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Testin, a novel binding partner of the calcium-sensing receptor, enhances receptor-mediated Rho-kinase signalling

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ABSTRACT

The calcium-sensing receptor (CaR) plays an integral role in calcium homeostasis and the regulation of other cellular functions including cell proliferation and cytoskeletal organisation. The multifunctional nature of the CaR is manifested through ligand-dependent stimulation of different signalling pathways that are also regulated by partner binding proteins. Following a yeast two-hybrid library screen using the intracellular tail of the CaR as bait, we identified several novel binding partners including the focal adhesion protein, testin. Testin has not previously been shown to interact with cell surface receptors. The sites of interaction between the CaR and testin were mapped to the membrane proximal region of the receptor tail and the second zinc-finger of LIM domain 1 of testin, the integrity of which was found to be critical for the CaR-testin interaction. The CaR-testin association was confirmed in HEK293 cells by coimmunoprecipitation and confocal microscopy studies. Ectopic expression of testin in HEK293 cells stably expressing the CaR enhanced CaR-stimulated Rho activity but had no effect on CaR-stimulated ERK signalling. These results suggest an interplay between the CaR and testin in the regulation of CaR-mediated Rho signalling with possible effects on the cytoskeleton.

1. Introduction

The calcium-sensing receptor (CaR) has a key role in extracellular calcium homeostasis but is also found in tissues unrelated to this process [1]. In addition to its primary physiological ligand (Ca^{2+}) the CaR responds to other di- and tri-valent cations, polypetides and aminoglycoside antibiotics and is modulated by ionic strength, pH and allosteric activators, including amino acids [2]. There is also great diversity in the intracellular signalling pathways (e.g. phospholipase C, extracellular-signal-regulated kinase (ERK) and Rho) it activates as well as the biological processes (e.g. secretion, proliferation, apoptosis and gene expression) that are regulated by the CaR [2]. The interaction of the CaR with accessory proteins is likely to be one important determinant of its biological response. Several protein partners have been identified using yeast two-hybrid screens based on cDNA libraries derived from tissues involved in calcium homeostasis and shown to influence receptor expression and/or function [3]. In order to identify protein binding partners with roles in CaR function unrelated to calcium homeostasis we performed a yeast two-hybrid library screen of a haemopoietic cell line (EMLC.1) library [4] using the CaR-tail as bait. Using this approach we identified the focal adhesion protein, testin, as a novel binding partner of the CaR.

Testin, also known as TES, has tumour suppressor activity, is present at the sites of focal adhesions and plays a role in cytoskeletal organisation [5]. The key structural features of testin are a prickle, espinas, testin (PET) domain and three Lin-11, Isl-1, Mec-3 (LIM) domains. Although there have been reports of testin interaction with proteins involved in intracellular signalling [5,6], we present evidence that testin can also interact with a cell surface receptor, the CaR. In addition, we found that overexpression of testin in CaR-expressing HEK293 cells significantly enhanced CaR-induced Rho kinase activity.

2. Materials and methods

2.1. Plasmids

The cDNA library amplified for use in this study was of an EMLC.1 mouse pluripotent haemopoietic cell line [4] kindly donated by Dr. S. Tsai, Fred Hutchinson Cancer Research Center,

Abbreviations: CaR, calcium-sensing receptor; ERK, extracellular-signal-regulated kinase; LIM, Lin-11∗ Isl-1∗ Mec-3; PET, prickle∗ espinas∗ testin; SRE, serum response element.

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Seattle, WA, USA. Library inserts were cloned into the LexA-based screening vector, pVP16. The bait construct used for the library screen (pBTM116-CaR[865–1078]) consisted of the human CaR intracellular tail (amino acids 865–1078) inserted into the bait vector pBTM116. For use in yeast two-hybrid mapping studies, the following bait deletion constructs were also generated: pBTM116-CaR[865–922], pBTM116-CaR[865–898], pBTM116-CaR[899–922], pBTM116-CaR[923–1078], pBTM116-CaR[965–1078] and pBTM116-CaR[987–1078]. The pVP16 construct isolated from the yeast two-hybrid screen that encoded the testin clone containing amino acids 148–357 was used as a template to produce a series of constructs containing various alanine substitutions generated using the QuikChange site-directed mutagenesis kit (Stratagene) as previously described [7]. For other studies, full length human testin was cloned from the MDA-MB-231 breast cancer cell line and inserted into the pcDNA3-EGFP V1 vector (kindly supplied by Professor K. Eidne, Western Australian Institute for Medical Research) (pcDNA3-EGFP-testin). The C-terminally FLAG-tagged CaR construct (pcDNA3.1-CaR-FLAG) has been described previously [7]. The serum response element (SRE)-luciferase reporter plasmid, pSRE-Luc, was a kind donation from Professor J. E. Pessin, Albert Einstein College of Medicine, New York, USA [8].

2.2. Yeast two-hybrid assay

The basic LexA screening procedure in which library and bait constructs cotransformed into yeast L40 are examined for the appearance of co-transformants exhibiting CaR-tail dependent transactivation of HIS or LacZ reporter genes, has been described previously [9]. Briefly, the lithium acetate method was employed to sequentially transform library plasmid DNA into yeast pretransformed with CaR-tail bait, after which the cells were amplified for 6 h, plated onto medium deficient in tryptophan, leucine, histidine, lysine and uracil and the plates examined after 3 days incubation at 30° C. Potential interacting clones were confirmed using a β-galactosidase colony lift assay to assess LacZ reporter gene activity, with times to colour development compared with known positive and negative cotransformant interactors – pBTM116-CyP40-(185–370) + pVP16-Hsp90-(520–724) and pBTM116-ARL-E1 + pVP16-Hsp90-(520–724), respectively [10]. Plasmid DNA extracted from positive colonies was used to amplify library inserts by PCR, which were then categorized by size and restriction enzyme profiling. Plasmid DNA from unique clones was then rescued in *Escherichia coli* [10] and the interaction of library protein with the CaR tail verified in yeast as described above. Library inserts were then sequenced and their identity determined by BLAST searches of the NCBI databases. For mapping studies, deletion constructs of the CaR-tail cloned into pBTM116 and pVP16 constructs containing either testin clone 148–357 or testin clone 254–419 were cotransformed into yeast, plated onto selection medium and cotransformant colonies examined for LacZ reporter gene activity using the β-galactosidase colony lift assay. All bait constructs were tested individually to ensure that they did not autonomously activate β-galactosidase.

2.3. Cell culture and transfection

HEK293 cells were obtained from Professor K. Eidne, Western Australian Institute for Medical Research and HEK293 cells stably expressing the CaR (HEK293-CaR) have been described previously [11]. Cells were propagated in DMEM containing 10% foetal calf serum, penicillin (100 Units/mL) and streptomycin (100 μg/mL) at 37° C in the presence of 5% CO_2 [7]. In addition, for HEK293-CaR, G418 sulphate at 100 μg/mL was included in the medium to maintain the stable expression of CaR. Cell monolayers were transfected at 60% confluency using Lipofectamine 2000 (Invitrogen) as previously described [12].

2.4. Co-immunoprecipitation studies

HEK293 cells cotransfected with pcDNA3.1-CaR-FLAG and pcDNA3-EGFP-testin were lysed 48 h later in buffer containing iodoacetamide and protease inhibitors and the extracted protein quantitated as described previously [7]. Non-specific binding proteins were removed from 2 mg of lysate protein by pre-clearing with 40 μl GammaBind G Sepharose beads (GE Healthcare) for 1 h at 4° C. Pre-cleared lysate was mixed by rotation overnight at 4° C with 5 μg of either a mouse monoclonal anti-FLAG antibody (Sigma–Aldrich) or a rabbit polyclonal anti-GFP antibody (Santa Cruz Biotechnology) for immunoprecipitation of CaR-FLAG or EGFP-testin, respectively after which, the antibody-protein complex was mixed by rotation for 4 h at 4° C with 40 μl of fresh GammaBind G Sepharose beads. Beads were then washed 6 times with cell lysis buffer (with the omission of iodoacetamide) and the bound protein was eluted with 50 μl sample buffer containing SDS and 2-mercaptoethanol. Proteins separated on a 7.5% or a 10% SDS–PAGE gel were blotted and probed respectively with mouse anti-FLAG monoclonal antibody (Sigma–Aldrich) or rabbit anti-GFP polyclonal antibody (Santa Cruz Biotechnology) and appropriate anti-species horseradish peroxidase-conjugated secondary antibody to detect co-immunoprecipitated CaR-FLAG or EGFP-testin. Signal was detected using Western Lightning *Plus*-ECL enhanced chemiluminescence substrate (PerkinElmer Life Sciences) as previously described [7].

2.5. Confocal microscopy

Cells transiently expressing CaR-FLAG and EGFP-testin were plated onto poly-L-lysine coated coverslips in a 6-well plate 24 h after transfection. Following a further 24 h incubation, cells were washed (3 × 3 min in PBS) at room temperature then fixed using 4% (v/v) formaldehyde in PBS for 20 min at room temperature. This was followed by further washing in PBS after which the cells were permeabilized in PBS containing 0.2% (v/v) Triton X-100 at room temperature for 20 min. Cells were washed again in PBS then incubated in blocking buffer (PBS containing 10% (v/v) goat serum and 1% (w/v) BSA) for 1 h at room temperature. Cells were incubated overnight at room temperature with FLAG antibody diluted 1/250 in blocking buffer, then washed in PBS and incubated with Alexa Fluor 546-conjugated goat anti-mouse antibody (Molecular Probes) diluted 1/400 in blocking buffer for 1 h at room temperature. Cells were then washed (5 × 3 min in PBS) at room temperature and the coverslips mounted onto slides with antifade mounting medium (50 mM Tris–PO_4, 50 mM NaH_2PO_4, 20% (v/v) polyvinyl alcohol, 30% (v/v) glycerol) and sealed with nail polish. CaR-FLAG and EGFP-testin were detected using appropriate excitation/emission filters on a Bio-Rad MRC1000/1024 UV confocal laser-scanning microscope.

2.6. ERK assay

The CaR-mediated phosphorylation of ERK was measured by Western analysis as described previously [11]. Briefly, HEK293-CaR cells in 25 cm^2 flasks were transfected with either pcDNA3-EGFP-testin or the vector control, pcDNA3-EGFP and 24 h later seeded into poly-L-lysine-coated 24-well plates in DMEM without $CaCl_2$ (Invitrogen) that was supplemented with 10% BSA, 100 Units/mL penicillin and 100 μg/mL streptomycin and 0.5 mM $CaCl_2$. Following incubation for 24 h, the medium was replaced with physiological saline solution (PSS) containing 0.2% BSA and 0.5 mM $CaCl_2$ and the cells incubated a further 1 h. Following