The PEF family proteins sorcin and grancalcin interact in vivo and in vitro

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Abstract   **The penta-EF hand (PEF) family of calcium binding proteins includes grancalcin, peflin, sorcin, calpain large and small subunits as well as ALG-2. Systematic testing of the heterodimerization abilities of the PEF proteins using the yeast two-hybrid and glutathione S-transferase pull-down assays revealed the new finding that grancalcin interacts strongly with sorcin. In addition, sorcin and grancalcin can be co-immunoprecipitated from lysates of human umbilical vein endothelial cells. Our results indicate that heterodimerization, in addition to differential interactions with target proteins, might be a way to regulate and fine tune processes mediated by calcium binding proteins of the penta-EF hand type.**
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Sorcin; Grancalcin; Apoptosis

1. Introduction

Calcium ions control a variety of cellular phenomena such as muscle contraction, proliferation, differentiation and apoptosis. The specificity of calcium signaling is obtained by the quality of the calcium signals, which has a temporal and spatial aspect and is dependent on the amplitude of the calcium transient [1]. In addition, the nature of the intracellular calcium receptor mediating signal transduction is crucial [2]. One structurally defined subgroup of calcium binding proteins is the PEF (penta-EF hand) family. This protein family comprises calpain large and small subunits, ALG-2 (apoptosis-linked gene 2), peflin, sorcin and grancalcin [3]. Based on sequence similarities, further subgroups can be made with pairs of the small/large calpain subunits, ALG-2/peflin and sorcin/grancalcin.

The best characterized PEF family proteins are the large and small subunits of calpain, which are known to heterodimerize to form an active protease [4,5]. A multitude of calpain enzymes with different properties and/or different expression patterns can be formed by heterodimerization of the small subunit with the many different large subunits which carry the sequence responsible for the catalytic activity [6].

Peflin and ALG-2 have been shown to interact, but the functional significance of this complex is so far unknown [4]. Interestingly, in contrast to all PEF family protein homodimers and the above mentioned calpain heterodimers, the ALG-2/peflin interaction only takes place in the absence of calcium. We have previously shown that two isoforms of ALG-2 exist [7]. The short form (ALG-2,1) lacks the two residues Gly[121] and Phe[122], present in the long form (ALG-2). In yeast two-hybrid experiments both ALG-2 isoforms homo- and heterodimerize, but only the long isoform of ALG-2 is able to interact with the target protein AIP1 [7]. Structural analysis of calpain [8], grancalcin [9], sorcin [10] and ALG-2 [11] has shown that the PEF family proteins dimerize by homotropic interaction in the fifth EF hand domain, which presumably does not bind calcium under physiological conditions.

Here we present a systematic analysis of heterodimerization of the PEF proteins using the yeast two-hybrid system. We found that sorcin, a modulator of the ryanodine receptor and expressed in many cell types [12], interacts in vitro and in vivo with grancalcin, a protein with so far undefined function, which is expressed in a restricted range of cell types, mostly of hematopoietic origin [13].

2. Materials and methods

2.1. Construction of GAL4 domain plasmids

pBDsorcin and *pADsorcin*: Sorcin cDNA was kindly provided by C.E. Creutz [14]. The cDNA was amplified by polymerase chain reaction (PCR) and cloned into the pBDGAL4 or pADGAL4 vector (Stratagene).

pBDgrancalcin and *pADgrancalcin*: Human grancalcin cDNA cloned into the pGEX2T vector was described in [15]. The cDNA was amplified by PCR and cloned into the pBDGAL4 or pADGAL4 vector.

*pAD*delta*peflin* and *pBDpeflin*: The pcDNA3 vectors containing an insert of the DNA sequence encoding the C-terminal 82 amino acids of murine peflin and full length peflin were kindly provided by P. Vito and subcloned into pADGAL4 or pBDGAL4.

2.2. Yeast strains and media

Two *Saccharomyces cerevisiae* strains were used in the two-hybrid assays: YRG-2 (Stratagene; described in [7]) and SFY-526 (Matα, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, canr,

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Abbreviations: AD or ADGAL4, transcriptional activation domain of GAL4; BD or BDGAL4, DNA binding domain of GAL4

gal4-542, gal80-538, URA3::UAS$_{GAL1}$-TATA$_{GAL1}$-lacZ). Yeast cultures were grown at 30°C in either YPD medium (2% peptone, 1% yeast extract, 2% glucose) or SD minimal medium (0.67% nitrogen base, 18.2% D-sorbitol, 2% glucose) supplemented with amino acids.

2.3. Yeast transformation and phenotype analysis

The transformation of yeast with plasmid DNA was done according to the lithium acetate TRAFO protocol [16]. The protein–protein interaction phenotype was analyzed by the ability of transformed yeast to grow on a medium lacking histidine and by the ability to activate expression of the β-galactosidase gene as described previously [7].

2.4. Immunodetection of the PEF proteins

Polyclonal antibodies against mouse ALG-2 were generated in rabbits and are described elsewhere [26]. Anti-grancalcin-1 rabbit antiserum was as described [15], anti-grancalcin-2 rabbit polyclonal was a generous gift from A.W. Segal, and anti-sorcin monoclonal antibody was from Zymed (San Francisco, CA, USA). All secondary horseradish peroxidase-conjugated antibodies were from Dako (DakoCytomation, Glostrup, Denmark).

Proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at reducing conditions and transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences, Sweden) according to standard procedures [17]. Membranes were blocked in TBS-T (20 mM Tris–HCl, pH 7.4, 150 mM NaCl), 5% non-fat milk and 0.05% Tween 20 and stained with antibody in the same buffer. Detection was performed using a chemiluminescent ECL substrate (Amersham Biosciences, Sweden) according to the manufacturer's manual.

2.5. Expression of GST fusion proteins and pull-down assay

Human sorcin cDNA cloned into the pGEX-KG bacterial expression vector was kindly provided by C.E. Creutz [14]. The BL21-CodonPlus(DE3)-RP Escherichia coli strain was transformed with either the pGEX-TK (ATCC) plasmid for glutathione S-transferase (GST) expression or with pGEX-KG/GST-sorcin for expression of the GST–sorcin fusion protein. Expression of the recombinant proteins was induced at OD$_{600}$ 1.0 by 1 mM isopropyl-1-thio-β-D-galactopyranoside. Bacteria were harvested after 2.5 h by centrifugation at $2500 \times g$ at 4°C for 15 min, and resuspended in 10 ml phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.3). Lysis was performed at room temperature for 25 min by adding 1 mg/ml lysozyme, followed by freezing/thawing and adding Triton X-100 to 1%. The bacterial debris was pelleted by centrifugation at $50\,000 \times g$ for 30 min at 4°C and the supernatant was further processed to purification on a 1 ml GSTRAP affinity column (Amersham Biosciences, Sweden) according to the manufacturer's protocol. The purified GST and GST–sorcin proteins were dialyzed against KTT buffer (140 mM KCl, 20 mM Tris–HCl, pH 7.4, 0.1% Triton X-100).

GST–sorcin or GST (5 μg) was incubated with 2.5 mg of glutathione-Sepharose 4B (Amersham Biosciences, Sweden) in the KKT buffer at 4°C overnight. The beads were spun down at $800 \times g$ for 1 min and washed once with KTT buffer. 15 μg of recombinant human grancalcin [18] or 15 μg ALG-2 [7] was added to the resin and the binding was performed in KTT buffer with either 1 mM CaCl$_2$ or 1 mM EDTA, overnight at 4°C. The beads were then washed five times with 1 ml NTT buffer (150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 0.1% Triton X-100), resuspended in 50 μl of KTT and 50 μl of 2×SDS–PAGE sample buffer (100 mM Tris–HCl, pH 6.8, 0.1% bromophenol blue, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and boiled for 10 min. The samples were subjected to SDS–PAGE followed by Western blot analysis.

2.6. Immunoprecipitation of the sorcin–grancalcin complex from human cells

Human high umbilical vein endothelial cells (HUVEC) were grown in 175 cm^2 cell culture flasks to sub-confluent density in EGM-2 Bullet kit medium (Clonetics, Belgium). The cells were lysed in a buffer containing 150 mM NaCl, 1 mM MgCl$_2$, 0.2% NP40, 25 mM Tris–HCl, pH 7.5 and proteinase inhibitor mix (Sigma, St. Louis, MO, USA). The extracts were pre-cleared by incubation with protein A-Sepharose beads (Amersham Biosciences, Sweden) for 1 h at 4°C The beads were incubated with either anti-grancalcin or anti-sorcin

antiserum for 90 min at 4°C and then washed four times with the lysis buffer. Precipitation of immune complexes was performed overnight at 4°C.

3. Results

Since heterodimerization among some of the PEF proteins had previously been reported, we tested whether the ALG-2 isoforms have dimerization partners within the PEF family. The cDNA encoding ALG-2, ALG-2,1, peflin, the 82 amino acid long C-terminal part of peflin (deltapeflin), grancalcin, sorcin, calpain small and large subunits were inserted into the pBDGAL4 and pADGAL4 vectors and expressed pairwise in the YRG-2 yeast strain. The interaction phenotype of the transformed yeast was analyzed by filter lift assay. We found that ALG-2 can bind the C-terminus of peflin
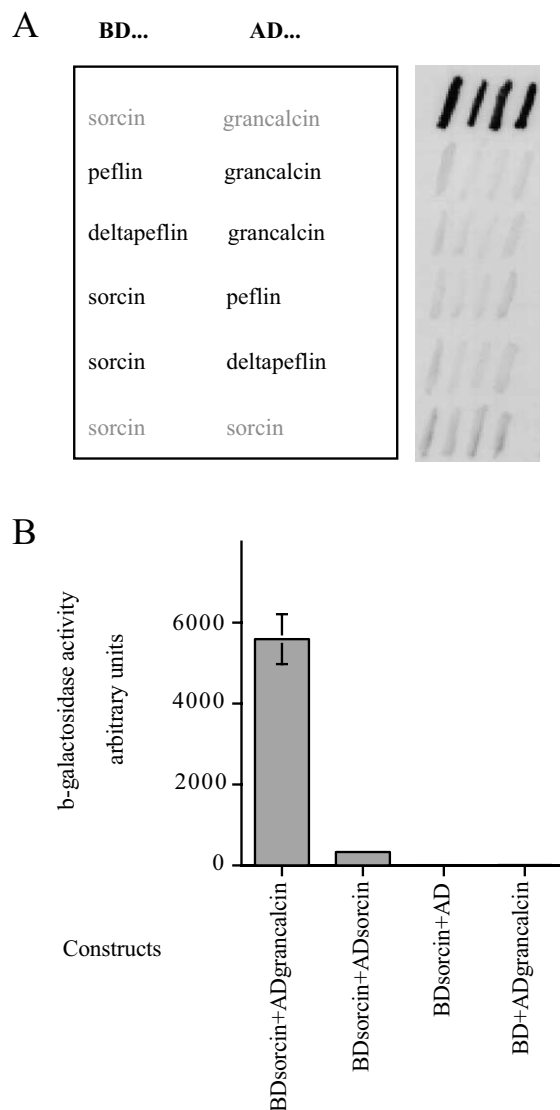

Fig. 1. Two-hybrid analysis of dimerization of the PEF proteins shows that grancalcin interacts with sorcin. A: Filter lift assay. Heterodimerization abilities of grancalcin, peflin and sorcin were tested. BD... and AD... indicate the fusion partners of the proteins. B: Luminescent β-galactosidase assay was applied to characterize the grancalcin/sorcin interaction. The enzyme activity was measured in lysates of four independent clones from each transformation. Values (mean ± S.D.) are given in arbitrary units.

(data not shown). This result was expected since ALG-2 interaction with this C-terminal fragment of peflin was initially observed by P. Vito, who provided this construct (unpublished data, with permission of the author). While we were performing these experiments, Maki et al. reported that peflin and ALG-2 indeed interact with each other, as shown by co-immunoprecipitation from different cell lines [4]. Surprisingly, no interaction between calpain large and small subunits was observed in either YRG-2 or SFY-526 yeast strains in contrast to the results published by Sorimachi et al. [6].

By systematically testing PEF proteins for the ability to heterodimerize (Fig. 1A) we found a so far undescribed interaction between sorcin and grancalcin (Fig. 1A,1). We also showed that sorcin homodimerizes in yeast (Fig. 1A,6), whereas grancalcin homodimerization cannot be tested since BDgrancalcin activates the lacZ reporter gene alone (data not shown).

The interaction of grancalcin with sorcin was quantitated by a liquid luminescent β-galactosidase reporter gene assay (Fig. 1B), which showed that heterodimerization is approximately 17 times stronger than sorcin homodimerization. To confirm these results, a pull-down assay was performed using recombinant GST–sorcin fusion protein and recombinant grancalcin. Fig. 2A shows that the recombinant proteins are able to heterodimerize in vitro in a calcium-independent manner. As a control for the specificity of the GST–sorcin–grancalcin interaction, the ability of ALG-2 to bind to GST–sorcin was also tested (Fig. 2B). Our results suggest that the sorcin/grancalcin interaction is specific.

The interaction between grancalcin and sorcin was further

WB: anti-sorcin

Fig. 3. Interaction of grancalcin and sorcin in HUVEC. Co-immunoprecipitation of grancalcin and sorcin. Pre-immune rabbit serum was used as a negative control (lane 1), immunoprecipitation with a specific anti-grancalcin-2 (lane 2) antibody, which in contrast to anti-grancalcin-1 showed no cross-reactivity with sorcin, HUVEC total lysate (lane 3). The immunostaining was performed with anti-sorcin antibody.

confirmed by immunoprecipitation from HUVEC, where both proteins were found to be expressed (Fig. 3). Thus, the two PEF proteins, grancalcin and sorcin, interact in vivo.

4. Discussion

By a systematic analysis of PEF protein heterodimerization we show here for the first time that sorcin and grancalcin physically associate in vitro in a calcium-independent manner in contrast to calpains or ALG-2/peflin heterodimers, whose association is influenced by calcium [4]. Moreover, sorcin and grancalcin are expressed in HUVEC, from which they can be co-immunoprecipitated. The finding of the grancalcin–sorcin interaction allows us to conclude that all so far known PEF proteins have the potential to heterodimerize with one other member of the PEF family.

Biological effects of the grancalcin–sorcin interaction remain to be investigated. The fact that these proteins are co-expressed at least in endothelial cells and cells of myeloid origin [15] indicates that the grancalcin–sorcin interaction may have a functional meaning.

It is well established that sorcin binds the ryanodine receptor at micromolar calcium concentrations. In the cardiac muscle this interaction leads to the inhibition of ryanodine binding [19]. In contrast, interaction of sorcin with the ryanodine receptor from skeletal muscle potentiates the binding of ryanodine [12]. Another interaction partner of sorcin is the L-type calcium channel [20]. Taken together, these data indicate that sorcin might be a modulator of the physical and functional link between calcium release channels of the plasma membrane and the sarcoplasmic reticulum. Grancalcin might influence modulation of the sorcin target proteins via high affinity heterodimerization with sorcin.

Only one grancalcin interacting partner, L-plastin [18], a leukocyte-specific protein with a calcium-dependent actin bundling activity [21], is known so far. L-plastin has been shown to be involved in integrin activation and subsequent increased adhesion of neutrophils after stimulation with inflammatory
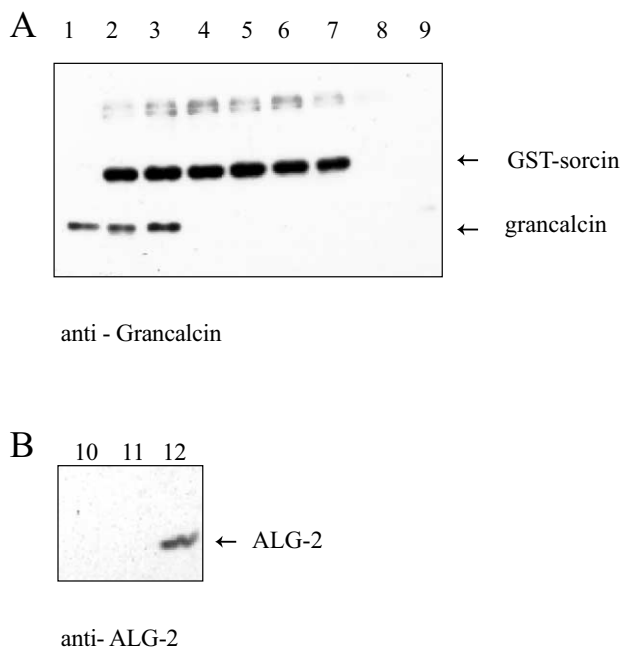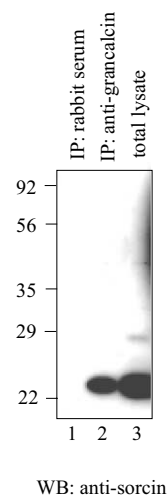

anti - Grancalcin

anti- ALG-2

Fig. 2. Grancalcin and sorcin interact in vitro. Pull-down assay. GST–sorcin (lanes 2–7) or GST (lanes 8,9) were bound to glutathione-Sepharose and incubated with either grancalcin (lanes 2,3,8,9) or ALG-2 (lanes 4,5,10,11) in the presence of either 1 mM $CaCl_2$ (lanes 2,4,6,8,10) or 1 mM EGTA (lanes 3,5,7,9,11). 10 ng grancalcin (lane 1) and 1.5 ng ALG-2 (lane 12) were run as controls for immunodetection. A: Immunodetection with anti-grancalcin-1 antibody. Note: anti-grancalcin-1 antibody cross-reacts with sorcin. B: Immunodetection with anti-ALG-2 antibody.

stimuli [22]. Interestingly, the grancalcin/L-plastin interaction is inhibited by calcium, reminiscent of the interaction between ALG-2 and peflin [4]. How sorcin/grancalcin heterodimerization could affect the biological function of L-plastin cannot be estimated since the functional consequences of grancalcin/L-plastin interaction have not been investigated so far. As it has been shown recently that grancalcin-deficient mice do not have major defects in vital leukocyte effector functions [23], it can be speculated that sorcin could possibly fulfill the function(s) of grancalcin.

It has to be emphasized here that the PEF proteins can share some of the targets. Both ALG-2 and sorcin interact with annexin VII [14], a protein which was suggested to play a role in apoptosis [24] and in the exocytotic membrane fusion process in chromaffin cells [25]. In many cell types sorcin and ALG-2 are co-expressed. However, ALG-2 does not form a stable complex with sorcin meaning that the two PEF proteins may independently modulate the activity of their shared target. In the presence of the heterodimerization partners (grancalcin for sorcin and peflin or ALG-2,1 for ALG-2) selection of the protein–protein interactions may occur. Thus, heterodimerization might be a way to regulate the recruitment of different PEF proteins in response to e.g. calcium transients in cells, which may allow a broad spectrum of biological responses such as apoptosis, cell motility or exocytosis to occur.

References

[1] Berridge, M.J. (1997) J. Physiol. 499, 291–306.
[2] Berchtold, M.W., Brinkmeier, H. and Muntener, M. (2000) Physiol. Rev. 80, 1215–1265.
[3] Maki, M., Narayana, S.V. and Hitomi, K. (1997) Biochem. J. 328, 718–720.
[4] Kitaura, Y., Matsumoto, S., Satoh, H., Hitomi, K. and Maki, M. (2001) J. Biol. Chem. 276, 14053–14058.
[5] Elce, J.S., Davies, P.L., Hegadorn, C., Maurice, D.H. and Arthur, J.S. (1997) Biochem. J. 326, 31–38.
[6] Sorimachi, H. et al. (1995) J. Biol. Chem. 270, 31158–31162.
[7] Tarabykina, S., Moller, A.L., Durussel, I., Cox, J. and Berchtold, M.W. (2000) J. Biol. Chem. 275, 10514–10518.
[8] Blanchard, H., Grochulski, P., Li, Y., Arthur, J.S., Davies, P.L., Elce, J.S. and Cygler, M. (1997) Nat. Struct. Biol. 4, 532–538.
[9] Jia, J., Han, Q., Borregaard, N., Lollike, K. and Cygler, M. (2000) J. Mol. Biol. 300, 1271–1281.
[10] Xie, X., Dwyer, M.D., Swenson, L., Parker, M.H. and Botfield, M.C. (2001) Protein Sci. 10, 2419–2425.
[11] Jia, J., Tarabykina, S., Hansen, C., Berchtold, M. and Cygler, M. (2001) Structure 9, 267–275.
[12] Lokuta, A.J., Meyers, M.B., Sander, P.R., Fishman, G.I. and Valdivia, H.H. (1997) J. Biol. Chem. 272, 25333–25338.
[13] Boyhan, A., Casimir, C.M., French, J.K., Teahan, C.G. and Segal, A.W. (1992) J. Biol. Chem. 267, 2928–2933.
[14] Brownawell, A.M. and Creutz, C.E. (1997) J. Biol. Chem. 272, 22182–22190.
[15] Lollike, K., Sorensen, O., Bundgaard, J.R., Segal, A.W., Boyhan, A. and Borregaard, N. (1995) J. Immunol. Methods 185, 1–8.
[16] Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. (1995) Yeast 11, 355–360.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[18] Lollike, K., Johnsen, A.H., Durussel, I., Borregaard, N. and Cox, J.A. (2001) J. Biol. Chem. 276, 17762–17769.
[19] Meyers, M.B., Pickel, V.M., Sheu, S.S., Sharma, V.K., Scotto, K.W. and Fishman, G.I. (1995) J. Biol. Chem. 270, 26411–26418.
[20] Meyers, M.B., Puri, T.S., Chien, A.J., Gao, T., Hsu, P.H., Hosey, M.M. and Fishman, G.I. (1998) J. Biol. Chem. 273, 18930–18935.
[21] Namba, Y., Ito, M., Zu, Y., Shigesada, K. and Maruyama, K. (1992) J. Biochem. (Tokyo) 112, 503–507.
[22] Jones, S.L., Wang, J., Turck, C.W. and Brown, E.J. (1998) Proc. Natl. Acad. Sci. USA 95, 9331–9336.
[23] Roes, J., Choi, B.K., Power, D., Xu, P. and Segal, A.W. (2003) Mol. Cell. Biol. 23, 826–830.
[24] Yu, F., Finley Jr., R.L., Raz, A. and Kim, H.R. (2002) J. Biol. Chem. 277, 15819–15827.
[25] Caohuy, H. and Pollard, H.B. (2002) J. Biol. Chem. 277, 25217–25225.
[26] La Cour, J.M., Mollerup, J., Winding, P., Tarabykina, S., Sehested, M. and Berchtold, M.W. (2003) Am. J. Pathology, in press.