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Supplemental materials and methods

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using the pLexA system according to the protocol of Golemis et al. (1997; Gyuris et al., 1993). The full ORF cDNA of rat VDAC1 (NM_031353) was cloned into the pGilda yeast-inducible expression vector fused with the pLexA DNA-binding domain. The EGY48 (*leu* −/−) yeast strain was transformed with the pGilda/VDAC1 vector using the lithium acetate method. The expression of the full-length VDAC1 protein was verified by immunoblotting, whereas its nuclear localization was shown by the negative repression test (Golemis et al., 1997). After the validation of the method, the pGilda/VDAC-containing yeasts were transformed with the *lacZ* reporter gene plasmid pSH18-34, followed by transformation of ~5 × 10^6 yeast cells with the pJG4-5 pLexA activation domain fusion vector cDNA libraries (from human embryonic kidney and adult liver; Origene). Positive clones were selected in two runs; first for their ability to grow on plates lacking leucine; and second, the remaining clones were assayed for β-galactosidase activity on medium supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). 372 and 104 positive clones were obtained from the kidney and brain cDNA libraries, respectively. The positive clones were first amplified by PCR using yeast stocks homogenized by three freeze/thaw cycles as template and primers annealing to the pJG4-5 vector (forward: 5′-CGTAGTGGAGATGCCTCC-3′; reverse: 5′-CTGGCAAGGTAGACAAGCCG-3′). The PCR reaction was performed using the Long term PCR kit (Roche). PCR products were first characterized by restriction enzyme mapping using the frequent cutter HaeIII enzyme. Clones were grouped according to their digestion patterns, verified by agarose gel electrophoresis, and one clone from each group was bidirectionally sequenced using the primers applied in the PCR reaction. Approximately 90% of the clones contained a sub-sequence of the ER-resident chaperone heat-shock 70-kd protein 5 (HSPA5; grp78; Table 1), probably reflecting the requirement of efficient folding of the VDAC1 protein. The results of sequencing of the remaining clones are shown in the table. One group of the putative interacting proteins were found to be cytoskeletal and signaling elements (shown in bold), whereas another group were found to be folding intermediates, presumably underlying the proper function of the VDAC OMM channel. To verify the interaction of VDAC1 with grp75 (heat-shock 70-kd protein 9b; HSPA9B, shown in bold/italics), the plasmid containing the positive cDNA was isolated from yeast. Cells of the positive clone were resuspended in 1 M sorbitol and 50 mM EDTA with the yeast lytic enzyme (2 mg/ml; MP Biomedicals). After 30 min at 37°C, the yeast cells were centrifuged, and the pellet was dissolved in Hirt's solution (10 mM Tris-HCl, pH 7.5, 50 mM EDTA, and 0.2% SDS) with 0.5 mg/ml proteinase K (Invitrogen) and incubated at 50°C for 6 h. The plasmid DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was precipitated with the same volume of 20% polyethylene glycol and 2.5 M NaCl, and the plasmid DNA was pelleted, washed with 70% ethanol, and resuspended in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The plasmid obtained was transformed into *Escherichia coli* strain DH5α and purified. The isolated PJG4-5/grp75(aa 471–681) plasmid was then cotransformed with the pGilda/VDAC1 to the EGY48 (*leu* −/−)/pSH18-34 yeast strain and verified to interact with VDAC1 by survival on *leu* – medium and by induced galactosidase activity on X-gal plates.

Subcellular fractionation and proteomic analysis

HeLa cells and rat liver were homogenized, and crude mitochondrial fraction (8,000 *g* pellet) was subjected to separation on a 30% self-generated Percoll gradient, as previously described (Vance, 1990). A low-density band (denoted as the MAM fraction) was collected and further purified by removing the pellet after a 10-min, 9,000 *g* centrifugation. The high-density Mito P fraction was pelleted by the same centrifugation. Microsomes were pelleted with 100,000 *g* centrifugation 90 min from the postmitochondrial supernatant. The resulting fractions were analyzed by immunoblotting and Blue native/SDS-PAGE 2D separation. For SDS-PAGE analysis, 10 μg of proteins of the different subcellular fractions were loaded on 10% SDS-polyacrylamide gels. For characterization of subcellular fractions, the following antibodies were used: αIP_3R (Calbiochem); αVDAC1 (Calbiochem); αgrp75 (Santa Cruz Biotechnology, Inc.; H-155, sc-13967); MnSOD (Santa Cruz Biotechnology, Inc.; N-20, sc-18503); and COXII (Santa Cruz Biotechnology, Inc.; N-20, sc-23984). For coimmunoprecipitation of grp75 with IP_3R and VDAC1, total cellular proteins were precipitated with 3 μg of a monoclonal grp75 antibody (MA3-028; Affinity BioReagents) and protein G (or A)–Sepharose (GE Healthcare) and washed with 50 mM Tris-HCl, 1% NP-40 (for the IP_3R), or 50 mM Tris-HCl (for VDAC1) according to the manufacturer's instructions. IP_3R1 was immunoprecipitated from the same total cellular protein extract with a rabbit polyclonal antibody (ab5908; ABCAM). For VDAC1 immunoprecipitation, an anti–human VDAC antibody was used (courtesy of V. De Pinto, University of Catania, Catania, Italy). In the case of IP_3R precipitation, the protein was overexpressed in HeLa cells using a mouse IP_3R-1 clone (courtesy of K. Mikoshiba). This was necessary because even if the immunoprecipitated IP_3R-1 was detectable without overexpression of the protein, it was not detectable in the input fractions with the available antibodies. The precipitated protein fraction was separated on 7 or 10% SDS-polyacrylamide gels and immunoblotted against IP_3R-3 (1:200; goat polyclonal; Santa Cruz Biotechnology,

Inc.;C-20; sc-7277), grp75, and VDAC1, as described. For Blue native and SDS-PAGE 2D separation of the MAM fraction proteins, the native MAM fraction was solubilized with 1 M aminocaproic acid and 2% dodecylmaltoside combined with 5% Serva Blue G and separated on a 4–12% acrylamide gradient gel in the first dimension. The gel was incubated with a dissociating solution (1% SDS and 1% mercaptoethanol), stacked over a 10% SDS-PA gel, and separated, and the proteins were immunoblotted against the IP_3Rs, grp75, and VDAC1 using the aforementioned antibodies.

$[Ca^{2+}]_c$ imaging

HeLa cells, which were transiently transfected with mRFP1-fused $IP_3R\text{-}LBD_{224\text{-}605}$ constructs, were loaded with 3 mM fura-AM (30 min at 37°C) and placed in a thermostatted chamber at 37°C in KRB/Ca^{2+} solution on the plate of an inverted microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.). A microperfusion system (ALA DVD-12; Ala Scientific) allowing rapid solution exchange was used to apply stimuli. Perfusion and image acquisition was controlled by the MetaFluor 5.0 software (Universal Imaging Corp.). The probe was excited at 340 and 380 nm, using a random access monochromator (Photon Technology International) and imaged through a Plan Neofluar 40×/1.30 NA oil objective (Carl Zeiss MicroImaging, Inc.) at 520 nm emission with a back-illuminated Cascade B512 camera (Photometrics). Excitation ratios were normalized to the initial ratio (ΔR/R).

References

Golemis, E.A., I. Serebriiskii, J. Gyuris, and R. Brent. 1997. Interaction trap/two-hybrid system to identify interacting proteins. *In* Current Protocols in Molecular Biology. F.M. Ausubel, R. Brent R, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl, editors. New York Greene Publishing/Wiley Interscience, New York.

Gyuris, J., E. Golemis, H. Chertkov, and R. Brent. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell*. 75:791–803.

Vance, J.E. 1990. Phospholipid synthesis in a membrane fraction associated with mitochondria. *J. Biol. Chem.* 265:7248–7256.

Supplementary Table 1.

Construct	$[Ca^{2+}]_m$ peak response	$[Ca^{2+}]_c$ peak value
control	81.5 ± 5.84	3.1 ± 0.15
$IP_3R\text{-}LBD_{224\text{-}605}$	114.3 ± 10.31*	2.4 ± 0.12*
$OMM\text{-}IP_3R\text{-}LBD_{224\text{-}605}$	121.7 ± 11.20*	2.9 ± 0.29
$OMM\text{-}IP_3R\text{-}LBD_{1\text{-}605}$	104.7 ± 5.29*	2.8 ± 0.21
$ER\text{-}IP_3R\text{-}LBD_{224\text{-}605}$	100.5 ± 5.98*	2.4 ± 0.24*
$OMM\text{-}IP_3R\text{-}LBD_{224\text{-}605}$(K508A)	110.5 ± 11.23*	3.0 ± 0.12
OMM-p130-PH	62.6 ± 6.01*	2.5 ± 0.28*
grp-75	51.9 ± 11.30	ND
siRNAc	46.4 ± 3.05	ND
siRNAc + $OMM\text{-}IP_3R\text{-}LBD_{224\text{-}605}$	65.2 ± 3.55**	ND
siRNAc + $ER\text{-}IP_3R\text{-}LBD_{224\text{-}605}$	59.2 ± 6.18**	ND
siRNA grp-75	42.2 ± 2.40	ND
siRNA grp-75 + $OMM\text{-}IP_3R\text{-}LBD_{224\text{-}605}$	48.8 ± 1.82	ND
siRNA grp-75 + $ER\text{-}IP_3R\text{-}LBD_{224\text{-}605}$	48.2 ± 2.40	ND

$[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ responses of HeLa cells expressing the denoted constructs and mtAEQmut or cytAEQ, respectively. Cells were stimulated with 100 μM histamine. Mean ± SEM values are shown. Values from > 10 traces of at least 3 separate experiments

* $p \leq 0.05$ with respect to control

** $p \leq 0.05$ with respect to siRNAc