Data in support of Rap2a GTPase expression, activation and effects in LPS-mediated innate immune response and NF-κB activation

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ABSTRACT

We present here the data to support the understanding of the implication of Rap2a GTPase in LPS-induced innate immune response and NF-κB activation. The data presented are related to molecular tools that were generated, acquired, optimized or validated to investigate Rap2a expression, activation and its effects in mammalian cells including RAW264.7 macrophages and THP-1 monocytes under inflammatory conditions. These data supplement important technical and biological information on immune function of Rap2a in macrophages activated by LPS, recently reported by us (Carvalho et al., 2019) [1].

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1. Data

Besides providing methods used for generating molecular tools to investigate Rap2a activation in cells this article supplements with biological data that support the GTPase expression and its implication in Toll-like receptor (TLR)-mediated innate immune response, recently reported by us (Carvalho et al., 2019) [1]. Fig. 1a–d depict the amplification and cloning of the Ras/Rap GTPase binding domain (RBD) of mouse Ral guanine nucleotide dissociation stimulator (RalGDS) from mammalian mouse embryonic fibroblasts (MEFs) and RAW264.7 macrophages. Recombinant expression of GST-RBD-RalGDS and its use in pull-down assays is validated in cell extracts from RAW264.7 macrophages (Fig. 2a). Analyses of the mRNA levels of Rap2a GTPase upon LPS or poly-IC stimulation of cells are shown in Fig. 3a–c. Validation of two distinct siRNAs targeting murine Rap2a and their effects are shown in Figs. 4 and 6. ELISA data for TNF in Rap2a siRNA-transfected RAW264.7 macrophages is shown in Fig. 5. The effects of Rap2a in NF-κB activity in human THP-1 monocytes upon LPS stimulation is shown (Fig. 7). Aminoacid and tree alignments data showing identity among Rap2 family members, and also among Rap1 members a and b are depicted in Fig. 8a–d.
2. Experimental design, materials and methods

2.1. Construction of pGEX plasmid containing the RBD from murine RalGDS

The Rap2 GTPase binding domain (RBD) of murine Ral guanine nucleotide dissociation stimulator (RalGDS) (GenBank accession no.: NM_009058.2) was cloned from mouse embryonic fibroblasts (MEFs). Briefly, total RNA was extracted from MEFs and subsequently transcribed into cDNA that was used as template in RT-PCR reactions using Q5 High fidelity DNA polymerase (New England Biolabs).
and the oligonucleotides RalGDS-Bam-Fwd: 5'-TCATGGATCCTCACTGCCTCTACA-3', and RalGDS-Eco-Rev: 5'-TTAGGAATTCGAAGATGCCTTTGGCA-3'. PCR-amplified cDNA fragment of 401-bp comprising the RBD-RalGDS mRNA coding sequence was cloned into pCR2.1 vector, and then a BamHI-EcoRI fragment was obtained by digestion, purified and finally subcloned in pGEX-6P-1 vector (GE Healthcare). The authenticity of the RalGDS-RBP insert sequence was confirmed by automated sequencing.

Fig. 2. Expression of GST-RBD-RalGDS and validation of affinity precipitation of Rap2a in mammalian cell extracts. (a) The expression of GST-RBD-RalGDS was obtained upon induction with 1 mM isopropyl-1-thio-beta-D-galactopyranoside (IPTG) for 2 hours of bacterial cell cultures transformed with pGEX-6P-1-RBD-RalGDS. Bacterial extracts were fractionated onto 10% SDS-PAGE, and followed by coomassie blue staining. (b) Coupling of GST-RBD-RalGDS to glutathione sepharose 4B (GS4B) beads. Suspensions of IPTG-induced bacterial cell cultures transformed with pGEX-6P-1 or pGEX-6P-1-RBD-RalGDS were centrifuged, lysed and sonicated. GST or GST-RBD-RalGDS fusion protein were mixed with GS4B. Bacterial cell lysates (lanes 1 and 2) and eluted samples (lanes 3–5) were separated by 10% SDS-PAGE, followed by coomassie blue staining. Two independent bacterial clones of pGEX-6P-1-RBD-RalGDS were used in lanes 4 and 5, respectively. (c) RAW264 cells were treated with PMA (100nM) as indicated. The lysates (500 μg) were then incubated with 100μl (~0.5mg) of bacterial lysates containing GST-RalGDS-RBD precoupled to GS4B beads. After washes, the beads mixtures were fractionated on 12% SDS–PAGE, transferred to PVDF membrane, and probed with anti-Rap2A antibody. Anti-Rabbit IgG (H + L), peroxidase conjugated was used as the secondary antibody. The detection was performed with Clarity Western ECL Blotting Substrate (BioRad) and followed by exposure to X-ray film. Densitometrical analysis of the western blots is shown on right where the densitometry values obtained for Rap2a were normalized to GST-RBD-RalGDS values in lanes 1–3. M, protein standards (250, 150, 100, 75, 50, 37, 25, 20, 10 KDa).
2.2. Expression and isolation of GST-RalGDS fusion protein

Procedures were followed as outlined by van Triest et al., 2001 [2] with some minor modifications. An overnight culture of BL21(DE3)pLysS bacteria bearing the plasmid pGEX-6P-1-RBD-RalGDS was diluted 1:50 (v/v) in one liter of LB (10 g/l (w/v) tryptone, 10 g/l (w/v) NaCl, 5 g/l (w/v) yeast extract) containing 50 µg/ml ampicillin and grown for a further hour at 37 °C. Protein synthesis was induced for 2 h with the addition of 1 mM IPTG. Cells were then pelleted (7700 × g at 4 °C, 10 min) and frozen at −80 °C overnight to break bacterial cell walls. The following day cells were resuspended in 25 ml of

![Expression analysis of Rap2a in LPS-treated RAW264.7 macrophages and human THP-1 monocytes. Non-quantitative RT-PCR and quantitative RT-qPCR analyses of Rap2a in RAW264.7 (a, b) and RAP2A in THP-1 (c) cells that were stimulated as indicated. Lower graph in (a) shows fold change expression after densitometric analysis of Rap2a:Gapdh ratio. Total RNA was extracted and subsequently transcribed into cDNA that was used as template in RT-qPCR reactions for detection of mRNAs. NT, not treated. NC, PCR negative control.](image-url)
cold 10 ml of 50 mM Tris—HCl pH 8.0, 20% (w/v) sucrose, 10% (v/v) glycerol, 2 mM dithiothreitol (DTT), 2 mM MgCl₂, containing protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, leupeptin 1 μg/ml, and aprotinin 2 μg/ml). Cell suspensions were sonicated on ice to shear DNA (10 × 15 sec bursts, at amplitude of 20–30%), and then cell debris was pelleted by centrifugation (12,000 × g at 4 °C, 60 min). The supernatant was collected and stored in aliquots of 1 ml at −80 °C until use. The presence of GST-RBD-RalGDS fusion protein in the clear was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Affinity precipitation of active GTP-bound Rap2a and immunoblotting

Bacterial lysates containing GST-RalGDS-RBD were used to determine the relative amount of active GTP-bound Rap2a in cell extracts of mammalian cell cultures. Detergent extracts of whole cells were prepared by solubilization in cold lysis buffer (Tris HCl 50 mM pH 7.4; NaCl 200 mM; 2.5 mM MgCl₂; 50 mM NaF; 10% v/v glycerol; 1% v/v nonidet P-40, and protease and phosphatase inhibitors) and centrifuged at 4 °C (10,000 × g, 10 min). Cell extracts (500 μg to 3 mg) were incubated with 200 μl (~1 mg) of bacterial lysates containing GST-RalGDS-RBD pre-coupled to glutathione sepharose 4B beads (30 μl per cell extracts) for 45 min at 4 °C under rotation. The beads were then washed four times in lysis buffer, followed by SDS–PAGE and immunoblotting with anti-Rap2a antibody (Thermo Fisher Scientific, Product # PA5-23298) used at a concentration of 1:500 (v/v).

Fig. 4. Rap2a mRNA levels are reduced after transfection with two different specific siRNAs. MEFs-SV40 were left untransfected or transfected with siRNA-GAPDH or two different siRNA targeting Rap2a for 24h. Then, cells were lysed to obtain total RNA transcribed into cDNA which was used as template in RT-qPCR reactions for detection of Rap2a.
2.4. End-point RT-PCR analysis

After treatment with TLRs agonists, medium was discarded and total RNA was obtained with Trizol following instructions of the manufacturer (Invitrogen). One microgram of total RNA was used to obtain the first strand cDNA in the presence of oligo-dT15, dNTPs, RNAsin, and reverse transcriptase (MMLV RT, Promega, Madison, WI, USA). One-tenth of first strand cDNA reaction was used as template in PCR reactions to amplify cDNA fragments corresponding to Rap2a and Gapdh mRNAs. The sequences of the primers used were as follows: Rap2a-F: 5’-CGATGCCGAGTACAAAGTG-3’ and Rap2a-R: 5’-GCCTAGACGAATCCTGTCCG-3’, annealing at 62 °C, 25 cycles. Gapdh-F: 5’-CCCTCAACTACATGCCAAAGT-3’ and Gapdh-R: 5’-CCTTCCACAATGCCCAAGT-3’, annealing at 52 °C, 25 cycles. Levels of the mRNA for the Gapdh were monitored in parallel to normalize the total amount of cDNA in each sample. The PCR products were fractionated onto 1.2% agarose gel, stained with ethidium bromide, visualized in UV transiluminator, and the images captured by a CCD camera in a photodumentation system.

2.5. RT-qPCR

The expression of mRNA for human RAP2A in THP-1 cells was analyzed with quantitative PCR performed with iQSybr Master Mix kit (BioRad) in the CFX96 Touch™ Real Time detection system (BioRad). The following primers for human RAP2A and GAPDH were used: RAP2A-FWD, 5’-ATGCCGAGTACAAAGTG-3’ and RAP2A-REV 5’-GGCACGAATCCACCTCAGT-3’; GAPDH-FWD, 5’-ACAGTCAGCCGATCTTCTT-3’ and GAPDH-REV, 5’-ACGACCAATCCGTGACTC-3’. Please refer to the research article “Both knock-down and overexpression of Rap2a small GTPase in macrophages result in impairment of NF-κB activity and inflammatory gene expression” for the analysis of expression of mRNA for mouse Rap2a (Carvalho et al., 2019) [1].

2.6. siRNA validation

Mouse embryonic fibroblasts (MEFs) (1 × 10^6 cells/well) were transfected with control (Sigma® MISSION® siRNA Universal Negative Control #1) and two different Rap2a-siRNA (Ambiom Silencer
select®; siRNA-I sequence CCUUCAUUGAGAAUACGAtt; or siRNA-II sequence GAUGAGCUCUUUGCAGAAAtt) at 100 nM using Lipofectamine2000 transfection reagents (Life Technologies) for 24h. Then, cells were lysed to obtain total RNA transcribed into cDNA which was used as template in RT-qPCR reactions for detection of Rap2a. Please refer to the research article “Both knock-down and overexpression of Rap2a small GTPase in macrophages result in impairment of NF-κB activity and inflammatory gene expression” for more data obtained with siRNA Rap2a I.

2.7. TNF cytokine assay

Supernatants from siRNA-control or siRNA-Rap2a (siRNA-I) transfected murine macrophages were harvested and the concentrations of TNF were measured by commercially available ELISA kit, according to the manufacturer’s instructions (R&D Systems).
2.8. Transfection of RAW264.7 and THP-1 cells and analysis of NF-κB activity by gene luciferase reporter

RAW264.7 and THP-1 cells (2 × 10^5/well) were transfected with endotoxin free preparations (GenElute™ Endotoxin-free Plasmid Midiprep Kit, Sigma-Aldrich) NF-κB reporter construct (400ng) and pRL-TK plasmid (100ng). RAW264.7 cells were then co-transfected with control and Rap2a-siRNA I and II at 100 nM using RNAiMax. THP-1 cells were co-transfected with 0.5 or 1.0 mg of Myc-Rap2a plasmid per well as indicated in Fig. 7. After one or two-day post-transfections, cells were stimulated with LPS. Cell lysates were harvested with passive lysis buffer (PLB1x, Promega) and assayed for Firefly and Renilla luciferase activities with Dual-Luciferase Reporter Assay Kit from Promega. The ratio of firefly luciferase to Renilla luciferase was calculated and the results presented as relative luciferase activity.

2.9. Sequence alignments

The Rap protein sequences which accession numbers on GenBank are AAM12628.1 (RAP2A); NP_083795.2 (Rap2a); CAJ18500.1 (Rap2b); AAH50056.2 (Rap2c); NP_663516.1 (Rap1a); NP_077777.1 (Rap1b) were aligned using the ClustalW software. The percentage of similarity was calculated and a tree alignment data was generated from the in silico analysis of ClustalW.
Fig. 8. Analysis of similarity between Rap proteins. The aminoacid sequence of murine Rap2a was aligned with human RAP2A (a), Rap1 members (b), and Rap2b and Rap2c (c). Tree alignment data of murine Rap proteins (d). Clustal W software was used to calculate the percentage of similarity and to generate tree alignment. Aminoacid sequences were obtained through NCBI (National Center for Biotechnology Information - USA) and the GenBank accession numbers for each protein are: AAM12628.1 (RAP2A); NP_083795.2 (Rap2a); CAJ18500.1 (Rap2b); AAH50056.2 (Rap2c); NP_663516.1 (Rap1a); NP_077777.1 (Rap1b).
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Transparency document

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References

[1] B.C. Carvalho, L.C. Oliveira, C.D. Rocha, H.B. Fernandes, I.M. Oliveira, F.B. Leão, T.M. Valverde, I.M.G. Rego, S. Ghosh, A.M. Silva, Both knock-down and overexpression of Rap2a small GTPase in macrophages result in impairment of NF-kB activity and inflammatory gene expression, Mol. Immunol. 109 (2019) 27–37.

[2] M. Van Triest, J. De Rooij, J.L. Bos, Measurement of GTP-bound Ras-like GTPases by activation-specific probes, Methods Enzymol. 333 (2001) 343–348.