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Stanniocalcin 2, forms a complex with heme oxygenase 1, binds hemin and is a heat shock protein

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ABSTRACT

Stanniocalcin 2 (STC2) is a homolog of stanniocalcin 1, a 56 kD glycoprotein hormone that originally was found to confer calcitonin-like activity in fish. Human STC2 is expressed in various tissues such as kidney, spleen, heart, and pancreas. STC2 has been demonstrated to be induced by different kinds of stress and display cytoprotective activity, but the molecular mechanism is poorly understood. Heme oxygenase 1 (HO1) degrades heme to biliverdin, carbon monoxide and free iron, and is a stress-responsive protein.

Using yeast two-hybrid screening we identified HO1 as a binding partner of STC2. The interaction was validated by in vivo co-immunoprecipitation and immunofluorescence. The binding site for HO1 was located to amino acids 181–200 of STC2. We also found that STC2 binds hemin via a consensus heme regulatory motif. Moreover, STC2 expression was induced by heat shock in HEK293 cells. Taken together, our findings point to three novel functions of STC2, and suggest that STC2 interacts with HO1 to form a eukaryotic 'stressosome' involved in the degradation of heme.

1. Introduction

Stanniocalcin 2 (STC2) is a homodimeric glycoprotein with approximately 34% homology to its paralog STC1, which is an anti-hypercalcemic hormone, first discovered in fish [1–3]. The mammalian STC2 gene is widely expressed in tissues such as kidney, spleen, heart, and pancreas. There are several evidences showing STC2 is involved in cancerous growth [4–8]. STC2 is also related to repression of cell growth in vivo. *STC2* knockout mice are 10–15% bigger and grow at a faster rate than wild-type mice [9] while overexpression of human STC2 cDNA in mice resulted in 45% smaller than wild-type [10]. Upregulated expression of STC2 is induced by stress caused by the unfolded protein response [11]. STC2 has recently been found to play a neuroprotective role against kainic acid toxicity in the hippocampus of ICR mice [12]. Taken together, these findings suggest that STC2 functions as a stress protein. However, the underlying molecular mechanism is still poorly understood, and no receptor for STC2 has been found.

Heme oxygenase 1 (HO1) catalyzes degradation of heme to generate carbon monoxide, free iron and biliverdin [13,14]. Besides heme, HO1 expression is induced by heavy metals, heat shock, inflammatory cytokines, prostaglandins and agents that cause oxidative stress [15,16]. HO1 is induced by hyperthermia in brain, testis, cochlea, intestine, hepatoma cells and glial cells of the hippocampus [17–22]. Additionally, HO1 has been found to have cytoprotective effect against oxidative, hypoxic, ischemic and inflammatory damage in multiple tissues and organs [23–25] but the detailed molecular mechanism is still unclear.

Heme, the substrate of heme oxygenases, is a prosthetic group that consists of an iron atom in the center of a porphyrin ring. In addition to hemoglobins and cytochromes, a number of proteins contain short heme regulatory motifs (HRM) which bind heme, such as yeast transcriptional activator HAP1, mammalian δ-aminolevulinate synthase, rabbit heme-regulated initiation factor (HRI) kinase, and iron regulatory protein 2 [26,27]. The conserved core of Cys-Pro motif in HRM binds heme by forming iron-cysteine thiol bonds [26]. A shift of the heme absorption spectrum is commonly used to detect heme binding of different proteins [26,27].

We report here that STC2 interacts with HO1 in vivo and that STC2 binds heme. We also show that STC2 can be induced by heat shock.

2. Materials and methods

2.1. Oligonucleotides and plasmid construction

In transient transfection experiments, human STC2 cDNA was cloned into the p3xFLAG-CMV-14 expression vector (Sigma) through *Eco*R1 and *Bam*H1 digestion sites to produce C-terminally FLAG-tagged *STC2*. Mutated STC2 cDNA fragments were amplified with the above construct as template, and cloned into the same expression vector at the same digestion sites. Human HO1 cDNA

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Table 1
Primer sequences for STC2 and HOI constructs and quantitative RT-PCR.

	Primer sequences
Constructs	
p3xFLAG-CMV-14-*hSTC2*	5′-GCGAATTCAATGTGTGCCGAGCGGCTGGGC-3′ 3′-ACTCATAAGACTATAGGCTCCCCTAGGCG-5'
p3xFLAG-CMV-l4-*hSTC2*Δ181–200	(first step, first part) 5′-GCGAATTCATTAACCACCATGTGTGCCGAGCGGCTGGGC-3′ 3′-AGCACTTGAACGACGACTGGACCCCTTCGG-5′ (first step, second part) 5′-GCTGCTGACCTGGGGAAGCCTGTGCTCCAT-3′ 3′-ACTCATAAGACTATAGGCCTCCCCTAGGCG-5′ (second step) 5'-GCGAATTCATTAACCACCATGTGTGCCGAGCGGCTGGGC-3′ 5′-ACTCATAAGACTATAGGCCTCCCCTAGGCG-5′
p3xFLAG-CMV-l4-*hSTC2*Δ191-200	(first step, first part) 5′-GCGAATTCATTAACCACCATGTGTGCCGAGCGGCTGGGC-3′ 3′-TCCACTTCCTCCGGTAGTGGACCCCTTCGG-5′ (first step, second part) 5′-GGCCATCACCTGGGGAAGCCTGTGCTCCAT-3′ 3′-ACTCATAAGACTATAGGCCTCCCCTAGGCG-5′ (second step) 5′-GCGAATTCATTAACCACCATGTGTGCCGAGCGGCTGGGG-3′ 3′-ACTCATAAGACTATAGGCCTCCCCTAGGCG-5′
p3xFLAG-CMV-14-*hSTC2*Δ129–190	(first step, first part) 5′-GCGAATTCATTAACCACCATGTGTGCCGAGCGGCTGGGG-3′ 3′-CGGCCTTCACGGGCCGGTAGGTGTCGCACG-5′ (first step, second part) 5′-GCCCGGCCATCCACAGCGTGCAGGTTCAGTG-3′ 3′-ACTCATAAGACTATAGGCCTCCCCTAGGCG-5′ (second step) 5′-GCGAATTCATTAACCACCATGTGTGCCGAGCGGCTGGGG-3′ 3′-ACTCATAAGACTATAGGCCTCCCCTAGGCG-5′
p3xFLAG-CMV-l4-*hSTC2*ΔC237–302	5′-GCGAATTCATTAACCACCATGTGTGCCGAGCGGCTGGGG-3′ 3′-ACCTGTCTTGGTTCGAGAGGTCCCCTAGGCG-5′
p3xFLAG-CMV-14-*hSTC2*ΔN1–101	5′-GCGAATTCATTAACCACCATGTTCATCAAAGACGCCTTGAAATG-3′ 3′-ACTCATAAGACTATAGGCCTCCCCTAGGCG-5′
pAMC-*hHO1*	5′-ATATCGTCGACATGGAAGCGTCCGCAACCC-3′ 3′-CCCGAAATACGGTACACTCGCCGGCGAATTC-5′
Quantitative RT-PCR	
human *STC*2	5′-ACTTTTCTGCACAACGCTGG-3′ 3′-ATGCACCTGGAGCACTTGAA-5′
human *STC*1	5′-ACAGCAAGCTGAATGTGTGC-3′ 3′-TGTCTGAACAGGCTTCGGAC-5′
human *Hsp*70	5′-TCCTTCGTTATTGGAGCCAG-3′ 3′-CCTGCGTCTAAACTACTACG-5′
human *GAPDH*	5′-GGTGAAGGTCGGAGTCAAC-3′ 3′-CTTCCGACCCCGAGTAAAC-5′

was amplified from whole BeWo cDNA sample and cloned into the pAMC expression vector (a gift from Dr. Tatiana Petrova, Molecular and Cancer Biology Program, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland) through *Sal*1 and *Not*1 digestion sites to produce N-terminally c-Myc-tagged *HO1*. The oligonucleotides used as PCR primers are listed in Table 1.

2.2. Cell culture and transient transfections

COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum (Gibco), 1 mM L-glutamine (Fluka, Buchs, Switzerland), 50 mg/ml penicillin (Orion, Espoo, Finland) and 50 mg/ml streptomycin (Sigma, St.Louis, MO, USA). HEK293 cells were cultured in RPMI 1640 medium (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, 50 mg/ml penicillin and 50 mg/ml streptomycin. All cell cultures were maintained in an atmosphere of 5% CO_2 in air at 37 °C. Transient transfections were performed with FuGENE™ HD (Roche) according to the manufacturer's instructions. The cells were harvested 24 h after transfection.

2.3. Yeast two-hybrid screening

Yeast two-hybrid screening was performed using Matchmaker™ Two-hybrid System 3 (Clontech). The full length human STC2 cDNA was cloned in-frame with the GAL4 DNA-binding domain of pGBKT7 vector and then used as bait construct. The bait construct was then transformed into yeast strain AH109. The yeast strain Y187 was pretransformed with human placenta Matchmaker™ cDNA library (Clontech) in the pACT2 vector. The two yeast strains can mate and then allow activation and expression of the reporter genes. Positive clones on selective media were further confirmed by monitoring the expression of the reporter genes. Finally, inserts of the positive clones were amplified by PCR and sequenced.

2.4. Immunofluorescence staining and confocal microscopy

Cells were grown on coverslips, fixed with 3.5% paraformaldehyde (PFA) for 20 min and permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 5 min at room temperature. After treatment with 10% human serum for 30 min, the cells were incubated with

primary antibodies and species-specific secondary antibodies conjugated with either TRITC or FITC. The samples were mounted with Mowiol (Calbiochem, La Jolla, CA, USA). Confocal imaging was carried out with a Leica TCS SP2 Laser Scanning system (Leica, Mannheim, Germany) attached to a DM IRE-2 inverted microscope with a 40× oil objective (numeric aperture 1.25). Images were assembled using Adobe Photoshop CS2 software.

2.5. Immunoprecipitation

Immunoprecipitation experiments were performed with the Immunoprecipitation Kit (Roche). Cells were lysed in phosphate-buffered saline containing 1% (v/v) Igepal CA-630 (Fluka), 50 mM NaF (Sigma), 200 μM Na_3VO_4 (Fluka) and 1× Complete Protease Inhibitor Cocktail (Roche). The Antibodies were added to supernatants of the whole cell lysates pre-cleared by incubation with homogeneous protein A- or G-agarose for 3 h at 4 °C on a rocking platform. After incubation on a rocking platform at 4 °C for one hour, protein A/G-agarose suspension was added for at least 3 h at 4 °C. Complexes were pelleted by centrifugation at 12,000 g for 20 s and washed with 1 ml wash buffer 1 (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and 1 Complete tablet) twice, 1 ml wash buffer 2 (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet P40, 0.05% sodium deoxycholate) twice and 1 ml wash buffer 3 (10 mM Tris–HCl, pH 7.5, 0.1% Nonidet P40, 0.05% sodium deoxycholate) once.

2.6. SDS–PAGE and western blot analysis

Samples were boiled for 5 min in 2× Laemmli sample buffer containing ß-mercaptoethanol. Total protein lysates were separated by 12% SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with Tris-buffered saline containing 5% (m/v) nonfat milk (Valio) at 4 °C overnight, incubated with primary antibodies and followed by donkey anti-mouse IRDye 800CW (Li-COR Biosciences) or goat anti-rabbit Alexa Fluor-680 (Invitrogen Molecular Probes) antibodies (both 1:20,000 dilution). An Odyssey Infrared Imager (Li-COR Biosciences) was used for visualization.

2.7. Quantitative real-time RT-PCR

Total RNA of cells was isolated using TRI Reagent RT (Molecular Research Center, Inc., Cincinnati, OH, USA) and cDNA was generated by reverse transcription using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) and amplified in a Maxima SYBR Green qPCR Master Mix (Fermentas GMBH, St. Leon-Rot, Germany) and LightCycler II instrument (Roche Diagnostics, Mannheim, Germany) according to the instructions. The data were analyzed using Light Cycler software, version 3.5 (Roche Diagnostics, Mannheim, Germany). The level of gene expression was normalized against human glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The quantitative RT-PCR primers are listed in Table 1.

2.8. Hemin binding assay

The hemin/peptide binding assay was performed with a 10-amino-acid STC2 peptide: aa122Ser-Arg-Lys-Cys-Pro-Ala-Ile-Arg-Glu-Met and a C125A mutated peptide: aa122Ser-Arg-Lys-Ala-Pro-Ala-Ile-Arg-Glu-Met (Genscript). A 2 mM stock solution of hemin (Sigma) in 20 mM NaOH was used to prepare a 20 μM

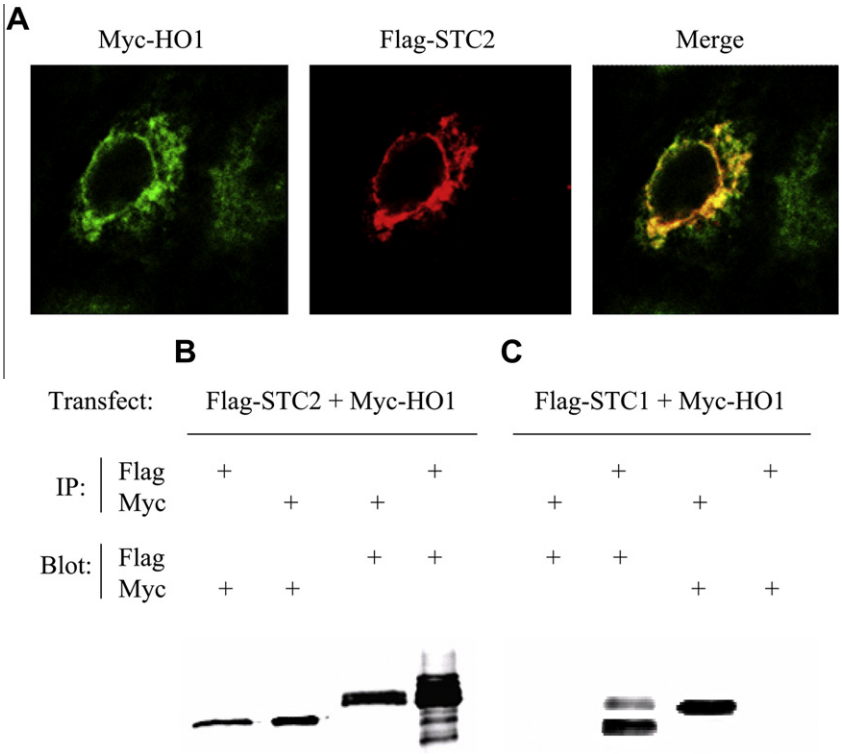

Fig. 1. Interaction of human STC2 and HO1 in COS-7 cells. (A) COS-7 cells were co-transfected with FLAG-tagged *STC2* and Myc-tagged *HO1*. After 24 h the cells were fixed, permeabilized and co-stained with rabbit anti-FLAG and mouse anti-Myc primary antibodies, followed by TRITC-conjugated swine anti-rabbit and FITC-conjugated goat anti-mouse secondary antibodies, respectively. Images were taken by confocal laser scanning microscopy. In the merged images, yellow indicates co-localization. (B) COS-7 cells were co-transfected with FLAG-tagged *STC2* and Myc-tagged *HO1*. The cells were harvested after 24 h and whole cell lysate was prepared. Rabbit anti-FLAG and rabbit anti-Myc primary antibodies were used to precipitate STC2-FLAG and HO1-Myc from the supernatant. The precipitates were analyzed with mouse anti-Myc and mouse anti-FLAG antibodies in western blot. (C) COS-7 cells were co-transfected with FLAG-tagged *STC1* and Myc-tagged *HO1*, and treated as in (B).

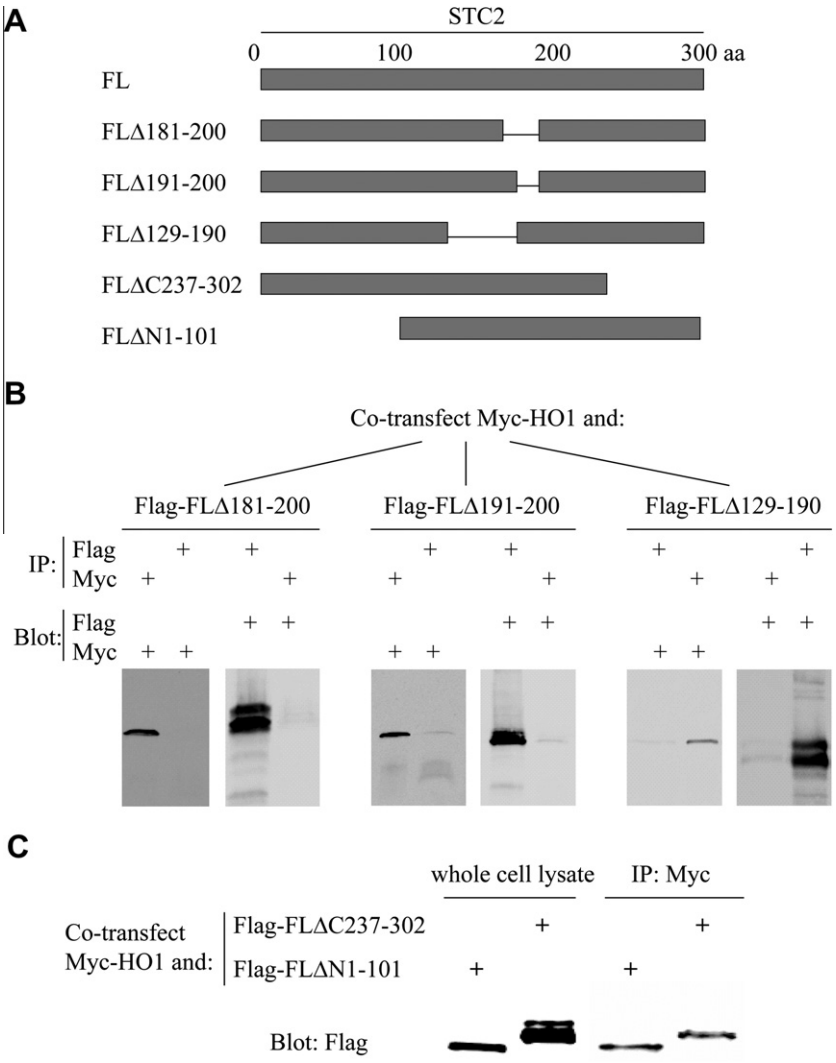

Fig. 2. Human STC2 181–200 amino acids contribute to the binding with human HO1. (A) Different deleted human STC2 cDNA constructs. (B) Each of the FLAG-tagged deleted human *STC2* constructs and Myc-tagged full length human *HO1* construct were co-transfected to COS-7 cells. The cells were harvested after 24 h, and whole cell lysates were prepared. Rabbit anti-FLAG and rabbit anti-Myc primary antibodies were used to precipitate deleted STC2 and full length HO1 from the supernatant. The precipitates were analyzed in western blotting with mouse anti-Myc and mouse anti-FLAG antibodies. (C) FLAG-tagged amino or carboxyl terminal deleted *STC2* constructs and Myc-tagged full length *HO1* construct were co-transfected to COS-7 cells. The cells were harvested after 24 h, and whole cell lysate was prepared. Rabbit anti-Myc primary antibody was used to precipitate HO1-Myc from the supernatant and the precipitates were analyzed in western blotting with mouse anti-FLAG antibody.

hemin solution in 0.1 M Tris–HCl (pH 7.5). Peptides were added directly to the diluted hemin solution, at a final concentration of 20 μM (1:1 hemin/peptide ratio). For each measurement the dilution and mixture was made freshly. An absorbance of 300–600 nm was scanned by a spectrophotometer (Ultrospec 3000pro, Amersham Pharmacia Biotech). The procedure of absorbance measurements with hemin and recombinant STC2 (BioVendor) was identical to that of peptides, except that rSTC2 was added at 2 μM final concentration to 2 μM hemin (1:1 hemin/rSTC2 ratio).

2.9. Chemicals

Chemicals and supplies, if not otherwise stated, were from Sigma (St. Louis, MO, USA). Oligonucleotides used as PCR primers were purchased from Oligomer (Helsinki, Finland). And the oligonucleotides used in quantitative real-time RCR were obtained from Sigma-Proligo. The restriction enzymes and T4 DNA ligase were from Fermentas. The following antibodies were used: mouse anti-FLAG, clone M2 (Sigma–Aldrich); rabbit anti-FLAG (Sigma–Aldrich); mouse monoclonal anti-c-Myc (Sigma–Aldrich); rabbit polyclonal anti-Myc-tag (MBL); swine anti-rabbit TRITC (Dako Cytomation, Glostrup, Denmark); goat anti-mouse FITC (Dako Cytomation); donkey anti-mouse IRDye 800CW (Li-COR Biosciences, Lincoln, NE, USA) and goat anti-rabbit Alexa Fluor-680 (Invitrogen Molecular Probes, Eugen, OR, USA).

3. Results

3.1. Human STC2 and HO1 co-localize in COS-7 cells

We used human STC2 cDNA as bait in a yeast two-hybrid screening of a human placenta cDNA library. HO1 was identified as one of the candidates. To study whether these two proteins interact in mammalian cells, FLAG-tagged *STC2* and Myc-tagged *HO1* constructs were co-transfected to COS-7 cells. Immunofluorescence stainings with antibodies to the tags were observed by confocal laser scanning microscopy. Fig. 1A shows that STC2 and HO1 co-localize in COS-7 cells, distributed predominantly in the perinuclear and endoplasmic reticulum regions.

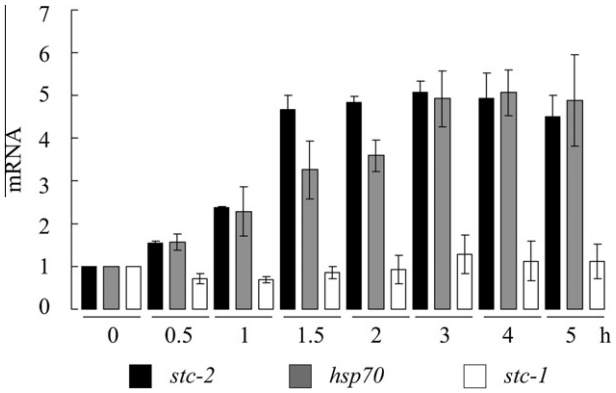

Fig. 3. STC2 mRNA is induced by heat shock in HEK293 cells. HEK293 cells were incubated at 42 °C for 0, 0.5, 1, 1.5, 2, 3, 4 and 5 h. Total mRNA was isolated and reverse transcribed to cDNA. The levels of *STC2*, *STC1* and *Hsp70* mRNA were measured by quantitative real-time PCR. *STC1* and *Hsp70* were used as negative and positive controls, respectively. Relative mRNA levels are expressed as the ratio of a mRNA level corresponding to that of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The data represent three independent experiments and were expressed as mean ± SD.

3.2. Human STC2 and HO1 interact directly in COS-7 cells

To demonstrate the direct binding of STC2 to HO1, we transfected COS-7 cells with FLAG-tagged *STC2* and Myc-tagged *HO1* constructs. Each protein was precipitated from lysates of the transfected cells, using the antibody to the respective tag and the presence of co-precipitate was analysed by western blotting. Myc-HO1 was detected in FLAG-STC2 precipitates and vice versa (Fig. 1B). Western blottings with the precipitating antibody were used as positive controls (Fig. 1B). A FLAG-tagged *STC1* construct was used to substitute the *STC2* construct as a negative control. There was no interaction between Myc-HO1 and FLAG-STC1 (Fig. 1C). These data show that human HO1 binds to STC2 but not to STC1.

3.3. Amino acids 181–200 of human STC2 contribute to the interaction with human HO1

To identify the site on STC2 that mediates binding of HO1 in vivo, COS-7 cells were co-transfected with several deletion constructs of human *STC2* (Fig. 2A) and the full length *HO1* construct. Immunoprecipitations from cell lysates were performed and analysed as described above. Human STC2 lacking amino acids 181–200 failed to bind HO1, whereas STC2 lacking either amino acids 181–190 or 191–200 displayed decreased ability to bind HO1 (Fig. 2B). Other deletion mutants were also tested. STC2 FLΔC237–302 and FLΔN1–101 displayed strong binding of HO1 (Fig. 2C). These results indicate that amino acids 181–200 of STC2 constitute the essential binding motif for HO1.

3.4. Human STC2 transcript is induced by heat shock in HEK293 cells

Since HO1 is known as a heat shock protein, we investigated whether its binding partner STC2 also responds to heat shock. HEK293 cells were exposed to 42 °C for 0–5 h and harvested immediately. STC2 mRNA was quantified by real time PCR. Hyperthermia for 3 h induced an over 5-fold increase in STC2 mRNA with a kinetics similar to that of Hsp70 (Fig. 3). Heat shock did not induce any increase, but rather a decrease in STC1 mRNA, similar to the reaction of most cellular proteins under hyperthermic conditions (Fig. 3).

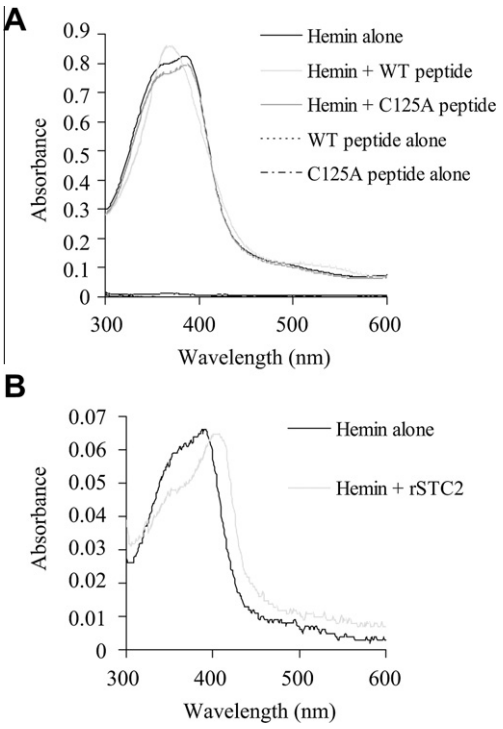

Fig. 4. Human STC2 interacts with hemin. (A) Hemin binding spectra of a peptide corresponding to the STC2 HRM (SRKCPAIREM) and a peptide with C125A mutation (SRKAPAIREM). Absorption spectrum of 20 μM hemin alone, 20 μM hemin added with either wild type STC2 or C125A mutation peptides at 1:1 M ratio. Both peptides were measured alone over the range of 300–600 nm using a spectrophotometer, and the results were recorded. (B) Hemin binding spectra of recombinant STC2. Absorption spectrum of 2 μM hemin alone and 2 μM hemin added with rSTC2 at 1:1 M ratio were measured and recorded as in (A).

3.5. Human STC2 binds hemin through a heme regulatory motif

We discovered a short sequence in STC2, namely, aa123Arg-Lys-Cys-Pro, which is homologous to previously reported heme regulatory motifs. We obtained a 10-residue peptide from aa122–aa131 (SRKCPAIREM) containing the RKCP motif and the corresponding peptide with a C125A mutation. The interaction between hemin and the peptides was assessed by the shift in the absorption spectrum of hemin. Hemin by itself displayed the anticipated absorption spectrum, with a peak at 385 nm and a shoulder (Fig. 4A). Addition of the wild type STC-2 peptide at an equimolar ratio to hemin caused a major shift of the soret band to a lower wavelength of 368 nm, and disappearance of the shoulder. The C125A mutated peptide did not change the position or the shape of the hemin soret band. To exclude the possibility that the observed effects were caused by the peptides alone, we demonstrated that none of the peptides showed any obvious absorption in the range of wavelengths measured (Fig. 4A). Furthermore, when recombinant STC2 protein was added at an equimolar ratio to hemin, we observed a 16 nm shift of the soret band to a higher wavelength and disappearance of the shoulder (Fig. 4B). These data demonstrate that human STC2 binds hemin through its HRM, and that cysteine125 is required for the binding.

4. Discussion

STC2 was identified over ten years ago, but its ultimate functions remain to be elucidated. Mice with deleted *stc* genes are viable and reproduce normally, indicating that stanniocalcins are not

indispensable for mammalian survival. An increasing body of evidence indicates that stanniocalcins belong to the group of stress-reactive proteins, the expression of which is triggered by hypercalcemia, hypoxic or oxidative stress [11,28]. Several recent reports also show that both STC1 and STC2 induce cytoprotective activity in cells under exogenous or endogenous stress [11,12,29,30]. The upregulated expression of STC2, in particular, found in various types of aggressive cancer could be related to enhancement of cancer cell survival. Its ultimate molecular mechanisms are still poorly understood.

We identified HO1 among the positive hits of yeast two-hybrid screening. The interaction of HO1 with STC2 is strong enough to allow co-immunoprecipitation from cell lysates. The intracellular co-localization of expressed HO1 and STC2 as detected by confocal microscopy of immunostained cells points to the in vivo interaction between the two proteins. By generating a series of deletion mutants of *stc2*, we were able to localize the motif mediating the HO1 binding to aa180–200. Stanniocalcins occur in vivo as homodimers [31]. The dimerisation does not appear to be essential for HO1 binding, since *stc2* mutant with deleted N-terminal 101 amino acids, which involve the homodimerisation site, still co-precipitated with HO1. This observation also suggests that one homodimeric STC2 molecule may bind two HO1 molecules.

We found that STC2 carries a HRM motif that binds hemin. We have previously reported that STC1 also carries a HRM with hemin-binding capacity [32]. The HRM of STC2 is not close to the amino acid sequence that mediates the binding to HO1. This suggests that STC2 can interact simultaneously with HO1 and hemin.

The functional implications of the interaction between HO1 and STC2 are still to be elucidated. HO1 and STC2 are stress-responsive proteins, both of which have been found to confer cytoprotective activity. Given this, the HO1-STC2 complex may represent a eukaryotic 'stressosome' in conditions where heme is liberated. STC2 appears to comprise a hemin scavenger that may provide substrate to HO1. It is also tempting to speculate that the motif of four histidine residues in the carboxyl terminus of STC2, that have been suggested to bind heavy metals [1,31], may function as a trap for the potential toxic free iron generated by HO1 in the HO1-STC2 complex.

In summary, we here report three previously unknown functions of STC2. It forms a complex with HO1, it can bind hemin, and its expression is induced by hyperthermia, indicating that STC2 is a potential member of the heat shock protein family.

Acknowledgments

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