Identification of BRAF as a new interactor of PLCε1, the protein mutated in nephrotic syndrome type 3

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Chai H, Hoskins BE, Ashraf S, Goyal M, Wiggins RC, Hildebrandt F. Identification of BRAF as a new interactor of PLCε1, the protein mutated in nephrotic syndrome type 3. Am J Physiol Renal Physiol 294: F93–F99, 2008. First published October 17, 2007; doi:10.1152/ajprenal.00345.2007.—Steroid-resistant nephrotic syndrome (NS) is characterized by late onset of proteinuria and slower progression to end-stage kidney disease (ESKD) (6–8). FSGS is characterized by late onset of proteinuria and slower progression to ESKD.

Recently, we identified mutations in the phospholipase C epsilon 1 gene (PLCE1) as a new cause of autosomal recessive NS in children that present with DMS or FSGS (9). Mutations in PLCE1 cause arrest of glomerular podocyte development at the S-shaped stage, thereby halting glomerular development and causing NS (9, 22). PLCε1 is a phospholipase enzyme that catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate and generates two second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (33). IP3 releases Ca2+ from intracellular stores, and DAG stimulates protein kinase C. Both messengers initiate a cascade of intracellular responses that result in differential gene expression, cell growth, and differentiation (33).

Most of the mutations identified in NS affect genes expressed in podocytes (30). Therefore, proteins expressed in the podocyte are of interest to elucidate the pathogenesis of NS. Discovering proteins expressed in podocytes that interact with PLCε1 will help to understand the signaling pathways and molecular mechanisms by which mutations in PLCE1 cause NS. We set out to identify a new interaction partner of PLCE1. PLCE1 was initially discovered as the C. elegans ortholog plc-1 (29). A database of putative protein interaction partners has been generated for C. elegans, using computationally integrated interactome, gene expression, phenotype, and functional annotation data from S. cerevisiae, C. elegans, and D. melanogaster (36). We hypothesized that proteins suggested by this database as interaction partners of C. elegans plc-1 may also be candidate interaction partners of the human ortholog PLCE1. The database relates plc-1 to the C. elegans ortholog (lin-45) of human BRAF by the criteria of “same C. elegans phenotype (sterile)” and “same C. elegans biological process (signaling).” We therefore examined whether human PLCε1 and BRAF interact within a protein complex. We demonstrate that BRAF is expressed in glomerular podocytes and is in fact in a complex with PLCE1.

MATERIALS AND METHODS

Cell culture. HEK293T and COS-7 cells between passage 15 and 20 were grown at 37°C as a monolayer culture in a humidified air atmosphere with 5% CO2 using minimal essential medium or DMEM (Invitrogen, Carlsbad, CA) supplemented with 1-glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin-neomycin.

Antibodies. Two rabbit polyclonal anti-PLCE1 antibodies, CS117 and RA1, have been characterized previously as described in Ref. 9. Mouse monoclonal anti-BRAF (sc-5284), rabbit polyclonal anti-BRAF (sc-166), mouse monoclonal anti-green fluorescent protein

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(GFP; sc-9996), and mouse monoclonal anti-glutathione S-transferase (GST; sc-138-HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary goat anti-rabbit-horseradish peroxidase (HRP) (cat. no. 31462) and goat anti-mouse-HRP (cat. no. 31432) antibodies were obtained from Pierce Biotechnology (Rockford, IL). For cell immunofluorescence studies, the fluorescence-conjugated secondary antibodies raised in goat, Alexa 488, and Alexa 594 were obtained from Molecular Probes (Carlsbad, CA). For immunohistochemistry studies, we used rabbit anti-PLCε1 antibody (RA1), mouse anti-BRAF antibody, and mouse monoclonal anti-podocalyxin antibody (2A4) (14, 15). Cy3-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse antibodies were used as secondary antibodies (Invitrogen).

Cloning vectors. The full-length human cDNAs of PLCε1 and BRAF were cloned into pENTR/D-TOPO vectors (Invitrogen). We then used the Gateway Technology to flip both cDNAs into different destination vectors. For yeast-2-hybrid screening, pDEST22 and pDEST32 vectors were used. The full-length human cDNA of BRAF and five partial constructs covering different domains of the full-length human cDNA of PLCε1 were cloned as bait and prey in pDEST32 and pDEST22 vectors, respectively. For GST pull down and coimmunoprecipitation, we used pDEST27 and pDEST53 to express GST-tagged and GFP-tagged proteins, respectively. Empty pDEST27 vector was used as a control.

GST pull down. The full-length human cDNAs of PLCε1 and BRAF genes were coexpressed in HEK293T as GST-tagged proteins together with GST-tagged proteins or with the GST empty control plasmid. Transfection was carried out using the FuGENE method (Roche Applied Science, Palo Alto, CA). After 48 h, transiently transfected cells were washed with cold PBS and then proteins were extracted using RIPA buffer with protease inhibitors (cat. no. 11836170001 Roche). Glutathione-agarose beads (cat. no. G4510, Sigma, St. Louis, MO) were added to the protein lysates and were incubated overnight at 4°C. After being washed several times with cold PBS with protease inhibitors, beads were suspended and denatured in Laemmli sample buffer and loaded onto SDS-polyacrylamide gels (4–15%). Gels were transblotted onto nitrocellulose filters. Filters were immunoblotted with mouse monoclonal anti-GFP antibody. The secondary antibody was an HRP-conjugated goat anti-mouse antibody. Blots were developed with ECL substrate (Santa Cruz Biotechnology sc-2048). For the GST pull down and transfection controls, the blots were stripped and reprobed with a mouse anti-GST-HRP antibody.

Coimmunoprecipitation. For HEK293T endogenous expression, cells were transfected with full-length human PLCε1 cDNA. After 48 h, cell lysates were precleared with protein A/G PLUS-Agarose beads from Santa Cruz Biotechnology (sc-2003) and incubated overnight at 4°C with either rabbit polyclonal anti-PLCε1 antibody (CS117) or normal rabbit IgG serum (sc-2027) as control, followed by incubation with A/G PLUS-Agarose beads for 3 h. The beads were washed extensively with cold PBS containing protease inhibitors, and bound proteins were boiled in sample buffer and resolved on a 4–15% SDG-PAGE gel and transblotted. The blots were immunoprobed with mouse anti-BRAF. For endogenous mouse kidney studies, kidneys obtained from a male adult 129S6 mouse were homogenized in 0.5% Triton X-100 with protease inhibitors and cleared by centrifugation. Lysates were further precleared using A/G PLUS-Agarose beads and immunoprecipitated with rabbit anti-PLCε1 (CS117) and immunoblotted with mouse anti-BRAF. For reverse coimmunoprecipitation, lysates were immunoprecipitated with mouse anti-BRAF and blots were immunoblotted with rabbit anti-PLCε1 (CS117).

Immunofluorescence in HEK293T and COS-7 cells. For exogenous expression of BRAF and PLCε1 in HEK293T and COS-7 cells, full-length human PLCε1 and BRAF cDNA were coexpressed in HEK293T and COS-7 as GST-tagged proteins. Transfection was carried out in four slide chambers using the FuGENE method (Roche Applied Science). After 48 h, transiently transfected cells were fixed in methanol for 15 min at −20°C. The cells were washed with PBS and then blocked with TBST containing 0.15% heat-inactivated fetal bovine serum for 1 h at room temperature. Double-immunofluorescence staining was performed with polyclonal rabbit anti-PLCε1 (CS117) and mouse monoclonal anti-BRAF. Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse were used as secondary antibodies. Slides were washed and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen). All images were captured with a Leitz DMRB microscope and an Optronics camera.

For endogenous expression of BRAF in HEK293T cells, cells were methanol fixed, washed, and images were captured as described in the exogenous studies. Immunofluorescence staining was performed with mouse monoclonal anti-BRAF. Alexa 594-conjugated goat anti-mouse was used as secondary antibody.

Fig. 1. Glutathione S-transferase (GST) pull down in HEK293T cell lysates of tagged phospholipase C epsilon 1 (PLCε1), tagged BRAF, and GST-empty vector. A: following cotransfection of HEK293T cells with green fluorescent protein (GFP)-PLCε1 and GST-BRAF (lanes 1, 2, 4, 5), GFP-PLCε1 was detected after GST-BRAF pull down (lane 4). Following cotransfection of HEK293T cells with GFP-BRAF and GST-PLCε1 (lanes 7, 8, 10, 11), GFP-BRAF was detected after GST-PLCε1 pull down (lane 10). Membrane was immunoblotted with mouse anti-GFP. B: blot of A was reprobed with mouse anti-GST antibody as control for loading and pull down efficiency.

WB: Mouse anti-GFP

WB: Mouse anti-GST

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A: Mouse anti-GFP

B: Mouse anti-GST
**Immunofluorescence in kidney sections.** Kidneys from 2-mo-old Fisher 344 rats were perfusion-fixed with periodate-lysine-paraformaldehyde as previously described (32). Cryostat-cut kidney sections were treated with Retrieve-All target unmasking reagent (Signet Laboratories, Dedham, MA) for 2 h at 90°C. Two-day-old Fisher 344 rat kidney sections (n = 1) were used for developmental studies. Sections were blocked with 10% goat serum in PBS. Double-immunofluorescence staining was performed with anti-PLCε antibody (RA1) and mouse anti-BRAF. We also used the 2A4 monoclonal anti-podocalyxin antibody as a marker of the apical surface of podocytes. Cy3-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse antibodies were used as secondary antibodies. Sections were costained with DAPI for nuclear staining.

The animal work was approved by the University Committee on Use and Care of Animals (UCUCA no. 7738, no. 8608).

**RESULTS**

**BRAF is in a complex of proteins with PLCε.** We evaluated whether BRAF would be part of a signaling complex with PLCε. We sought to investigate the interaction by GST pull down assay. Following cotransfection of HEK293T cells with the full-length human cDNA encoding PLCE1 fused with GFP (GFP-PLCE1) and the full-length human cDNA of BRAF fused to GST (GST-BRAF), we demonstrated by GST pull down that GST-BRAF can pull down GFP-PLCE1 (Fig. 1A, lane 4). Reciprocally, GST pull down of GST-PLCE1 yielded GFP-BRAF in the pull down as shown in Fig. 1A, lane 10. We therefore demonstrated that BRAF interacts with PLCε and we conclude that BRAF can form a complex with PLCε.

**BRAF and PLCε proteins colocalize in HEK293T and COS-7 cell lines.** First, we evaluated the subcellular localization of endogenous BRAF expression in HEK293T cells. Following immunostaining with mouse anti-BRAF, we demonstrated a cytoplasmic subcellular localization of BRAF in HEK293T cells (Fig. 2A, a and b). The signal is specific to BRAF as this staining pattern was not seen in the “secondary only” control (Fig. 2A, c).

To further evaluate the identification of BRAF as an interaction partner of PLCε, we studied the subcellular localization of exogenous BRAF and PLCε proteins. We evaluated the distribution of both proteins in two cell lines, HEK293T and COS-7.

![Fig. 2. Subcellular localization of PLCε1 and BRAF in HEK293T and COS-7 cells. A: subcellular localization of endogenous BRAF in HEK293T cells. HEK293T cells were immunostained with mouse anti-BRAF following with a goat anti-mouse (Alexa 594), showing cytoplasmic localization of endogenous BRAF in HEK293T (red, a). A merged image with DNA stained with DAPI (blue) is shown in b. A staining control with secondary goat anti-mouse only and DAPI is shown in c. B: colocalization of PLCε1 with BRAF in HEK293T and COS-7 cells. Expression of PLCε1 (green; a and d) and BRAF (red; b and e) was seen in HEK293T and COS-7 cells. Whenever cells are transfected with both proteins, there is full colocalization of the 2 proteins as seen in the merged images (c and f) as yellow. Nuclei (DNA) were stained with DAPI (blue).](http://ajprenal.physiology.org/)}
and COS-7. Following the expression of GST-PLCε1 and GST-BRAF fusion proteins, and double-immunofluorescence analysis with rabbit anti-PLCε1 and mouse anti-BRAF, we demonstrated an overlap of cytoplasmic subcellular distribution of BRAF and PLCε1 (Fig. 2B). The expression patterns of BRAF and PLCε1 proteins were overlapping in both cell lines, compatible with an interaction of BRAF and PLCε1 in both cells.

**Endogenous BRAF interacts with endogenous PLCε1.** As both studies carried out above are in vitro studies, we examined protein-protein interaction of PLCε1 and BRAF at the endogenous level by immunoprecipitation. HEK293T cells express endogenous BRAF (Fig. 3A); however, the endogenous expression of PLCε1 in HEK293T cells is low or undetectable (9). To study the interaction between endogenous BRAF and overexpressed PLCε1, we expressed GFP-PLCε1 fusion protein in HEK293T cells and immunoprecipitated with rabbit anti-PLCε1 (CS117). Immunoblotting analysis revealed that endogenous BRAF can be immunoprecipitated by the overexpressed PLCε1 but not by a control IgG (Fig. 3A). We therefore demonstrated that endogenous BRAF interacts with the exogenous PLCε1.

To study the coimmunoprecipitation of endogenous PLCε1 and endogenous BRAF, we performed coimmunoprecipitation studies in mouse kidney lysates. Lysates from mouse kidneys were immunoprecipitated overnight with polyclonal rabbit anti-PLCε1 antibody (CS117). Immunoblotting analysis revealed that endogenous BRAF can be coimmunoprecipitated by an antibody to endogenous PLCε1 but not by a control IgG (Fig. 3B). We therefore demonstrated that the endogenous PLCε1 coprecipitates with endogenous BRAF. However, we were not able to demonstrate the reciprocal coimmunoprecipitation. The reason for this result is not known, but it may be due to the BRAF antibody masking the interaction epitope by a third protein since the interaction is not direct. Another possibility is that a conformational change in BRAF may occur, making the epitope less accessible to the BRAF antibody.

**BRAF is an indirect interaction partner of PLCε1.** PLCε1 contains multiple known protein-protein interaction domains, suggesting that it may represent a scaffolding protein of signaling processes. To test whether the interaction of PLCε1 and BRAF may be direct, we performed yeast-2-hybrid studies using five different subclones of PLCε1 as bait and full-length BRAF as prey. Analysis of these studies failed to show any direct interaction between the five partial domains of PLCε1 and BRAF (data not shown). No interaction was seen when the clones were reversed, using BRAF as bait and PLCε1 subclones as prey. Therefore, we conclude that BRAF does not interact directly with PLCε1 (data not shown).

**Expression and colocalization of endogenous BRAF and PLCε1 protein in rat podocyte glomerulus.** To further examine BRAF expression in renal glomerulus, we performed immunohistochemistry studies in newborn and adult rat kidney sections. In these studies, the podocyte apical marker podocalyxin was used to delineate the glomerular structure (28). Using a monoclonal mouse anti-BRAF antibody, we demonstrated for the first time that BRAF is expressed in podocytes at the S-shaped stage of glomerular development in the newborn rat (Fig. 4, a–c), and in adult rat glomerulus (Fig. 4, d–f), as demonstrated by the colocalization with the podocyte podocalyxin marker (Fig. 4, c and f). BRAF expression was not limited to podocytes as it was present in tubular epithelial cells in both newborn developing kidneys and to a lesser extend in adult kidneys (Fig. 4, a and d). In contrast, PLCε1 expression was restricted to podocytes in both developing and adult kidneys implying that BRAF may have functions other than through its interactions with PLCε1. PLCε1 is known to be expressed in the podocyte, and mutations in PLCε1 lead to an arrest of glomerular development (9). To further evaluate the identification of BRAF as an interactor of PLCε1, we examined the colocalization of BRAF and PLCε1 in developing podocytes of rat kidney sections (Fig. 4, g–i). Using mouse anti-BRAF and rabbit anti-PLCε1 (RA1), we demonstrated a colocalization of both proteins in developing podocytes (Fig. 4i).

**DISCUSSION**

We utilized a *C. elegans* genetic interaction database to generate further candidate interaction partners for PLCε1/plc-1 (36). Using a GST pull down assay, we were able to demonstrate that BRAF and PLCε1 are in the same protein complex. By immunofluorescence studies we showed subcellular colocalization of both proteins in two cell lines, HEK293T and COS-7, supporting the interaction of both exogenous proteins. Furthermore, we demonstrate that exogenous PLCε1 is able to precipitate endogenous BRAF in HEK293T cell lines and endogenous PLCε1 can precipitate endogenous BRAF in

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**Fig. 3.** Coimmunoprecipitation of PLCε1 and BRAF in HEK293T cells and mouse kidney lysates. A: exogenous PLCε1 immunoprecipitates endogenous BRAF in HEK293T cells. HEK293T cells were transfected with GFP-PLCε1 and endogenous human BRAF was detected after immunoblotting with mouse anti-BRAF. Rabbit IgG was used as control for the immunoprecipitation. B: endogenous PLCε1 immunoprecipitates endogenous BRAF in mouse kidney lysates. Adult mouse kidney lysates were immunoprecipitated with polyclonal rabbit anti-PLCε1 (CS117) and endogenous mouse BRAF was detected after immunoblotting with mouse anti-BRAF. Rabbit IgG was used as control for the immunoprecipitation.
mouse kidney, providing evidence of in vivo interaction between BRAF and PLCε1. Using immunohistochemistry studies in rat kidney, we demonstrated for the first time that BRAF is expressed in podocytes and colocalizes with PLCε1, supporting their interaction in glomerular podocytes. We thereby identify BRAF as a new interaction partner of PLCε1. A negative result in yeast-2-hybrid interaction studies is compatible with the hypothesis that the interaction of PLCε1 and BRAF may be indirect.

PLCε1 contains several protein domains: RasGEF_CDC25 (guanine nucleotide exchange factor for Ras-like small GTPases domain), PH domain (pleckstrin homology domain), EF hand, phospholipase catalytic domains (PLC_X and PLC_Y), C2 motif (protein kinase C conserved region 2, subgroup 2), and RA1 and RA2 domains (RasGTP binding domain from guanine nucleotide exchange factors). Most of the predicted domains and motifs of human PLCε1 are highly conserved in plc1 orthologs of evolutionarily distant organisms such as D. rerio and C. elegans, suggesting a conserved function of the domain assembly within PLCε1 (9).

Fig. 4. Immunofluorescent photomicrographs showing the distribution of BRAF in rat glomeruli. a–c: Newborn rat kidney at the S-shaped stage of glomerular development demonstrating that BRAF is widely distributed (a). b: Podocalyxin (green) marks the apical surface of developing podocytes (arrowhead). Nuclei are stained in blue with DAPI. The merged image (c) shows that developing podocytes contain BRAF. d–f: Adult rat glomerulus. d: Cells in the glomerulus contain BRAF (arrows). e: Nuclei (blue) and podocalyxin (green) to delineate the glomerular structures and to identify podocytes whose cell surface is decorated with podocalyxin (arrows). f: (Merge) shows that BRAF is present in podocytes in the adult glomerulus. g–i: Colocalization of BRAF with PLCε1 in a developing glomerulus. g: Distribution of BRAF (green) in podocytes (arrowhead). h: Distribution of PLCε1 (red) and nuclei (blue) in the glomerulus. The merged image i shows as yellow color where BRAF and PLCε1 codistribute in developing podocytes.

BRAF is localized on human chromosome 7q34. The BRAF gene was initially discovered as a transforming gene in NIH3T3 cell transfection assays with human Ewing sarcoma DNA (10). BRAF belongs to the RAF family of genes. In mammals, there are three highly conserved RAF genes, ARAF (otherwise known as A-Raf), BRAF (B-Raf), and CRAF (C-Raf or Raf-1) (5). BRAF encodes a serine-threonine kinase protein of 766 amino acid residues that contains a Raf-like Ras-binding domain (RBD) and a protein kinase C-conserved region 1 domain (C1). The RBD domain of BRAF binds directly to MEK1/2, and ERK1/2 signaling within the Ras/MAP kinase pathway. Recently, we identified IQGAP1 (IQ motif-containing GTPase-activating protein 1) as an interactor of PLCε1 (9). IQGAP1 has also been shown to interact with the critical slit diaphragm protein nephrin, another protein defective in NS (16). IQGAP1 is coexpressed with PLCε1 in the S-shaped and capillary loop stages of glomerular development (9, 17, 18). Since IQGAP1 is a known scaffold protein for MAP kinase signaling (26, 27), it represents the second protein of this pathway after H-Ras that interacts with PLCε1 (13, 33). Recently, IQGAP1 has been shown to interact directly with BRAF (24).
BRAF INTERACTS WITH PLC1

H-Ras (21). A defect in BRAF is involved in a wide range of cancers (4). The most common mutation, which accounts for more than 90% of cancer cases caused by mutations in BRAF, is a glutamic acid for valine substitution at position 600 (V600E). BRAF/V600E is activated and induces constitutive ERK signaling through hyperactivation of RAS-MEK-ERK pathways, and constitutive NF-κB, stimulating proliferation, survival, and transformation (5).

BRAF was identified as a major MEK activator in neuronal tissue (2, 11, 19, 31). It is involved in the transduction of mitogenic signals from the cell membrane to the nucleus. BRAF serves as a central intermediate in many signaling pathways by connecting upstream tyrosine kinases with downstream serine/threonine kinases, such as mitogen-activated protein kinase (MAPK) and MAPK kinase (MKK, also known as MEK) (3, 25). We identified BRAF, a protein that has been implicated in the MAP kinase pathway, as a third interaction partner of PLCε1 in addition to H-Ras and IQGAP1. This opens the MAP kinase signaling pathway as an avenue of interest to study how defects in this pathway may be involved in the pathogenesis of NS. Identification of additional proteins that are expressed in the podocyte and interact directly or indirectly with PLCε1 will help in the understanding of how mutations in PLCε1 cause NS.

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