Human homolog of mouse tescalcin associates with Na^+/H^+ exchanger type-1

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Received 10 September 2001; revised 24 September 2001; accepted 24 September 2001

First published online 18 October 2001

Edited by Gianni Cesareni

Abstract A novel regulatory protein, tescalcin (TSC), recently isolated from mouse embryonic testes, has been implicated in gonadal differentiation. Employing the yeast two-hybrid system with the Na^+/H^+ exchanger type-1 (NHE1) carboxyterminal domain as a bait we have identified a novel NHE1-associated protein of 214 amino acid residues representing the human homolog of mouse TSC (96.7% identity). Co-precipitation experiments demonstrated the interaction of human TSC with NHE1 in vitro and in vivo, and $^{45}Ca^{2+}$ overlay assay revealed that TSC binds Ca^{2+}. Immunofluorescence studies indicated that TSC is prominent in cellular lamellipodia where it colocalizes with NHE1. Abundant expression of TSC mRNA in the heart suggests that TSC may play important role(s) in concert with NHE1 in cardiac tissues.

Key words: Tescalcin; Na^+/H^+ exchanger type-1; Calcineurin homologous protein; Yeast two-hybrid system

1. Introduction

Na^+/H^+ exchangers (NHEs) form a family of membrane proteins that exchange extracellular sodium ions for intracellular protons thereby removing H^+ from the cytosol of actively metabolizing cells [1,2]. In addition to maintaining intracellular pH homeostasis, NHEs are involved in regulating cell volume and transepithelial Na^+ transport. To date six distinct isoforms of NHE have been described [1,2]. The 'housekeeping' NHE isoform type-1 (NHE1) is expressed ubiquitously in virtually all species, tissues and cell types. Intracellular acidosis is the major stimulus for NHE1 activation, but a variety of mitogenic and non-mitogenic signals increase the affinity of the proton sensor, mostly by phosphorylation of the antiporter [3–6]. NHE-associated proteins have been identified which further modulate NHE1 activity without changing its phosphorylation state, e.g. the elevation of cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ promotes the binding of Ca^{2+}-calmodulin to an autoinhibitory site at the cytosolic tail region of NHE1 which activates the antiporter via de-inhibition [7,8]. Calcineurin homologous protein (CHP), a 24-kDa protein closely related to the Ca^{2+} binding B subunit of the heterodimeric phosphatase calcineurin acts as a crucial co-factor of NHE1 activity through direct interaction with the carboxyterminal tail region of NHE1 [9,10].

In the present study we have employed the yeast two-hybrid system to search for novel NHE1-associated proteins. We identified a new CHP-related protein of 24 kDa representing the human homolog of mouse tescalcin (TSC) [11]. Human TSC specifically interacts with the cytosolic tail domain of NHE1 and its corresponding mRNA is highly expressed in the adult human heart thus pointing to a potential role of this novel protein in the regulation of NHE1 in cardiac tissues.

2. Materials and methods

2.1. Materials

Yeast two-hybrid system vectors, pEG202 and pJG4-5, were kindly provided by Roger Brent (Harvard Medical School, Boston, MA, USA). pECE/NHE1-GFP was a kind gift from Jacques Pouysségur (Centre National de la Recherche Scientifique, Nice, France). pGEX2T was obtained from Pharmacia, and pcDNA3.0 and pcDNA3.1A from Invitrogen. Human placenta Matchmarker® cDNA library and Multiple Tissue Northern (MTN®) blots were purchased from Clontech (Palo Alto, CA, USA). Polyclonal antisera to human TSC (α-TSC) and human NHE1 (α-cdNHE1) were raised in rabbits against glutathione *S*-transferase (GST) fusion proteins with TSC (GST-TSC) and the carboxyterminal domain of NHE1 (GST-cdNHE1), respectively, following standard immunization protocols. Mouse monoclonal antibody to hemagglutinin (α-HA) was obtained from BabCo, Pansorbin® cells from Calbiochem (San Diego, CA, USA), GSH-Sepharose from Pharmacia, and chemiluminescence kit from Amersham (Amersham, UK).

2.2. Yeast two-hybrid screening and bacterial expression of GST fusion proteins

For identification of proteins interacting with the carboxyterminal cytosolic domain of human NHE1 (cdNHE1) we employed the yeast two-hybrid system [12] using *Saccharomyces cerevisiae* strain EGY48 with reporter plasmid pSH18-34. To construct the LexA-fused bait protein human cdNHE1 (corresponding to amino acid residues 503–815) was amplified by polymerase chain reaction (PCR) using forward primer 5′-GGCGAATTCATTATGGCCCTGGTAGACCTGTTGG-CTGTG-3′ with an *Eco*RI restriction site, and reverse primer 5′-GGGGACTCGAGCTGCCTGCTGGCCCTGGCGTTACTGCC-3′ holding an *Xho*I restriction site. The construct was inserted into the *Eco*RI and *Xho*I restriction sites of pEG202 (pEG-cdNHE1$_{503–815}$). EGY48/pSH18-34 containing pEG-cdNHE1$_{503–815}$ was transformed with the human placenta Matchmarker cDNA library, and approximately 1×10^7 transformants were plated on selection medium (leu2). Appearing colonies were screened by the β-galactosidase reporter gene assay (lacZ) using X-gal plates, and plasmids of positive yeast clones were rescued by transformation of *Escherichia coli* KC8 strain. To test

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Abbreviations: CHP, calcineurin homologous protein; CNB, calcineurin B subunit; DIG, digoxigenin; GST, glutathione *S*-transferase; HA, hemagglutinin; NHE1, Na^+/H^+ exchanger type-1; PBS, phosphate-buffered saline; TSC, tescalcin

0014-5793/01/$20.00
PII: S0014-5793(01)02986-6

the specificity of protein–protein interaction EGY48/pSH18-34 was retransformed with the isolated library plasmids. The yeast reporter strain contained pEG-cdNHE1$_{503-815}$ as the bait and empty pEG202 or pEG-eNOS$_{1-486}$ (encoding the oxygenase domain of endothelial NO synthase) as controls. Transformants were tested for leu2 and lacZ activity. DNA sequences of selected library plasmids were determined by dideoxy chain termination method (MWG Biotech).

For bacterial expression as GST fusion proteins of identified cdNHE1 interaction partners, the inserts of the isolated library plasmids were excised by *Eco*RI and *Xho*I and ligated into pGEX2T previously modified for in-frame expression of *Eco*RI/*Xho*I fragments. *E. coli* BL21 strain was transformed with recombinant pGEX2T plasmids, and GST fusion proteins from exponentially growing bacteria were purified by GSH-Sepharose affinity chromatography according to the manufacturer's instructions (Pharmacia).

2.3. Construction of expression plasmids and transient transfection

For expression of carboxyterminally myc-tagged human TSC (TSC-myc), human TSC cDNA was amplified by polymerase chain reaction using forward primer 5′-CCCGCCGCGGAATTCATGGGCGCTG-CCCACTCCGCGTCT-3′ with an *Eco*RI restriction site, and reverse primer 5′-GTGCAGTTTCTCCGCGGATCTAGAGGTGGGTCCG-TGGCAG-3′ with an *Xba*I restriction site. TSC cDNA was 3′-terminally myc-tagged by insertion into the *Eco*RI and *Xba*I restriction sites of pcDNA3.1A. For transient expression of N-terminally HA-tagged cdNHE1 (HA-cdNHE1) the cdNHE1$_{503-815}$ cDNA fragment was ligated into pcDNA3.0 previously tailored for the expression of HA-tagged proteins.

LAP1, i.e. a mouse fibroblast cell line lacking endogenous NHE1, and COS7 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum. COS7 cells were transiently transfected using the DEAE-dextran transfection method [13]. For LAP1 cells we used FUGENE-6 according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

2.4. Immunoprecipitation and immunoblotting

To study protein–protein interactions in vitro we used COS7 cells expressing TSC-myc or HA-cdNHE1. Two days after transient transfection the cells were washed three times with ice-cold phosphate-buffered saline (PBS), lysed for 1 h on ice in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.4 mM PMSF (lysis buffer), and centrifuged at 14 000×*g* for 10 min at 4°C. GST-cdNHE1 or GST-TSC (each 10 μg/ml) were added to the respective supernatants, and the mixtures were incubated with 100 μl each of a suspension of GSH-Sepharose for 1 h at 4°C under rotation in the presence of 0.1% Triton X-100 (final concentration). The beads were washed three times each with lysis buffer containing 0.1% Triton X-100. Protein complexes bound to GSH-Sepharose were dissolved in SDS buffer, separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted by monoclonal α-myc or α-HA, followed by a peroxidase-labelled secondary antibody and the chemiluminescence detection assay.

To analyze the in vivo interaction of proteins, COS7 cells transiently co-expressing TSC-myc and HA-cdNHE1 were washed 48 h after transfection and lysed as above. Immunoprecipitation was done with α-TSC or α-cdNHE1, the precipitates were resolved by SDS–PAGE, and co-immunoprecipitated HA-cdNHE1 or TSC-myc was detected as detailed above.

2.5. Calcium binding

Ca^{2+} binding to mouse TSC has not been demonstrated experimentally, and therefore we tested human TSC by the $^{45}Ca^{2+}$ overlay assay [14]. Briefly, duplicate samples of GST fusion proteins (5 μg each) were run on SDS–PAGE. One half of the gel was Coomassie-stained, and the other half was blotted onto nitrocellulose. The membrane was washed three times in 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole HCl, pH 6.8 for 1 h, incubated for 10 min in the same buffer containing 1 μCi/ml of $^{45}CaCl_2$, and then rinsed twice in distilled water to remove non-specifically bound $^{45}Ca^{2+}$. After air drying for 3 h, $^{45}Ca^{2+}$ specifically bound to protein bands was visualized by exposure to a Kodak SB-5 film for 3 days.

2.6. Northern blot analysis

The Multiple Tissue Northern (MTN®) blot was hybridized with a digoxigenin (DIG)-labelled RNA probe for TSC according to the manufacturer's instructions (Roche Diagnostics). A PCR fragment of TSC including the T7 promoter of pJG4-5 was used as template. The RNA probe was hybridized at 68°C in DIG Easy Hyb solution, and the blot washed twice at room temperature in standard saline-citrate (SSC) including 0.1% SDS, followed by high stringency washes in 0.1×SSC/0.1% SDS at 68°C. After incubation with CDP-*Star* a Lumi-Imager was used for detection.

2.7. Immunofluorescence

For immunolocalization studies LAP1 fibroblasts co-expressing human NHE1-GFP and TSC-myc were grown on chamber slides. The cells were washed with PBS 30 h after transient transfection and fixed with ice-cold methanol for 10 min. After rinsing with PBS the cells were incubated with α-myc (1:100) in PBS containing 1% bovine serum albumin (v/v). Cells were washed with PBS and incubated for 1 h with cy3®-conjugated α-mouse Ig. After washing with PBS the stained cells were inspected by a confocal laser scanning microscope. NHE1-GFP and TSC immunostaining was documented at randomly selected areas with a 63× lens using a CCD camera.

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hTSC   MGAAHSASEEVR---ELEGKTGFSSDQIEQLHRRFKQLS-GDQPTIRKENFNNVPDLELN 56
mTSC   MGAAHSASEEVR---ELEGKTGFSSDQIEQLHRRFKQLS-GDQPTIRKENFNNVPDLELN 56
CHP    MGSRASTLLRDEELEEIKKETGFSHSQITRLYSRFTSLDKGENGTLSREDFQRIPELAIN 60
CNB    MGNEASYPL--------EMCSHFDADEIKRLGKRFKKLDLDNSGSLSVEEFMSLPELQQN 52
       **   *           :  : *. .:* :*  **..*. .:. ::  *:*  :*:*  *

hTSC   PIRSKIVRAFFDNRNLRKGPSGLADEINFEDFLTIMSYFRPIDTTMDEEQVELS-----R 111
mTSC   PIRSKIVRAFFDNRNLRKGSSGLADEINLEDFLTIMSYFRPIDTTLGEEQVELS-----R 111
CHP    PLGDRIINAFFPEG---------EDQVNFRGFMRTLAHFRPIEDNEKSKDVNGPEPLNSR 111
CNB    PLVQRVIDIFDTDG---------NGEVDFKEFIEGVSQFSVKGDKEQ------------- 90
       *: .:::  *  :           .:::::. *:  :: *     .

hTSC   KEKLRFLFHMYDSDSDGRITLEEYRNVVEELLSGNPHIEKESARSIADGAMMEAASVCMG 171
mTSC   KEKLKFLFHMYDSDSDGRITLEEYRNVVEELLSGNPHIEKESARSIADGAMMEAASVCVG 171
CHP    SNKLHFAFRLYDLDKDEKISRDELLQVLRMMVGVN--ISDEQLGSIADRTIQEAD----- 164
CNB    --KLRFAFRIYDMDKDGYISNGELFQVLKMMVGNN--LKDTQLQQIVDKTIINAD----- 141
         **:* *::** *.*  *:  *  :*:. ::. *  :.. .  .*.* :: :*

hTSC   QMEPDQVYEGITFEDFLKIWQGIDIETKMHVRFLNMETMALCH 214
mTSC   QMEPDQVYEGITFEDFLKIWQGIDIETKMHIRFLNMETIALCH 214
CHP    ----QDGDSAISFTEFVKVLEKVDVEQKMSIRFLH-------- 195
CNB    ----KDGDGRISFEEFCAVVGGLDIHKKMVVDV---------- 170
           .:    *:* :*  :   :*:. ** : .
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Fig. 1. Human TSC is homologous to calcineurin B-like proteins. The deduced amino acid sequences of human TSC (hTSC), mouse TSC (mTSC), CHP and human CNB were aligned using the ClustalW1.7 software. Identical amino acid residues (*) and conserved exchanges (.) are indicated; gaps (-) have been introduced to optimize the alignment. EF-hand motifs are shaded and the motif present in all four proteins is boxed.

3. Results

3.1. Identification of human TSC as an NHE1-associated protein

In an effort to identify novel NHE1-interacting proteins we screened a human placenta cDNA library by the yeast two-hybrid system utilizing a cDNA fragment encoding the carboxyterminal cytosolic domain of NHE1 as the bait. Approximately 1×10^7 co-transformants were screened and identified positive clones were tested for specific interaction in a re-screen with pEG202-cdNHE1 and unrelated baits, pEG202 and pEG-eNOS$_{1-486}$, for negative control. Among the positive clones that were subjected to sequence analysis was CHP, an established NHE1-interacting protein [15], thus confirming the validity and specificity of our screening assay. In addition we obtained a DNA sequence coding for an open reading frame of 642 nucleotides preceded by a 'perfect' Kozak sequence [16]. Database analysis demonstrated that the predicted protein sequence of 214 amino acid residues shares 99.5% homology and 96.7% identity with mouse TSC, a novel protein containing a single EF-hand motif that has recently been identified in mouse developing testes [11]. The human TSC homolog is identical to the hypothetical protein FLJ20607 of unknown function whose sequence has been deposited in the database by the NEDO human cDNA sequencing project (GenBank accession number: NM017899).

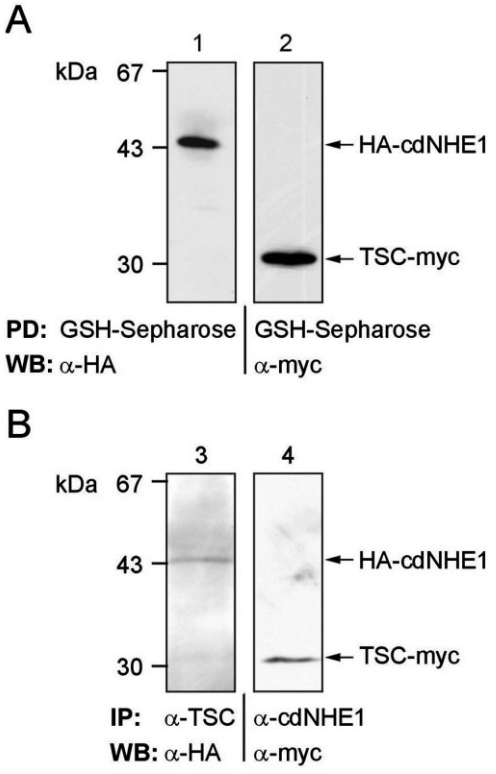

Fig. 2. TSC interacts with NHE1 in vitro and in vivo. A: COS7 cells overexpressing HA-cdNHE1 (lane 1) or TSC-myc (lane 2) were lysed in buffer containing Triton X-100, and 10 μg/ml GST-TSC (lane 1) or GST-cdNHE1 (lane 2) was added. Pull-downs (PD) were done with GSH-Sepharose followed by Western blotting with α-HA (lane 1) or α-myc (lane 2). B: COS7 cells overexpressing TSC-myc and HA-cdNHE1 were lysed in buffer containing Triton X-100. Immunoprecipitation (IP) was done with α-TSC (lane 3) or α-cdNHE1 (lane 4) followed by Western blotting with α-HA (lane 3) or α-myc (lane 4). Experiments were repeated three times with similar results.

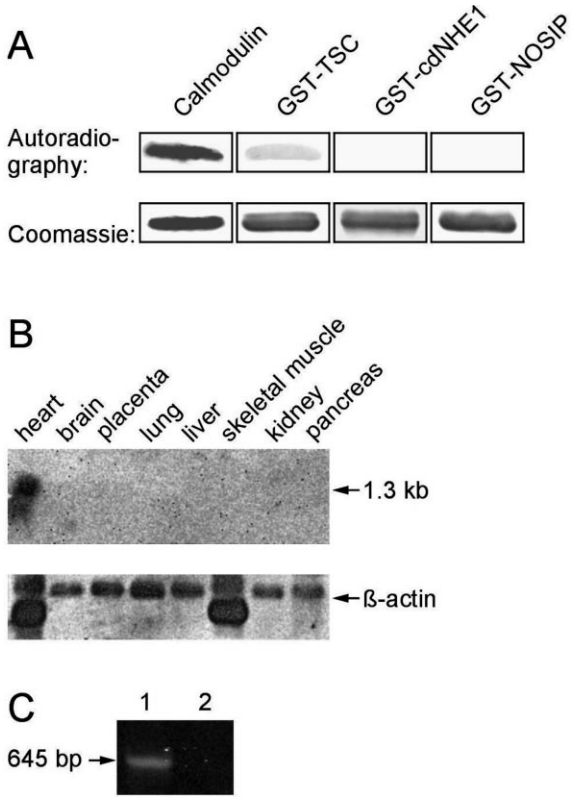

Fig. 3. Characterization of human TSC. A: Ca^{2+} binding to TSC: samples (5 μg each) of calmodulin, GST-TSC, GST-cdNHE1, and GST-NOSIP were applied in two identical sets to SDS–PAGE. One set of proteins was stained with Coomassie brilliant blue to monitor protein loading (bottom); the other set of proteins was transferred to nitrocellulose, followed by incubation with $^{45}Ca^{2+}$ and autoradiography (top). B: Tissue distribution of TSC: Northern blot hybridization of poly(A^+) RNA from various human tissues was performed with a DIG-labelled RNA probe of human TSC generated by in vitro transcription. A DIG-labelled β-actin RNA probe was used for control. C: Human placenta cDNA library was applied for the amplification of full-length TSC cDNA by PCR (lane 1); control was done in the absence of a template (lane 2).

Human TSC is the third member of a subfamily of Ca^{2+} binding proteins including CHP (56% homology and 34% identity to human TSC) and calcineurin B, the regulatory subunit of calcineurin phosphatase (46% homology and 26% identity). Analysis of the predicted human TSC amino acid sequence revealed an N-myristoylation consensus site, M*G*AAHSA, and a region comprising a consensus sequence defined for a Ca^{2+} binding EF-hand motif (Fig. 1). Human TSC does not confer a typical signal sequence or nuclear localization sequence, and therefore likely represents a cytosolic protein.

3.2. Specificity of the human TSC–NHE1 interaction

To demonstrate the specific interaction between human TSC and NHE1 in vitro, extracts of COS7 cells overexpressing HA-tagged cdNHE1 were incubated with GST-TSC fusion protein isolated from bacterial overexpression systems. GST-TSC was pulled-down by GSH-Sepharose, and co-precipitation of HA-cdNHE1 was demonstrated by Western blotting using antibodies to the HA-tagged fusion protein (Fig. 2A, lane 1). Vice versa, complexes of GST-cdNHE1 and myc-tagged TSC (myc-TSC) were pulled down by GSH-Sepharose

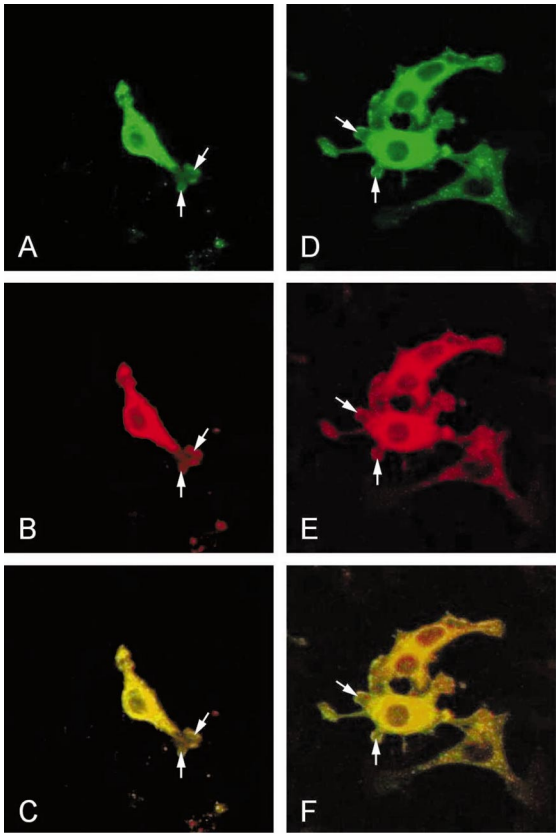

Fig. 4. TSC colocalizes with NHE1 in mouse fibroblasts. Cells overexpressing NHE1-GFP and TSC-myc were fixed with ice-cold methanol and incubated with a mouse α-myc followed by visualization of TSC by a cy3®-coupled α-mouse immunoglobulin. A: NHE1-GFP; B: TSC-myc; C: overlay of both images. Arrows point to lamellipodia.

from lysates of COS7 cells overexpressing myc-TSC and supplemented with purified GST-cdNHE1 (Fig. 2A, lane 2). To demonstrate binding of TSC to NHE1 in a cellular context ('in vivo') we constructed COS7 cells co-expressing TSC-myc and HA-cdNHE1. Immunoprecipitations were done with anti-TSC or anti-cdNHE1, and co-immunoprecipitated HA-cdNHE1 (Fig. 2B, lane 1) or TSC-myc (Fig. 2B, lane 2) was detected by anti-HA or anti-myc, respectively. These results demonstrate the specific interaction of cdNHE1 and TSC in a mammalian cell line.

3.3. Calcium binding properties of TSC

Calcium binding of the GST-TSC fusion protein was examined in a $^{45}Ca^{2+}$ overlay assay. GST-cNHE1 and GST-NOSIP [16] served as negative controls, and human calmodulin was the positive control. The proteins were applied to SDS–PAGE, and two identical gels were produced. One replica was Coomassie-stained (Fig. 3A), and the other one was blotted and incubated with $^{45}CaCl_2$. Autoradiography revealed a clear signal for GST-TSC indicative of $^{45}Ca^{2+}$ binding by TSC, most probably through its single EF-hand-like motif that is also present in CHP and calcineurin B (Fig. 3A). Expectedly calmodulin exposing four Ca^{2+} binding sites produced a strong signal whereas the negative controls, i.e. GST-cdNHE1 and GST-NOSIP, failed to show significant Ca^{2+} binding.

3.4. Tissue distribution of human TSC mRNA

To examine the relative expression levels of TSC mRNA, we performed Northern blot analysis with selected human tissues using an in vitro transcribed DIG-labelled TSC RNA probe. A 1.3 kb mRNA was strongly expressed in human heart whereas Northern blotting did not reveal appreciable amounts of the TSC mRNA in brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Fig. 3B). Our preliminary data indicate that TSC is also expressed in adult testes though at a much lower level than in the heart, and in salivary glands (data not shown). PCR analysis using the human placenta Matchmarker cDNA library and primers flanking the full-length TSC cDNA indicated that low levels of the TSC transcript are present in tissues such as placenta (Fig. 3C).

3.5. Immunolocalization of NHE1 and TSC

LAP1 fibroblasts transiently co-expressing TSC-myc and full-length human NHE1 fused to GFP were used to analyze the subcellular localization of the two interacting proteins in intact cells. Strong labelling due to overexpression of NHE1 and TSC was seen throughout the cytosol (Fig. 4). Remarkably lamellipodia protruding from the cell surface showed a distinct colocalization of NHE1 and TSC suggesting that the interaction between the two proteins also occurs in intact cells.

4. Discussion

In a recent study Perera et al. demonstrated by RT-PCR that TSC mRNA is present in the developing gonads of mouse embryos suggesting that TSC could be involved in gonadal differentiation [11]. Though the authors noted that TSC mRNA was most abundant in the heart of adult mice they did not offer an explanation for this puzzling finding. Here we demonstrate that the human homolog of TSC is associated with the cytosolic tail domain of the sodium-proton exchanger NHE1 known to be abundant in the heart [17].

TSC belongs to a family of proteins with homology to the regulatory subunit calcineurin (CN) phosphatase, referred to as calcineurin B subunit (CNB). All known members of CNB-like proteins contain at least one EF-hand motif for high affinity Ca^{2+} binding, and typically their biological activity is regulated by the cytosolic Ca^{2+} concentration. Among the known CNB-like proteins, rat CHP has been shown to undergo conformational change(s) upon Ca^{2+} binding to its EF-hand motifs [18], and human CHP was demonstrated to bind to the carboxyterminal region of NHE1 [10]. Thus CHP may act as a Ca^{2+} sensor translating changes of $[Ca^{2+}]_i$ into altered NHE1 activity. Given the structural homology of CHP and TSC we hypothesize that TSC may regulate NHE1 activity in a Ca^{2+}-dependent manner though this hypothesis awaits experimental confirmation. It will be interesting to learn whether CHP and TSC compete for the same binding site on the NHE1 cytosolic tail domain, or whether they bind to distinct regions.

A previous study has demonstrated a protein of 24 kDa ('p24') to be associated with NHE1, however, the structure and function of p24 have remained unknown [19]. Our co-immunoprecipitation experiments demonstrating the specific interaction of TSC and the C-terminal domain of NHE1 in vitro and in vivo establish an apparent molecular weight of 24 kDa for human TSC, and therefore it is well possible that

TSC and p24 are identical proteins. Given the extensive sequence similarity of human and mouse TSC with CHP and the fact that CHP is an established modulator of NHE1 activity [10], one is tempted to speculate that TSC may also function as a modulator of NHE1 activity. This notion is emphasized by our findings that human TSC mRNA is abundantly expressed in cardiac tissues where NHE1 is also prominent [17], and that TSC localizes to the same cellular compartments, i.e. lamellipodia, where full-length NHE1 resides [20]. Thus TSC may play important role(s) by reversibly associating with the regulatory tail domain of NHE1 [21] thereby modulating the activity, location and/or degradation of NHE1 in cardiac tissues. Because NHE1 is involved in the regulation of cell proliferation, differentiation, and neoplastic transformation [22–24] it is well conceivable that NHE1 is a major target for TSC in developing gonads.

In the human heart NHE1 is the predominant isoform [17] that is involved in the adaptation to intracellular acidosis during ischemia and in the homeostasis of intracellular pH following reperfusion [1,2]. Accumulating evidence suggests that NHE1 activity can also lead to adverse effects during ischemia and reperfusion due to proton export and cytosolic Na^+ accumulation promoting Ca^{2+} influx via the Na^+/Ca^{2+} exchanger and thus leading to Ca^{2+} overload, cell necrosis and arrhythmia [4,25]. Studies are underway aiming at the elucidation of the role(s) that TSC might play in concert with NHE1 under (patho)physiological conditions.

Acknowledgements: This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. We wish to thank Drs. B. Schölkens and A. Busch (Aventis, Frankfurt) for generous support of these studies.

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