones. When cell lysates were subjected to immunoprecipitation with HA followed by immunoblotting with HIS, STC2M-HIS protein (30 and 35 kDa) was found to be immunoprecipitated by RanBPM-AD-HA (64 kDa) and vice versa, whereas no band was detected in the control cell lysates (Fig. 1D). These results demonstrated that STC2 interacted with RanBPM in 293T cells.

Subcellular distribution of STC2 and RanBPM
To examine the subcellular localizations of STC2 and RanBPM, 293T cells were cotransfected with the STC2M-HIS and RanBPM-AD-HA expression vectors followed by indirect immunofluorescence detection (Fig. 2A). The red fluorescence of STC2M-HIS showed strong immunoreactivity in the cytoplasm (Fig. 2B). Immunoreactivity for RanBPM-AD-HA was observed in both the nucleus and cytoplasm (Fig. 2C). Merged images showed that STC2 and RanBPM were co-localized in the cytoplasm (Fig. 2D).

Effect of STC2 on RanBPM-mediated nuclear receptor transactivation
Several nuclear receptors (NRs) interact with RanBPM, and the transcriptional activities of NRs are stimulated by RanBPM in a ligand-dependent manner (18, 19). To understand the role of STC2 in the transcriptional regulation of NRs, we tested whether STC2 activated RanBPM-mediated NR transactivation. Similar to previous reports, RanBPM enhanced transactivation of rat androgen receptor (rAR), rat thyroid receptor β (rTRβ), rat glucocorticoid receptor (rGR), and mouse estrogen receptor β were co-transfected with RanBPM-activating domain-hemagglutinin (RanBPM-AD-HA) and STC2 mature peptides-histidine (STC2M-HIS) expression vectors, or their maternal ones. When cell lysates were fixed and incubated with HIS (for STC2M) or HA (for RanBPM-AD) antibody, followed by incubation with red-fluorescent Alexa Fluor 594 (for STC2M-HIS) (B) or green-fluorescent Alexa Fluor 488 (for RanBPM-AD-HA) (C) conjugated secondary antibody. The cells were examined by confocal laser scanning microscope. Merged and phase-contrast images (D). Number of immunoreactive or co-immunoreactive cells / number of total cells.
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Fig. 3. STC2 enhances the effect of RanBPM-induced AR activation. 293T cells were transfected with pcDNA3-STC2M-HIS (50-200 ng), pcDNA3-RanBPM-HA (50-200 ng), NR expression vectors including rAR (10 ng) (A), rTRβ (5 ng) (B), rGR (100 ng) (C), or mERβ (100 ng) (D), their respective luciferase reporters (MMTV-Luc, TRE-Luc, and ERE-Luc reporter gene; 200 ng each), along with LacZ expression vector (20 ng). After transfection, cells were treated for 24 h with T (10⁻⁹, 10⁻⁸ M), T3 (10⁻⁸, 10⁻⁷ M), DEX (10⁻⁸, 10⁻⁷ M), or E2 (10⁻⁹, 10⁻⁸ M). Luciferase activity was measured and normalized to the internal β-galactosidase control. Data are shown as means ± SEM of triplicate separate transfections. Differences were considered significant at P < 0.05. All lowercase letters (a-g) indicates significant differences through post hoc comparisons.

Fig. 4. Liganded AR enhances interaction of RanBPM with STC2. (A) Association of RanBPM with liganded or unliganded AR. pcDNA3-RanBPM-HA (1 μg) and AR (0.5 μg) expression vectors were co-transfected into 293T cells and treated with T (10⁻⁹ M). After an additional 18 h, products immunoprecipitated with the AR antibody were analyzed with the HA antibody (for RanBPM). Control lysate (CL) from 293T cells co-transfected with pcDNA3 empty vector was also immunoprecipitated with the AR antibody. (B) Association between STC2 and AR. pcDNA3-STC2M-HIS (1 μg) and pcDNA3-AR (0.5 μg) expression vectors were co-transfected into 293T cells and treated with T (10⁻⁸ M). After an additional 18 h, products immunoprecipitated with the HIS antibody (for STC2M) were analyzed with the AR antibody. Control lysate (CL) from 293T cells co-transfected with pcDNA3 vector or pcDNA3-AR was immunoprecipitated with the HIS antibody, followed by immunoblotting with the AR antibody. (C) Effects of AR on the interaction between STC2M and RanBPM in the absence or presence of T. Total cell lysates were prepared from 293T cells that were co-transfected with STC2M-HIS (1 μg), RanBPM-HA (1 μg), and AR expression vectors (0.5 μg), and treated with T as indicated for 18 h. Immunoprecipitated products with HIS antibody were analyzed by immunoblotting with HA for RanBPM or AR antibody. (D) A putative working model for how T may affect the steady-state AR-STC2-RanBPM complex. In the absence of T, AR preferentially interacted with RanBPM. Upon ligand binding (T-activated state), AR appeared to mediate the association of the STC2-RanBPM.

DISCUSSION
In this study, we demonstrated that STC2 is a RanBPM-interacting protein. Although the biological role of RanBPM remains unclear, it interacts with Ran-GTPase, which functions in mi-
toxic spindle formation, microtubule formation, and nucleocytoplasmic transport (15). RanBPM also interacts with several NRs and the interaction upregulates transactivation in a ligand-dependent manner (18, 19). Based on these observations, we hypothesized that STC2 is implicated in RanBPM-regulated NR transactivation. As expected, overexpression of RanBPM augmented liganded AR, TRβ, GR, and ERβ transactivation. Although RanBPM induced AR- and GR-, but not ER-mediated transactivation (19), our results showed that ERβ transactivation was also influenced by the overexpression of RanBPM. Collectively, the present study suggests that STC2 enhances RanBPM-mediated AR transactivation in a T-dependent manner, but not for liganded TRβ, GR, or ERβ. Further studies with an available STC2 antibody to detect endogenous STC2 expression would be important in delineating the effect of STC2 for the AR/RanBPM interaction in the presence of T.

Liganded AR was also co-purified with RanBPM/STC2, suggesting that STC2 is a tentative partner of the RanBPM/AR complex. STC2 was initially identified as a cytoprotective protein in hypercalcemia (20), hypoxia (21, 22), endoplasmic reticulum stress (13, 20, 23), and oxidative stress related to the unfolding protein response (UPR) (13). To examine the forming complex of RanBPM/STC2, we preferentially explored the involvement of intracellular calcium in the interaction of STC2 with RanBPM. Although both STC2 and RanBPM have been shown to be important regulators of calcium signaling (20, 24, 25), no notable effect was detected in the interaction between the two proteins by calcium chloride treatment (Supplementary Fig. 2). These results suggest that the STC2/RanBPM complex is at least in part not influenced by an influx of calcium.

Androgens and AR are key molecules in development of the prostate and need to interact with coactivators to control their physiological function. AR transactivation is mediated by AR nuclear importing via Ran-GTP (26) and interacting with coregulators, such as Ran/ARA24 (27) and ARA267-α (28), which interact with RanBPM. RanBPM has been reported to interact with many proteins, activating a broad spectrum, and diverse biological functions have been suggested according to the different partners (29). Considering that RanBPM can interact with apoptotic activators, such as actylcholinesterase (AChE) (30) and cyclin-dependent kinase 11 (CDK11, p46) (31), in the cytoplasm, it may be that STC2 is able to recruit liganded AR/RanBPM as a stress-responsive complex to protect androgen-sensitive cells and/or tissues, such as the testis and prostate.

In summary, we demonstrated that STC2 is an interacting partner of RanBPM that can stimulate RanBPM-induced AR transactivation. Moreover, we showed that STC2 is able to recruit the RanBPM/AR complex in a T-dependent manner (illustrated in Fig. 4D), although there were slight differences between AR-STC2 direct interaction and the tentative ternary complex AR-RanBPM-STC2 in T-dependent action. Our data provide insight into the physiological function of STC2 in AR-mediated gene expression and its cytoprotective role in androgen-sensitive cells.

MATERIALS AND METHODS

Yeast strains, cDNA library, and bait cDNA

The yeast strain AH109 and a pretransformed human testis cDNA library in the vector pACT2 (Clontech, CA, USA) were used in the yeast two-hybrid screenings. Yeast extract-peptone-dextrose (Clontech) was used as the standard medium for yeast cell culture. As a bait, cDNA corresponding to the mature peptide region of human STC2 (STC2M; Fig. 1A) was PCR-amplified using forward (5’-CGCGAATTCCGGAGCCGACCAACCA-3’) and reverse (5’-TCTGATATCCGGAGGTGAGGATCACCAC-3’) primers with human STC2 cDNA (gift from Regeron Inc., Chuncheon, Republic of Korea) as a template. The product was subcloned into pGBT9 (Clontech) using primer-derived EcoRI and Xhol sites to construct pGHT9-STC2M.

Yeast two-hybrid screening

AH109 cells were transformed with pGHT9-STC2M expression vector and mated with Y187 yeast cells containing a human testis cDNA library according to the manufacturer’s protocol (Clontech). The mated yeast cells were plated on minimal medium lacking histidine, tryptophan, and leucine (SD/His, Trp, Leu-) in the presence of 20 mM 3-amino-1,2,4-triazole (Sigma-Aldrich, St. Louis, MO, USA). Prey vectors from the surviving yeast clones were isolated using the phenol:chloroform extraction method, retransformed into Y187 cells, and checked by a colony-lift β-galactosidase expression assay according to the manufacturer’s instructions (Clontech; Fig. 1B). The DNA sequences of the positive clones were analyzed using an ABI 3100 DNA sequencer (Applied Biosystems, CA, USA).

Mammalian expression vectors

STC2M cDNA (837 bp) was excised from pGBT9-STC2M using EcoRI and Xhol. Next, using primer set including hexahistidyl1 peptide (HIS-Tag) (5’-CGCGAATTCCGGAGCCCCACCAAACCC-3’ and 5’-TATTCTGATATCCGGAGGTGAGGATCACCAC-3’) and sequence encoding HA (5’-CGCGAATTCCGGAGCCCCACCAAACCC-3’ and 5’-TATTCTGATATCCGGAGGTGAGGATCACCAC-3’), we subcloned the STC2M fragment into pcDNA3 (32), resulting in a HIS-tagged STC2M expression vector. RanBPM cDNA including the activation domain (RanBPM-AD) was amplified using a primer set containing restriction sites for EcoRV and Xhol and sequence encoding hemagglutinin (HA-Tag) (5’-CGCGAATTCCGGAGCCCCACCAAACCC-3’ and 5’-TATTCTGATATCCGGAGGTGAGGATCACCAC-3’) into the isolated RanBPM partial cDNA as the template. Full-length cDNA encoding RanBPM cDNA (2190 bp) was amplified from pCMV-SPORT6-RanBPM (Benebiosis Co., Seoul, Republic of Korea) as a template. The DNA sequences of the positive cDNA clones were analyzed using an ABI 3100 DNA sequencer (Applied Biosystems, CA, USA).

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AD and RanBPM cDNAs were inserted into the EcoRV and Xhol sites of pcDNA3 to construct HA-tagged RanBPM-AD and RanBPM (Fig. 1C).

**Immunoprecipitation and immunoblotting**

293T (human embryonic kidney) cells were cultured in 6-well plates in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA, USA) in a humidified atmosphere containing 5% CO₂ at 37°C. The cells (2 × 10⁶/well) were transiently transfected with pcDNA3-RanBPM-AD-HA (1 μg), pcDNA3-STC2M-HIS (1 μg), and/or pcDNA3-AR (0.5 μg) using the polyethylenimine reagent (Sigma-Aldrich). At 24 h after transfection, T (Sigma-Aldrich) was applied to the culture medium at the indicated concentrations. After an additional 18 h, the cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in 0.5 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 170 mM NaCl, 5 mM EDTA, 0.5% Nonidet-P40, and 1 mM DTT) supplemented with a protease inhibitor cocktail (including EDTA, disodium salt; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cell lysates were precleared with protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 4 h. The pre-cleared lysates were then incubated with 1 μg of HA antibodies (for RanBPM; Sigma-Aldrich), HIS antibodies (for STC2M; Santa Cruz Biotechnology), or AR antibodies (Santa Cruz Biotechnology) overnight at 4°C, after which the immunocomplexes were adsorbed to protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 4 h. The resins were washed three times with lysis buffer, and the bound proteins were detected by SDS-PAGE and Western blot analysis as described in [32, 33].

**Immunofluorescence**

293T cells were washed twice with cold PBS and fixed in 4% paraformaldehyde at room temperature for 15 min. The cells were subsequently washed twice with PBS and treated with a permeabilization solution (0.1% Triton X-100 in PBS) on ice for 5 min and incubated in PBS containing 3% bovine serum albumin (BSA) for 1 h. The cells were then incubated with antibodies against HA (1 : 200) or HIS (1 : 100) in PBS containing 3% BSA overnight at 4°C. After incubation, the cells were washed and incubated with green-fluorescent Alexa Fluor 488 or red-fluorescent Alexa Fluor 594 (Invitrogen) in the dark at 4°C for 1 h. After washing, the cells were mounted in mounting solution (Sigma-Aldrich) and observed by confocal laser scanning microscopy (Fluoview FV300; Olympus, Tokyo, Japan).

**Luciferase assay**

293T cells were cultured with DMEM supplemented with 10% charcoal-stripped FBS (Invitrogen) for 24 h in a humidified atmosphere containing 5% CO₂ at 37°C. Transfection, ligand treatment, and a luciferase assay were performed as described previously [32]. Briefly, 5 × 10⁴ cells were plated in 24-well plates and transiently transfected with LacZ expression vector (pRSV-β-galactosidase) and luciferase reporter vector (mouse mammary tumor virus, MMTV-Luc, thyroid hormone response element, (TRE-Luc, and estrogen response element, ERE-Luc), along with the indicated amounts of various mammalian expression vectors (RanBPM-HA, STC2M-HIS, rAR, rTRβ, rGR, and mERβ) using the polyethylenimine reagent (Sigma-Aldrich). The mammalian expression vectors used in this study encoding MMTV-Luc, TRE-Luc, ERE-Luc, LacZ, rAR, rTRβ, rGR, and mERβ were described previously [33, 34]. At 24 h after transfection, T, E2, dexamethasone (DEX), triiodothyronine (T3), or the same amount of dimethyl sulfoxide (Sigma-Aldrich) was applied to the culture medium at the indicated concentrations. After 18 h, the cells were harvested and the level of luciferase activity was assayed using a microplate luminometer (Berthold Co., TN, USA) and normalized by the β-galactosidase values. All data shown are means ± SEM of at least triplicate separate transfections. All analyses were performed using the SPSS software (ver. 18.0; SPSS Inc., IL, USA). For multiple comparisons, a one-way ANOVA followed by a post hoc Tukey test was used. Differences were considered significant at P < 0.05.

**ACKNOWLEDGEMENTS**

This work was supported by the National Research Foundation of Korea (NRF) grant, funded by the Korean government (MEST: 2012R1A1A2044506; NRF-2009-0070344), and Gangneung-Wonju National University (2013 visiting scholar program).

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