Supplementary Materials and Methods

Antibodies and reagents

FITC-labeled anti-human CD97 mouse monoclonal antibody (clone VIM3b) was from BD Pharmingen. Anti-αtubulin mouse monoclonal antibody (clone DM1A) and monoclonal anti-vinculin (clone VIN-11-5) were from Sigma. Anti-myc tag monoclonal antibody (clone 9E10) and anti-myc tag polyclonal antibody were from Upstate Cell Signaling Solutions. Anti-hemagglutinin (HA) mouse monoclonal (clone 12CA5) was from Roche. Alexa Fluor conjugated anti-HA mouse monoclonal and Rhodamine conjugated Phalloidin were from Invitrogen Molecular Probes. Rabbit monoclonal antibodies directed against ERK (Cat# 4695), pERK (Cat# 4370), AKT (Cat# 2938), and pAKT (Cat# 4058) were from Cell Signaling Technology. The PI3 kinase inhibitor (LY294002) was from Calbiochem and the cell permeable Rho inhibitor (CT04) was from Cytoskeleton, Inc. Lysophosphatidic acid (1-Oleoyl-2-Hydroxy-*sn*-Glycero-3-Phosphate) was from Avanti Polar Lipids, Inc. Hepatocyte growth factor (HGF) and human SDF-1α were from PeproTech. Rabbit polyclonal anti-lysophosphatidic acid receptor Edg2 (Cat# NLS211) was obtained from Novus Biologicals. All FITC and Rhodamine conjugated secondary antibodies were from Jackson ImmunoLaboratories, Inc. The anti human CD97 rabbit polyclonal antibody was produced against a recombinant peptide encompassing residues 26 through 308 of the extracellular domain.

Expression constructs

The SRE-luciferase construct, pCEFL/Gα_{12} (L229), pCEFL/Gα_{13} (L226), pCEFL/Gα_q (L209), and TOPO pCDNA3.1/p115 RGS (aa 1-252) were from Silvio Gutkind (National Institutes of Health, NIDCR, Bethesda, MD, USA). RhoA^{V14} was

from Alan Hall (University College London, London, UK). Full length CD97-3EGF, myc-tagged CD97-3EGF and CD97-5EGF, CD97-TM1 and myc-tagged CD97β were cloned into pCDNA3.1. All myc tags were added at the encoded C-terminus of the indicated protein. The pEGFPN1/RGS16 WT, 3XHApCDNA3.1/Rho WT, and myc-tagged C3 exoenzyme constructs were a gift from Kirk Druey (National Institutes of Health, NIAID, Bethesda, MD, USA). Lentiviral expression constructs for CD97 (Grp78>Hs.CD97 3EGF-myc), LPAR1 (Grp78>Hs.3xHA-LPA1), and CXCR4 (Grp78p>Hs.3xHA-CXCR4) were generated by the Protein Expression Laboratory, SAIC-Frederick, Inc. All HA tags were added at the N-terminus of the indicated protein.

Immunohistochemistry

A prostate cancer tissue array with matched normal adjacent tissue and metastatic bones Cat# PR954 (US Biomax, Inc) was subjected to a graded alcohol deparaffinization, and was incubated in methanol containing 0.3% H_2O_2. Steam antigen retrieval was done for 15 minutes using Target Retrieval solution (Dako North America). Slides were incubated with Background Buster solution (Innovex Biosciences) for 40 minutes at room temperature, followed by an overnight incubation in the primary antibody at 4 ºC. Following washes, the sections were treated with a biotinylated secondary antibody for 30 minutes at room temperature, followed by 30 minute streptavidin horseradish-peroxidase incubation. All antibodies were diluted in Background Reducing Diluent (Dako North America). Immunoreactive cells were visualized with 3 3' diaminobenzidine tetrahydrochloride and a hematoxylin counterstain. Images were captured using an Aperio ScanScope digital slide scanner at 20X.

SRE-luciferase reporter assay

COS 7 cells were plated on 6-well cell culture dishes and transiently transfected the following day with mixtures of the designated expression plasmids (550ng), RGS-p115, C3 exoenzyme or empty vector (550ng), the SRE-firefly luciferase reporter plasmid (750ng) and the internal control *Renilla* luciferase expression plasmid pRL-TK (Promega) (150ng) using Lipofectamine Plus (Invitrogen). The total DNA concentration was kept constant by adding empty vector when necessary. Twenty-four hours post-transfection, cells were prepared using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Assays were repeated at least three times.

Facs analysis and cell sorting

Cells were trypsinized, quenched with complete medium and allowed to rock gently for 2-4 hours at room temp. One million cells were stained with 20µl of Alexa Fluor® 647 conjugated mouse anti-human CD97 antibody clone VIM3B (BioLegend). Staining of HA-tagged LPAR1 and CXCR4 was carried out using 20µl PE conjugated anti-HA mouse monoclonal (Miltenyi Biotec). FACS analysis was performed on the samples using the BD Cellquest Pro program on a FACSCalibur (Bectin Dickenson). The acquired data was analyzed and quantified using FlowJo Version 8.8.3 flow cytometry software (Tree Star, Inc). Transfected LNCaP cells were sorted to enrich for expression of CD97, LPAR1, and CXCR4. Positive cells were collected using a FACSAria Cell Sorter (BD Bioscience) and cultured as usual.

CD97 silencing

ShRNA and non-silencing shRNA were introduced by lentiviral infection using standard procedures. The CD97 silencing plasmid was pLK0.1 clone TRCN0000008235 (shRNA) and the control plasmid that did not effect CD97 expression was pLKO.1 clone TRCN0000008238 (NS ShRNA) from Open Biosytems. To rescue CD97 expression in stable CD97 depleted DU145, the cells were infected with lentivirus containing an shRNA nontargetable mutant of CD97 and selected with 1μg/ml blasticidin (InvivoGen). The nontargetable CD97 expression construct was generated using the QuikChange II site-directed mutagenesis kit (Stratagene) to introduce 3 silent point mutations into the region targeted by the CD97 shRNA. The primers used to generate the nontargetable mutant were forward, 5'-GCGATCCTTATGGCACACTACGACGTG-3': and reverse, 5'-CACGTCGTAGTGTGCCATAAGGATCGC-3'.

In situ proximity ligation assay (Duolink)

The protein-protein interaction assay in LNCaP and DU145 cells was performed using the Duolink II 563 detection kit according to the manufacturer's instructions (O-Link Bioscience). Primary antibodies directed against LPAR1-HA or CXCR4-HA (anti-HA mouse monoclonal) and CD97 (rabbit polyclonal) were used. Control experiments used only one primary antibody or cells expressing only one receptor. Images were captured using a Zeiss M1 Axio fluorescence microscope equipped with a 20X objective and analyzed using the Duolink Image Tool.