JPET Fast Forward. Published on May 1, 2006 as DOI: 10.1124/jpet.105.098848
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JPET #98848

LYSOPHOSPHATIDIC ACID BINDS TO AND ACTIVATES GPR92, A G PROTEIN-COUPLED RECEPTOR HIGHLY EXPRESSED IN GASTRO-INTESTINAL LYMPHOCYTES

by

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Running title: LPA activates and binds to GPR92

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text pages: 35

number of tables: 3

number of figures: 8

number of references: 35

number of words in Abstract: 123

number of words in Introduction: 858

number of words in Discussion: 2012

Non-standard abbreviations:

GPCR G protein-coupled receptor

IEL intra-epithelial lymphocytes

LPA Lysophosphatidic acid

LPL lamina propria lymphocytes

ORF open reading frame

S_1P sphingosyl-1-phosphate

Section: Cellular and Molecular

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Abstract

Here, the ligand binding, activation, and tissue distribution of the orphan G protein-coupled receptor (GPCR) GPR92 were studied. GPR92 binds and is activated by compounds based on the lysophosphatidic acid (LPA) backbone. The binding of LPA to GPR92 was of high affinity (K_D = 6.4 ± 0.9 nM) and led to an increase in both phosphoinositide hydrolysis and cAMP production. GPR92 is atypical in that it has a low sequence homology with the classical LPA_{1-3} receptors (21-22%). Expression of GPR92 is mainly found in heart, placenta, spleen, brain, lung and gut. Notably, GPR92 is highly expressed in the lymphocyte compartment of the gastrointestinal tract. It is the most abundant GPCR activated by LPA found in the small intestinal intraepithelial $CD8^+$, cytotoxic T-cells.

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Introduction

A large number of physiological events involve intercellular signaling through G protein-coupled receptors (GPCR). Furthermore, many drugs used in medicine today interact with such receptors. However, only a fraction of all potential receptors encoded in the genome have been assigned a cognate ligand. Therefore, the remaining orphan receptors constitute a vast reservoir of potential drug targets for therapeutic development. This makes the identification of ligand receptor pairs (de-orphanization) one of the most important efforts in medicine today.

The human GPR92 gene is located on chromosome 12, region p 13.31 and contains an intronless open reading frame that encodes 372 amino acid residues. It belongs to the rhodopsin sub-family of GPCR and is structurally most closely related to GPR23/LPA_4, a recently identified as a receptor for lysophosphatidic acid (LPA) (Noguchi et al., 2003).

LPA, also known as monoacyl-*sn*-glycero-3-phosphate, is a lipid signaling mediator with a wide spectrum of actions. Originally described as an intermediate in intracellular lipid biosynthesis, LPA is now accepted as a pluripotent extracellular mediator with mitogenic effects in almost every tissue (Moolenaar et al., 2004). At a physiological level, LPA influences such diverse biological processes as tissue re-modelling, neurogenesis, myelination, and olfaction as well as reproductive and immune functions (Mills and Moolenaar, 2003; Ishii et al., 2004).

From a pathophysiological point of view, increasing evidence indicates that LPA has an important role in wound healing by attracting macrophages and by stimulating migration and proliferation of mesenchymal and epithelial cells (Balazs et al., 2001; Sturm and Dignass, 2002). It also seems to participate in the development of

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atherosclerosis as an active constituent of oxidized LDL (Natarajan et al., 1995; Hayashi et al., 2001; Siess and Tigyi, 2004).

The diverse actions of LPA together with the fact that LPA is a major serum constituent at >1μM (Goetzl and Lynch, 2000), prompted research towards the identification of high-affinity LPA receptors. To this date, four LPA receptors, all belonging to the genetic super-family of G-protein coupled receptors, have been described: LPA_1 (Edg-2), LPA_2 (Edg-4), LPA_3 (Edg-7), and the recently described structurally divergent GPR23/LPA_4 (Chun et al., 2002; Anliker and Chun, 2004a; Anliker and Chun, 2004b)). While LPA_1 is the receptor with the widest distribution, the expression of LPA_2 and LPA_3 are somewhat more restricted and GPR23/LPA_4 even more so being expressed almost entirely in the ovary (Noguchi et al., 2003).

This study describes the identification of the previous orphan receptor GPR92 as a putative novel LPA receptor. This receptor is highly expressed in placenta, lung, brain, heart, spleen and immune cells, particularly those associated with the gastrointestinal tract.

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Methods

Chemicals – Lysophosphatidic acid, 1-oleoyl [oleoyl-9, 10-^{3}H(N)] (48.6 Ci/mmol) and inositol, myo-[2-^{3}H(N)] (18.5 Ci/mmol) were obtained from PerkinElmer Life Sciences, Inc. (Boston, MA). Sphingosine-1-phosphate (S1P 18:1) and oleoyl lysophosphatidic acid (LPA 18:1) were obtained from Larodan Fine Chemicals (Malmö, Sweden). Stearoyl lysophosphatidic acid (LPA 18:0), palmitoyl lysophosphatidic acid (LPA 16:0), myristoyl lysophosphatidic acid (LPA 14:0), oleoyl lysophosphatididylcholine (LPC 18:0), arachidoyl lysophosphatidyl choline (LPC 20:0), oleoyl lysophosphatididylethanolamine (LPE 18:1), oleoyl lysophosphatididylserine (LPC 18:1), oleoyl lysophosphatididylglycerol (LPG 18:0) and diacylglycerol pyrophosphate (DGPP 8:0) were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were from Sigma-Aldrich.

Cell culture – The mouse cell line mICc12 derived from the small intestinal epithelium was obtained from Dr. A. Vandevalle (INSERM, Paris, France) and was grown as described by Bens *et al*. (Bens et al., 1996). MODE-K, another mouse small intestinal cell line, was grown in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate (Invitrogen). The mouse T-cell line 2017 and the S194 mouse plasmacytoma cells (both from ATCC) were grown in RPMI 1640 supplemented with 2% sodium pyruvate, 1% Hepes, 10% FBS at 37°C 5% CO_2. The reporter cell lines HF1 (Kotarsky et al., 2001) and HF1-GPR92 (stably expressing human GPR92) were grown in DMEM containing 10 and 3% FBS, respectively. CHO K1 cells were grown in DMEM containing 10% FBS. Stable CHO cell lines were transfected with the plasmid pIRESpuro-GPR92 (see below) or a plasmid coding for green fluorescent protein

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instead, using Metafectene (Biontex; Munich, Germany) and selected for 3 weeks by addition of puromycin.

The following blood cell lines were studied: Nalm6, CCR-CEM, T45, U937, Molt-4, SKO007, TF1, TMβ1 and HL60 (kind gifts from Dr. Magnus Lindvall, Lund University, Sweden), all grown in RPMI 1640 containing 10% FBS. The HL60 cells were differentiated by 1% DMSO for 7 days and the Nalm6 and CCR-CEM cells with 1 μM retinoic acid for 7 days.

Construction of Reporter Cells – The gene for the human GPR92 was isolated from genomic DNA by PCR using Pfx polymerase (Invitrogen) and the primers 5´ GCGAATTCACCATGTTAGCCAACAGCTCCTCA 3´ and 5´ GCGAATTCAGAGGGCGGAATCCTGGGGACA 3´ In short, 100 ng of human gDNA (Promega) was amplified with 20 μM of primers as described by the manufacturer, the product was subcloned in the pIRESpuro vector (Clontech-Invitrogen) and confirmed by sequencing. The reporter cell line HF1-GPR92 was constructed, using the HF1 host reporter cell line and the pIRESpuroGPR92 vector, essentially as described by Kotarsky *et al.* (Kotarsky et al., 2001).

Reporter Assay – HF1 and HF1-GPR92 reporter cells were seeded (1.6 x 10^4 per well) in 96- well plates in 100 μl medium. After 72 hr, test substances diluted in PBS without Ca^{2+} or Mg^{2+} were added to the wells. After another 16 hr, incubation was interrupted by the removal of the medium. Ten μl per well of lysis buffer were added and the plates were stored at –80°C until analysis. Luciferase activity was measured with a Luciferase assay kit (BioThema, Sweden) according to the manufacturer's instructions. All samples were run in triplicate and repeated 2 to 6 times. The

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luminescence assays were done in a BMG Lumistar Galaxy luminometer as previously described (Kotarsky et al., 2001; Kotarsky et al., 2003b).

Phosphoinositide (PI) Hydrolysis – HF1 and HF1-GPR92 cells were assayed essentially as described (Kang and Leeb-Lundberg, 2002), with a few modifications. Briefly, cells grown in 6-well dishes were incubated with 5 µCi/ml *myo*-[^{3}H]inositol in DMEM, 5% heat-inactivated horse serum at 37°C for 24 h in 10% CO_2. Prior to experimentation, the cells were washed four times with 1 ml of Leibovitz's L-15 medium at room temperature and incubated in Leibovitz's L-15 medium, 1 mM LiCl for 30 min. Following replacement with 2 ml of the same medium, the cells were incubated with increasing concentrations of LPA at 37°C for 30 min. Inositol phosphates were then extracted and isolated using anion exchange chromatography.

cAMP production - HF1 and HF1-GPR92 cells were grown in 6-well dishes to approximately 70% confluency. After stimulating the cells for 20 min with LPA the cell layer was washed and then extracted for 20 min with 300 µl 0.1 M HCl per well. The cell extracts were analyzed using a cAMP ELISA kit (Cayman) according to the manufacturer's instructions.

Membrane Preparation for Radioligand Binding Studies – Membrane preparations were essentially done as previously described (Owman et al., 1997). Briefly, HF1 and HF1-GPR92 cells grown on 10-cm petri dishes were first washed twice with ice-cold phosphate-buffered saline (PBS). The cells were then resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and homogenized using an ultra-turrax tissue homogenizer (IKA Werke GmbH Staufen, Germany) at 20,500 rpm for 10-15 sec. Membranes were isolated by centrifugation and

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the pellets were then resuspended in the above buffer and frozen at approximately 9 mg/ml.

Radioligand Binding – Membranes were diluted in PBS/0.1% BSA to a concentration of approximately 0.2 mg of protein /ml. Binding assays were then performed by incubating about 80 μg of protein with various concentrations of [^{3}H]LPA in a total volume of 0.5 ml. Non-specific binding was determined in the presence 1 μM non-radioactive LPA. After incubation for 60 min at room temperature, assays were terminated by dilution with 4 ml of ice-cold PBS/0.3% BSA and rapid vacuum filtration on Whatman GF/C filters previously soaked in 1% polyethyleneimine. The trapped membranes were washed with an additional 2 x 4 ml of ice-cold PBS/0.3% BSA. The filters were counted for radioactivity in a Beckman LS5000TD scintillation counter. No specific [^{3}H]LPA binding was detected in mock-transfected cells. Binding constants were calculated using the Sigma plot program. Association rate experiments were done by incubating membranes for various times after adding [^{3}H]LPA, whereas dissociation rate experiments were done by incubating membranes with [^{3}H]LPA for 30 min followed by addition of 1 μM LPA and incubation for various times thereafter.

Northern Blot – Total RNA was isolated from 10^7 cells by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Twenty-five micrograms of total RNA was separated by electrophoresis and transferred to a nylon membrane according to standard procedures. The membrane and two pre-made tissue blots (Clontech-Invitrogen) were hybridized with the cloned human GPR92 ORF according to standard procedures (50% DMF at 42˚C) and subjected to autoradiography.

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Quantitative real-time PCR – Real time quantitative PCR was performed in a LightCycler system (Roche) using the SYBR Green I detection method. The reactions were performed in a total volume of 20 µl containing 5 µl of diluted cDNA (1:20), dH_2O (negative control) or purified gene specific PCR product (for standard curves), 1 x PCR buffer (Invitrogen), 3 mM $MgCl_2$, 7% DMSO, 0.5 µM of each primer, 200 µM of each dNTP, 0.5 µg/µl BSA, 1:30 000 dilution of SYBR Green I and 0.5 units Platinum TAQ enzyme (Invitrogen). Following denaturation at 94°C for 2.5 min, a total of 40 cycles were run (each 10 s at 96°C, 10 s at 57°C, 20 s at 72°C). Melting curve profiles were analyzed and the specificity of the bands further verified by electrophoresis on agarose gels. Primers used for qRT-PCR were constructed in accordance to Peixoto (Peixoto et al., 2004) and are listed in Table 2.

Isolation of Cells from Mouse Intestine and Lymphoid Organs – Epithelial cells and intraepithelial lymphocytes from the mouse intestinal mucosa were prepared as described by Svensson *et al.* (Svensson et al., 2002). Briefly, dissected mouse small intestines were flushed with PBS, opened, and after the removal of the Peyer´s patches, cut into small pieces. Epithelial cells and crypts were released by six consecutive EDTA treatments of 20 min each. The fractions were pooled and the cells counted in an inverted microscope. Intraepithelial lymphocytes were enriched by Percoll-gradient centrifugation. Lamina propria lymphocytes were isolated by two consecutive treatments with collagenase in the presence of Ca^{2+} ions and Percoll-gradient centrifugation (Svensson et al., 2002). Lymphocytes from mesenteric lymph nodes and spleen were isolated by passing the organs through a tissue strainer.

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Purification of CD8α^+ IEL from mouse small intestine – Intraepithelial lymphocytes purified by percoll gradient centrifugation (see above) were incubated with blocking buffer (PBS, anti-FcRII/III antibody (2.4G2), 2% FBS) for 20 min on ice, followed by an incubation with biotin coupled anti-CD8α antibody and labeling with anti-biotin coupled paramagnetic beads (Miltenyi Biotech). Cells were separated on a LS column (Miltenyi Biotech) according to the manufacturer`s protocol. The purity of the cell preparation was determined to 95- 97% CD8α^+ as verified by staining the cells with FITC-labeled anti-CD8α antibody (BD Bioscience) and analysis in a flow cytometer (FACS Calibur; BD Bioscience).

RT-PCR Analysis – Total RNA was isolated from $2x10^6$ cells prepared from the mouse tissues as described above using the Absolutely RNA Mini kit (Stratagene). Total RNA (4 μg RNA for cell lines and 1 μg RNA of tissue lymphocytes) was used to synthesize cDNA with Superscript III (Invitrogen). The cDNA preparations of CD8$^+$ intraepithelial lymphocytes (Fig. 8A) (Marsal et al., 2002) were a kind gift from Dr. Markus Svensson, Lund University. Total RNA of K46, 40eI, and J558 B-cell lines were a kind gift of Dr. Mikael Sigvardsson, Lund University.

One microliter of the cDNA and a 10x dilution were used for PCR with 20 μM of the following PCR primers: Mouse GPR92 forward: 5´ TCTGCGGCCGCGACATGATGTTTGCCAATTCTTCAG 3´; Mouse GPR 92 reverse: 5´ AGGGAATTCTACGTTCACTTAGACTGCGTCCTCTCAG 3´. The following primers for mouse β-actin were used to assess the total amount of RNA present in the samples: β-actin forward 5´ GGTGGGAATGGGTCAGAAGGACT 3´; β-actin reverse 5´ CCACGCTCGGTCAGGATCTTCAT 3´. The PCR cycling conditions were as follows: 94°C 2 min; 96°C 30 sec; 57°C 30 sec; 70°C 1 min; 35 cycles; 72°C 7 min.

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Statistical Analysis – All data shown are calculated as means ± SEM. The level of significance between groups was assessed by unpaired Student's t-test (95% confidence interval) using the GraphPad PRISM or InSTAT software packages. Dose-response curves were plotted, EC_{50} and IC_{50} values were calculated using GraphPad PRISM software. In the figures, *** = $p<0.001$, ** = $p<0.01$ and * = $p\leq0.05$.

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Results

Sequence Analysis of the Orphan Receptor GPR92 – Phylogenetically, the orphan receptor GPR92 maps to the "purinocluster" of GPCRs (Fredriksson et al., 2003) where one of the closest neighbors is GPR23/LPA_4, recently reported as an atypical LPA receptor (Noguchi et al., 2003). GPR92 displays 31% homology to GPR23/LPA_4, and between 21.3 and 22.6% homology with the classical LPA family of receptors (Fig. 1).

Identification of LPA as a Putative Ligand for GPR92 – In order to search for the cognate ligand for GPR92, the GPR92 cDNA was stably introduced into the reporter cell line HF1. This cell line was chosen since it has been used earlier to identify ligands acting on orphan G protein-coupled receptors (Kotarsky et al., 2003a; Nilsson et al., 2003). The HF1 reporter cell line uses a synthetic enhancer region composed of six TPA-response elements and a chimeric EGFP/luciferase reporter gene. HF1 cells are primarily activated by GPCRs coupling to G-proteins of the Gαq and Gαi classes (Kotarsky et al., 2001).

Since the GPR92 gene displayed high expression in leukocyte cell lines (see below), the presence of a natural ligand in serum was investigated. It was found that serum was able to strongly activate the receptor in a concentration-dependent and specific manner (Fig. 2A). Attempts to purify the active component from bovine serum enriched an active fraction containing serum albumin (data not shown). As lipid compounds can bind to serum albumin (Pages et al., 2001), different lipid mediators were checked for their ability to activate GPR92 expressing reporter cells. LPA and S1P were likely serum-derived candidate ligands. Consequently, the GPR92 reporter cells were

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challenged with 3 μM LPA 18:0 and 3 μM sphingosine-1-phosphate (S1P). Exposure to LPA 18:0 caused a statistically significant (p= 0.0014) activation of the reporter cells expressing GPR92, whereas no response was observed in the parent cells (Fig. 2B). On the other hand, S1P produced no significant difference (p=0.5922) compared to HF1-GPR92 reporter cells stimulated with PBS (Fig. 2C). A small stimulation by S1P was observed in the control HF1 cell line (Fig. 2C).

To further study the specificity of the GPR92 activation by LPA, HF1 reporter cells were stimulated with increasing concentrations of LPA 14:0 in the presence or absence of the LPA_1 /LPA_3 antagonist diacylglycerolpyrophosphate (DGPP 8:0) (Sardar et al., 2002). The presence of 1 μM DGPP did not shift the dose-response curve of LPA 14:0 (Figure 2D). This was further confirmed by the observation that increasing concentrations of DGPP had no effect on the LPA 14:0 activation of HF1pGPR92 reporter cells (Fig. 2E).

After transfection of GPR92 cDNA into the reporter cells, a significant amount of activity was observed in the presence of PBS only (Fig. 2A). This activity was not due to the presence of remaining serum, since it was also found after incubation in serum-free medium or after prolonged cultivation in medium supplemented with delipidated FBS (data not shown).

To exclude the possibility that introduction of the GPR92 cDNA into the reporter cells had changed the expression of another LPA receptor subtype, we analyzed the expression of all known LPA receptor subtypes in HF1 and HF1pGPR92 cells by quantitative RT-PCR. Whereas LPA_1 and GPR23/LPA_4 transcripts could not be detected at all, transcripts for LPA_2 and LPA_3 were present at low levels. Importantly, overexpression of GPR92 did not alter the expression levels of any of the other LPA receptor subtypes (LPA_1, LPA_2, LPA_3 and GPR23/LPA_4) (Fig. 2F).

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In order to investigate the specificity of the GPR92/LPA interaction, a number of compounds including lipid mediators and purinoceptor ligands were tested on HF1 and HF1pGPR92 reporter cells. Whereas the positive controls, LPA 14:0 and fetal bovine serum at 15%, activated GPR92 transfected reporter cells, none of the purinoceptor ligands tested resulted in a specific activation of GPR92 transfected cells (Fig. 3). In fact, the structurally related lipid mediator platelet-activating factor (PAF), at 1 μM, was the only mediator that caused a weak but specific activation of GPR92 transfected cells (10-15% of 1 μM LPA 14:0) (Fig. 3).

In order to investigate the pharmacological profile of GPR92, 11 different lipid analogs, all structurally related to LPA 18:1, were tested. It was found that mediators based on the LPA structure were by far the most effective lipids (Table 1). LPA with acyl chains comprising 14 or 16 carbon atoms displayed the highest potency (Table 1). Among ligands based on structures other than LPA, only lysophophatidylserine (LPS 18:1) and lysophophatidylcholine (LPC 18:1) were able to activate GPR92 (Table 1), albeit at concentrations 10-100 times higher than those required for activation by LPA.

Activation of GPR92 by LPA: PI Hydrolysis – Stimulation of HeLa HF1 and HF1pGPR92 cells with increasing concentrations of LPA 18:1 for 30 min in the presence of 1 mM LiCl resulted in a concentration-dependent increase in PI hydrolysis in the GPR92 transfected cells (Fig. 4A). However, no EC_{50} value could be calculated since the response was still increasing at the highest LPA concentration used, which was 30 μM. Nevertheless, the curve was similar to that observed using the reporter assay. No statistically significant decrease in the response was observed following treatment with 100 ng/ml pertussis toxin (165±15% basal PI hydrolysis for pertussis toxin treated and 182±8% of basal PI hydrolysis for untreated controls).

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To confirm the results obtained in HeLa cells, GPR92 was also stably expressed in CHO cells. Only the GPR92-transfected cells responded to LPA with an increase in PI hydrolysis (Fig. 4B). Pretreatment with pertussis toxin again had no effect on the response (Fig. 4B).

Activation of GPR92 by LPA: cAMP Production - Stimulation of HeLa HF1pGPR92 cells with increasing concentrations of LPA 18:1 for 20 min in the presence of 1 mM IBMX resulted in a concentration-dependent increase in the intracellular cAMP level (Fig. 4A). LPA had again no effect on control transfected HeLa cells. The cAMP response curve was very similar to both the PI hydrolysis and the reporter cell response curve.

Binding of LPA to GPR92 – To directly determine if LPA binds to GPR92, radioligand binding using [^{3}H]LPA 18:1 was performed on a particulate preparation of HF1 and HF1pGPR92 cells. Figure 5A shows that [^{3}H]LPA interacted with specific binding sites in the HF1pGPR92 membrane preparation. Analysis of equilibrium binding revealed that the radioligand bound with high affinity (K_D = 6.4 ± 0.9 nM) to an apparently single finite population of binding sites (B_{max} = 3.35 ± 0.36 pmol/mg of protein, n=3) (Fig. 5B). The [^{3}H]LPA binding to GPR92 was completely reversible with a rapid association rate (k_1 = 1.25 ± 0.39 x 10^7 M^{-1} min^{-1} (n=3)) and a slow dissociation rate (k_{-1} = 7.75 ± 0.72 x 10^{-3} min^{-1} (n=3)), which corresponds to a K_d of 0.62 nM (Fig. 5C). While the reason for the slightly lower K_d value calculated from the rate constants of the LPA binding as compared to that obtained from equilibrium binding is currently not known, both K_d values indicate a high affinity interaction in the nanomolar range. Analysis of the competition of [^{3}H]LPA by several LPA analogs revealed that GPR92 has a strong preference for the LPA backbone regardless of the

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length of the acyl side chain (Table 2). Neither S1P (data not shown), nor the LPA_3/LPA_1 antagonist DGPP 8:0 (Sardar et al., 2002) competed for the LPA binding site present in GPR92-transfected cells. These results show directly that LPA is a specific, high affinity ligand for GPR92.

Cell and Tissue Distribution of GPR92 – Northern blot analysis of a set of human tissues showed that the GPR92 gene is primarily expressed in heart, placenta, brain, gut and spleen (Fig. 6A). The results from spleen suggested a possible expression in blood cells. When a select number of leukocyte cell lines were subjected to Northern blot analysis, it was revealed that human TF1, Nalm6, and undifferentiated HL60 cells all strongly expressed GPR92 (Fig. 6B). Bands of different sizes were observed in both blots, which suggests that the GPR92 receptor can be expressed from different transcripts. Interestingly, the entry BC033571 in Genebank suggests a transcript length of 2352 bp for a GPR92 mRNA, which corresponds well to a predominant transcript detected. In order to obtain quantitative expression data for the GPR92 receptor gene, total RNA was isolated from a number of selected mouse tissues and the level of receptor gene expression determined by qRT-PCR. The highest expression was found in heart and small intestine. Lower levels were found in the other tissues examined (Fig. 6C).

Cellular Expression of Mouse GPR92 in Gut – Various approaches were used in attempts to identify the cell types expressing GPR92 in the gastrointestinal tract. First, PCR analysis was performed on cDNA prepared from two cell lines, MODE-K and mICc12, both derived from the mouse small intestinal epithelium. Neither cell line expressed the receptor (Fig. 7A). On the other hand, expression was verified in the murine B-cell plasmacytoma cell lines S194, 40eI, and J558 and in the mouse pro T-

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cell lymphocytoma cell line 2017 (Fig. 7A). Since the results indicated lymphocytes as the source of the receptor expression, we next analyzed different lymphocyte populations with semi-quantitative PCR. The lymphocytes were isolated from mouse colon and small intestine and included: intra-epithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), Peyers's patches lymphocytes (PP) and lymphocytes from mesenteric lymph nodes (MLN). High levels of mouse GPR92 expression were observed in all populations. Interestingly though, the IEL compartment of the small intestine showed a stronger expression of GPR92 than the LPL compartment (Fig. 7B).

The next experiment aimed to determine the major cellular expression site of mouse GPR92 in the small intestinal epithelium. Because the IEL compartment mainly consists of $CD8^+$ T-cells, we subjected MACS purified $CD8\alpha^+$ IEL, Peyer`s patches lymphocytes, and an epithelial cell preparation to quantitative RT-PCR. The highest expression level was observed in $CD8\alpha^+$ IEL followed by Peyers's patches lymphocytes and epithelial cells (Fig. 7C).

The $CD8^+$ cells present in the small intestinal epithelium consist of different sub-populations defined by their expression of different CD8- and TCR- chains. Three sub-populations of $CD8^+$ T-cells present in the epithelium, $CD8\alpha\alpha^+$ TCRαβ, $CD8\alpha\beta^+$ TCRαβ and $CD8\alpha\alpha^+$ TCRγδ, were investigated for their expression of GPR92 by semi-quantitative PCR. Indeed, all were found to be positive for GPR92 expression (Fig. 8A). To strengthen the relevance of the finding that GPR92 is expressed on the $CD8^+$ cells, we analyzed the relative expression of all known LPA receptor subtypes on these cells. The results showed that the GPR92 transcript is by far the most abundant transcript. Transcripts for LPA_3 and GPR23/LPA_4 could not be detected at all, and LPA_2 and LPA_1 transcripts were present at lower levels (Fig. 8B).

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Discussion

LPA is the simplest glycerophospholipid and mediates a wide range of cellular responses, including platelet aggregation, cell proliferation, modulation of chemotaxis smooth muscle cell contraction, protection from apoptosis, and transcellular migration (van Leeuwen et al., 2003; Moolenaar et al., 2004). LPA has been shown to interact with at least four GPCRs known as LPA_1, LPA_2, LPA_3, and GPR23/LPA_4 (Chun et al., 2002; Noguchi et al., 2003). In the present study, we identify the orphan receptor GPR92 as a putative additional LPA receptor.

The LPA receptor family can be divided into two subfamilies where one is composed of the first three members, LPA_1, LPA_2 and LPA_3, while the other subfamily consists of GPR23/LPA_4 and GPR92. The fact that the latter subfamily of LPA receptors is structurally more related to the purinoreceptor cluster of GPCRs than to the original LPA receptors indicates different ancestor genes. All of the human LPA receptor genes are localized to different chromosomes but the ones forming the original subfamily belong to the same paralogon group (Fredriksson et al., 2003), which suggests that they have a common ancestral gene. Although LPA_4 and GPR92 share a structural similarity, they are part of different paralogons.

LPA bound to GPR92 with an affinity in the low nanomolar range. This affinity is higher than that of the previously described GPR23/LPA_4 (Noguchi et al., 2003). On the other hand, the affinities of LPA binding to the classical LPA receptors have not been reported. The pharmacological binding profile revealed that GPR92 was unable to discriminate between different LPA analogs varying in the length of their acyl-groups. The LPA inhibitor DGPP failed to displace radio-labeled LPA from GPR92 membrane

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preparations. DGPP at 1 μM has been shown to inhibit signaling on the classical LPA receptors LPA_3 and LPA_1 (Sardar et al., 2002; Ohta et al., 2003).

GPR92-mediated LPA stimulation leads to increases in PI hydrolysis and cAMP production suggesting that this receptor is coupled at least to the G_q and possibly also to the G_s class of G-proteins in HeLa cells. This signaling pattern is similar to that of GPR23/LPA_4, but differs from the classical LPA receptors, which preferentially couple to G_i, $G_{12/13}$ and/or G_q. The potencies of LPA to stimulate both of these cellular responses as well as the reporter assay were considerably lower than the affinity of LPA for the receptor measured in particulate membranes. The reason for this difference is currently unknown. LPA at higher concentrations acts as an agonist through this receptor since it stimulates both PI hydrolysis and cAMP production in a receptor dependent manner. While our results clearly show that LPA is a ligand for GPR92, the details of the LPA signal behavior for this receptor clearly requires further investigation. The fact that the LPA1/LPA3 antagonist DGPP failed to inhibit LPA-stimulation of GPR92 is consistent both with its inability to displace radio-labeled LPA from GPR92 and, to the structural difference between LPA1/LPA3 and GPR92. The order of potencies for LPA analogs with different chain length to stimulate the expression of the reporter gene showed that, GPR92 was most efficiently activated with LPA analogs with an acyl chain shorter than 18 carbon atoms. This is in apparent contrast to the previously identified GPR23/LPA_4 (Noguchi et al., 2003) and the classical LPA receptors (Bandoh et al., 2000), which are more efficiently activated by long acyl-chain LPA analogs. However, it must be noted that these results originate from different experimental assay systems. Nevertheless, it has been reported that a substantial amount of LPA consists of LPA 16:0 both in plasma (Baker et al., 2001) and brain tissue (Sugiura et al., 1999). A physiological difference between different LPA analogs has so far not been established. However, there is a discrepancy between

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the binding results that indicate that chain length is unimportant for the displacing ability of the LPA –analogs and the activation results showing a preference for shorter chain-lengths. A possible interpretation of this observation could be the presence of two binding states for the GPR92 receptor with only the lower affinity state being coupled to activation of the receptor.

In summary, GPR92 seems to be most related to GPR23/LPA_4 since they share structural similarity, a similar signaling pattern, and a comparable affinity for LPA. The LPA receptors differ with respect to their distribution in various tissues (see ref. (Anliker and Chun, 2004a; Ishii et al., 2004) for review). While LPA_1 is broadly expressed throughout the body, the expression of LPA_2 and LPA_3 is more restricted. GPR23/LPA_4, the first member of the purinoreceptor-related subfamily, is expressed almost entirely in the ovaries (Noguchi et al., 2003). Expression of the classical LPA receptors (LPA_{1-3}) has been studied in human leukocytes (Goetzl et al., 2000; Zheng et al., 2000). Whereas the LPA_3 receptor could not be detected on any of the cell populations studied, the LPA_2 receptor was mapped to $CD19^+$ B-cells and $CD4^+$ T-cells, and the LPA_1 receptor was expressed by $CD4^+$ T-cells and $CD14^+$ monocytes (Goetzl et al., 2000; Graler and Goetzl, 2002). Interestingly, none of the LPA receptor subtypes studied were found in $CD8^+$ T-cells isolated from human blood (Goetzl et al., 2000; Zheng et al., 2000).

In regard to GPR92, although we detected a high expression also in the heart, we decided to focus on its expression in lymphocytes, especially of those in the gastrointestinal tract. The intestine can be divided in an epithelial layer and the underlying lamina propria. The major cell types present within the epithelium includes; the epithelial cells and intraepithelial lymphocytes that consist mainly of $CD8^+$ T-cells. Therefore, we purified $CD8^+$ lymphocytes and epithelial cells and quantified GPR92 mRNA abundance. Highly purified $CD8^+$ IEL derived from the small intestinal

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epithelium expressed significantly higher levels of GPR92 than percoll gradient purified epithelial cells. The fact that GPR92 could not be found in two cell lines derived from the mouse small intestinal epithelium substantiates the idea that GPR92 is predominantly expressed in the lymphocyte compartment.

The $CD8^+$ T-cell preparation was further tested for the presence of other LPA receptor subtypes and it was found that GPR92 was the most abundant, whereas LPA_2 and LPA_1 were found at lower levels. Although, this is the first report showing that $CD8^+$ T cells express LPA receptors an exact mapping of the receptor expression in the different cell populations would be facilitated by specific antibodies.

In conclusion we have identified the former orphan receptor GPR92 as a putative LPA receptor, highly expressed in the heart and gastrointestinal tract. GPR92 is in many respects similar to the previously identified GPR23/LPA_4. It binds LPA with high affinity and activates PI hydrolysis and cAMP production. However its expression pattern markedly differs from GPR23/LPA_4. GPR92 is highly expressed in lymphocytes, especially in the gastrointestinal tract. It is co-expressed with two classical LPA receptors in intraepithelial $CD8^+$ T-cell present in the small intestinal mucosa.

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Footnotes

a. This work was supported by GS Development, the Segerfalk Foundation, Crafoord Foundation, Kock Foundation, Swedish Society for Medical Research, Royal Physiographic Society, the Juhlin Foundation, Medical Faculty at Lund University and the Swedish Research Council. K.K. was supported by a post-doctoral fellowship financed by Swegene and the Wallenberg Foundation.

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Figure Legends

Fig. 1. **Phylogenetic tree constructed for the human GPR92 and selected human GPCRs.** The amino acid sequences were aligned using ClustalX and the phylogenetic tree created with a bootstrap value of 1000.

Fig. 2. **GPR92 expressing HF1 reporter cells are activated by LPA.** Cells were seeded at 1.6 x 10^6 cells per well in 100 µl medium. After 72 h, serum or various lysophospholipids diluted in PBS in a volume of 25 µl were added. After another 18 h, induced luciferase activity was measured and expressed in relative luminescence units (RLU). *A* shows the effect of serum. *B* shows concentration response curves for 2 different LPA analogs (LPA 14:0 and LPA 18:0). One experiment out of six performed in triplicate or quadruplicate values is shown. Mean (± S.E.M). *C* the effect of sphingosine-1-phosphate (3µM) on HF1 and HF1pGPR92. n=3 Mean (± S.E.M). n.s. = not significant. *D* shows the effect of 1 µM diacylglycerolpyrophosphate (DGPP) on increasing concentrations of LPA 14:0, n=2; Mean (± S.E.M) *E* reveals the effect of increasing DGPP concentrations on cells stimulated with 660 nM LPA 14:0. n=2; Mean (± S.E.M) *F* expression pattern of the different LPA receptor subtypes in the HF1 reporter cells. Data represent the mean (± S.E.M) from three to four experiments run in duplicate values.

Fig. 3. **Screening of lipid mediators and ligands to purinoceptors on GPR92 expressing reporter cells and the parenteral cell line HF1.** Reporter cells were cultured as described in Material and Methods and test compounds were added for 12 h and luciferase activity measured as described. Relative response was calculated

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as the ratio between the value of the test compound minus the background value of unstimulated cells and the maximal response value (1 μM PMA) minus the background value. The following concentrations of test compounds were used: LPA 14:0 1 μM, fetal bovine serum (FBS) 15 μl, platelet activating factor (PAF) 1 μM, prostaglandin E2 (PGE2) 1 μM, leukotriene B4 (LTB4) 1 μM, fMLP 1 μM, linoleic acid (LA) 100 mM, prostaglandin J2 (PGJ2) 1 μM, deoxyribonucleic acids (dNTPs) 1 mM, leukotriene D4 (LTD4) 1 μM, uridine-5-diphosphoglucuronic acid (U2PG) 100 mM, adenosine 5´-diphosphoribose (ADP-ribose) 100 mM, adenosine-triphosphate (ATP) 100 mM, adenosine 10 mM, uridine 10 mM, guanosinemonophosphate (GMP) 10 mM, guanosinediphosphate (GDP) 10 mM, guanosine-5´diphospho-d-mannose (G2P-Man) 10 mM. Results shown are one experiment representative of three performed.

Fig. 4. **LPA-dependent stimulation of PI hydrolysis and cAMP production.** Intact cells were incubated with the indicated concentrations of LPA and assayed for PI hydrolysis as described under ”methods”. The results are presented as “% of Basal”, where 100% is the amount of PI hydrolysis and cAMP production in the absence of LPA. The results are mean ± S.E.M. of 3 independent experiments with each point assayed in duplicate *A,* PI hydrolysis and cAMP production in HeLa HF1pGPR92 cells. *B,* PI hydrolysis in CHO cells stably transfected with GPR92 or empty control plasmid stimulated with 5 μM LPA or control in the presence or absence of 100 ng/ml pertussis toxin.

Fig. 5. **[^{3}H]LPA binding to particulate preparations of HeLa HF1pGPR92 cells.** *A,* Saturation binding isotherm. Particulate preparations (160 μg protein) were

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incubated with increasing concentrations of [^{3}H]LPA, and the binding assays were performed as described under "methods". In *A*, total binding, non-specific binding, determined in the presence of 1 μM LPA, and specific binding are shown. The result is representative of 3 experiments with each point assayed in duplicate. In *B*, Scatchard analysis of the specific binding in *A* is shown (K_D = 5.6 nM); (B_{max} = 402 fmol). *C*, Association and dissociation binding curves. Preparations (160 μg protein) were incubated with a constant concentration of [^{3}H]LPA (2-3 nM), with and without 1 μM LPA, for various times upon which they were terminated to determine the association of specific binding (●). In a separate experiment, particulate preparations were incubated with a constant concentration of [^{3}H]LPA (2-3 nM) for 60 min. At this time, 1 μM LPA was added (*arrow*). The binding was then terminated at various times thereafter to determine dissociation of specific binding (○). The results are presented as "% of Total" where 100% is the specific binding of [^{3}H]LPA at 60 min of incubation. The results are mean ± S.E.M. of 3 independent experiments with each point assayed in duplicate.

Fig. 6. **Analysis of GPR92 mRNA expression in human and mouse tissues and human blood leukocyte cell lines.** *A*, A commercial human tissue blot probed with GPR92 ORF. *B*, total RNA (25 μg) derived from the indicated cell lines was separated electrophoretically, and transferred to a nylon membrane. The membranes were hybridized with [^{32}P]dCTP-labeled full-length human GPR92 probe. The positions to which ribonucleic acid standards of known molecular mass migrated are indicated to the right. The putative GPR92 transcripts are on the left (open arrow head not found in Genebank, black arrow agrees with Genebank (BC033571). *C*, Quantitative real time PCR was performed on a selected number of mouse tissues.

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The samples were run in duplicate or triplicate and the experiment repeated three or four times with samples derived from four different mice. Values shown are the mean of all experiments performed and the error bars indicate the ±SEM between the different experiments performed.

Fig. 7**. Expression of mouse GPR92 mRNA in cell lines and cell populations present in the gut**. Semi-quantitative RT-PCR was performed on RNA isolated from the indicated cells. Results from one experiment out of 3-4 are shown. 1:10 indicates a ten fold dilution of the cDNA (1:1) used in the experiment. *A,* two epithelial and five lymphoid cell lines tested. *B,* lymphocyte populations isolated from mouse tissues (LPL = lamina propria lymphocytes; IEL = intra-epithelial lymphocytes; co = colon; SI = small intestine; PP ly = Payer`s patch lymphocytes; MLN = mesenteric lymphnode lymphocytes; spleen = spleen derived lymphocytes). *C,* GPR92 mRNA abundance was assessed in highly purified $CD8^+$ T-cells, payers patches lymphocytes and epithelial cells. Three experiments were performed in duplicate or triplicate values. Values shown are the mean of all experiments performed and the error bars indicate the ±SEM between the different experiments.

Fig. 8. **Expression of GPR92 in different subpopulations of $CD8^+$ IEL and quatitative LPA receptor expression in $CD8^+$ IEL.** *A,* The different subpopulations of $CD8^+$ intra-epithelial lymphocytes present in the small intestinal epithelium were tested for the expression of GPR92 by semi-quantitative PCR. The cDNA was run undiluted (1:1) and as a ten fold dilution (1:10). *B,* RT-PCR quantitation of LPA receptor expression in MACS purified, $CD8^+$ IEL from the mouse small intestine. The experiment was repeated three times with IEL preparations from 3 different mice

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each evaluated in duplicate or triplicate. Values shown are the mean of all experiments performed and the error bars indicate the ±SEM between the different experiments.

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TABLE 1

Potency and relative efficacy of phospholipids on GPR92

LPA 18:1	5.03 ±0.13	100	6
LPA 18.0	5.12 ±0.19	127±14.6	6
LPA 16:0	6.40 ±0.36	117±21	6
LPA 14.0	6.08 ±0.31	95±19	5
LPC 18:1	4.13 ±0.37	n.d.	3
LPC 20:0	<4.00	n.d.	3
LPE 18:1	<4.00	n.d.	3
LPS 18:1	4.4 and 4.1	n.d.	2
LPG 18:1	<4.00	n.d.	3
S1P 18:1	<4.00	n.d.	3
DGPP 8:0	<4.00	n.d.	3

[a] pEC_{50}, -log molar concentration of substance resulting in 50% of maximal reporter gene expression. Average value ±standard error of the mean; number of experiments performed. Relatve efficacy of LPA 18:1 was set to 100%. d.n.=not determined.

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TABLE 2

IC_{50} values for various lipid analogs on [^{3}H]LPA binding to particulate preparations of HeLa HF1pGPR92 cells.

Analog	IC_{50}
	μM[a]
LPA 14:0	0.103 ± 0.044
LPA 16:0	0.075 ± 0.011
LPA 18:0	0.113 ± 0.048
LPA 18:1	0.059 ± 0.002
LPE 18:1	>>1
LPS 18:1	>>1
LPG 18:1	>>1
LPC 18:1	>>1
LPC 20:0	>>1
DGPP	>>1

[a] The values were calculated by the nonlinear regression program GraphPad Prism with data obtained from competition binding experiments using approximately 2 nM [^{3}H]LPA 18:1 as described under Methods. The results are presented as means ± SEM of three experiments.

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TABLE 3

Primer sequences used for quantitative real-time PCR

β-actin fw	CCGGGACCTGACAGACTA
β-actin rev	GTTTCATGGATGCCACAGGAT
hmLPA1fw	CTGAAGACTGTGGTCATTGTGC
hmLPA1rev	AACCACAGAGTGGTCATTGCTG
hLPA2fw	GTCAAGACTGTTGTCATCATCCT
hLPA2rev	GGAAGCATGATGCGAGTGCG
mLPA2fw	GTCAAGACGGTTGTCATCATTCT
mLPA2rev	GAAGCATGATCCGCGTGCT
mLPA3fw	ACAAAGCTTGTGATCGTCCTGT
mLPA3rev	TCATGATGGACATGTGTCTTTCC
hLPA3fw	GATTGTTTTGTGTGTTGGGACG
hLPA3rev	TGGTCAGGTTGCTATGGACC
hGPR23fw	CACCAATCTAGCTGTCTCTGATT
hmGPR23rev	GCATTGTTGACATTAGTGGTGGA
mGPR23fw	AACCTGGCCCTCTCTGATTT
hGPR92fw	CTCTCCTACGCACTGCACCACT
hGPR92rev	GAAGCTCTCGAAGCATAGGCGCA
mGPR92fw	CCGTACATGTTCATCTGGAAGAT
mGPR92rev	CAGACTAATTTCTCTTCCCACCT

Sequences are listed 5`to 3`end. The primers are named according to the receptor assessed. m = mouse and h = human; fw = forward and rev = reverse primers.

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Figure 1

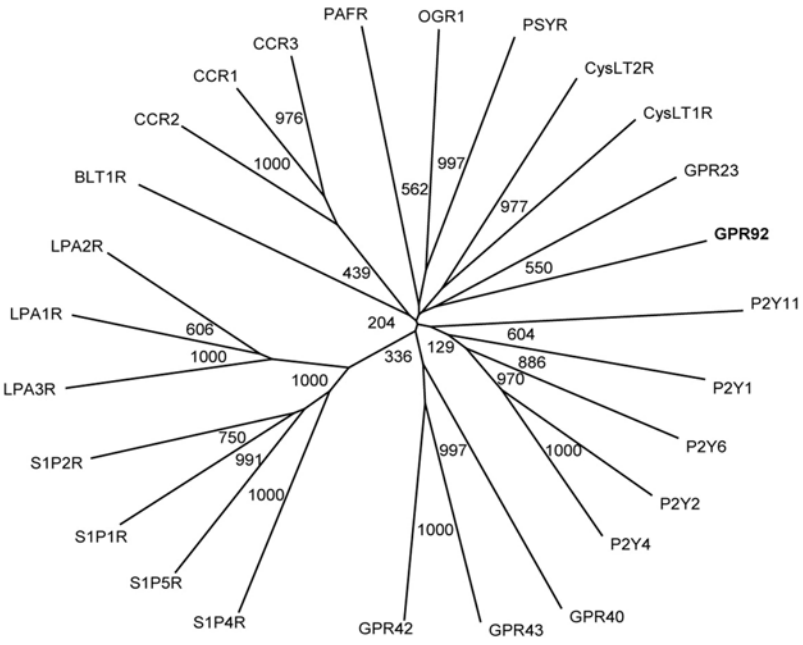

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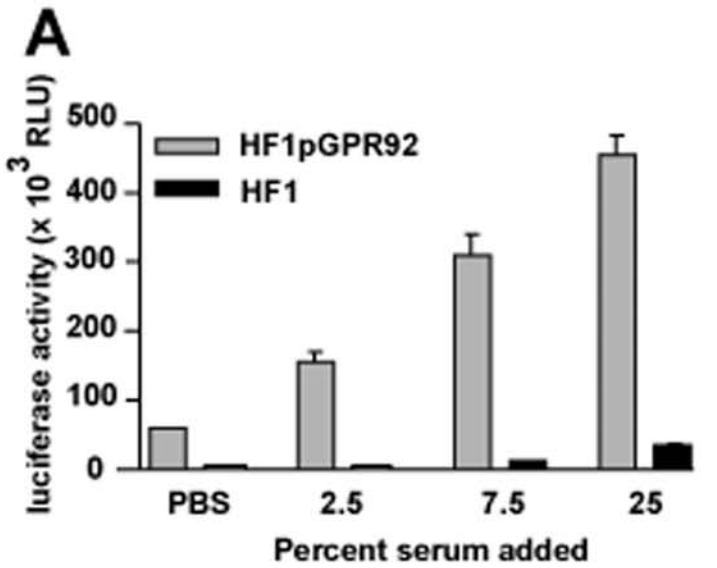
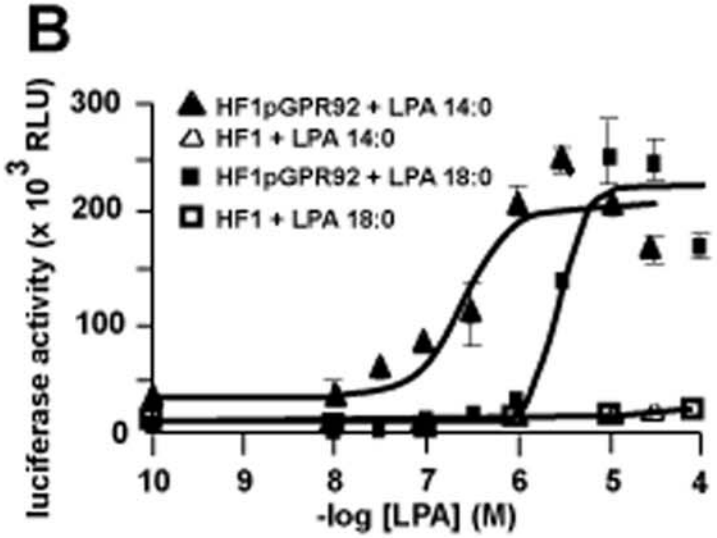
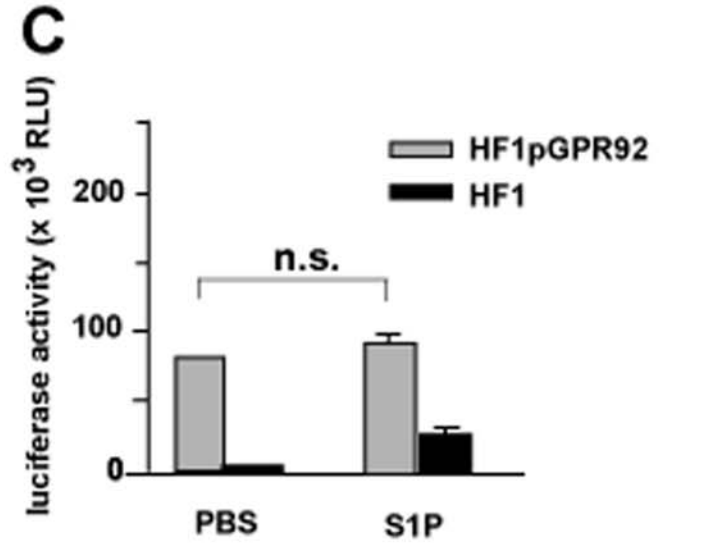
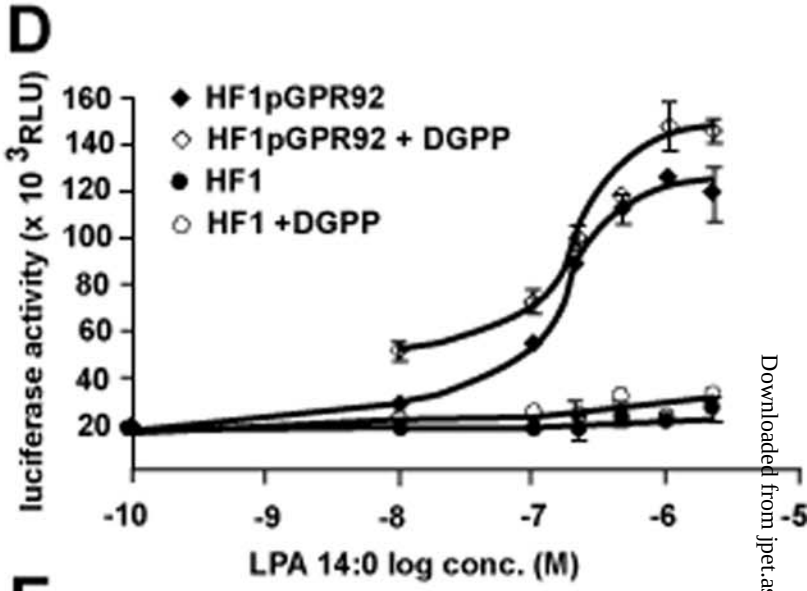
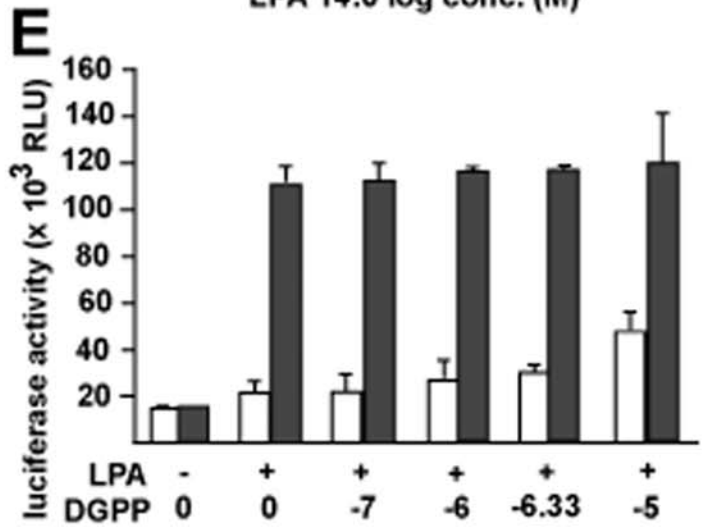
Figure 2











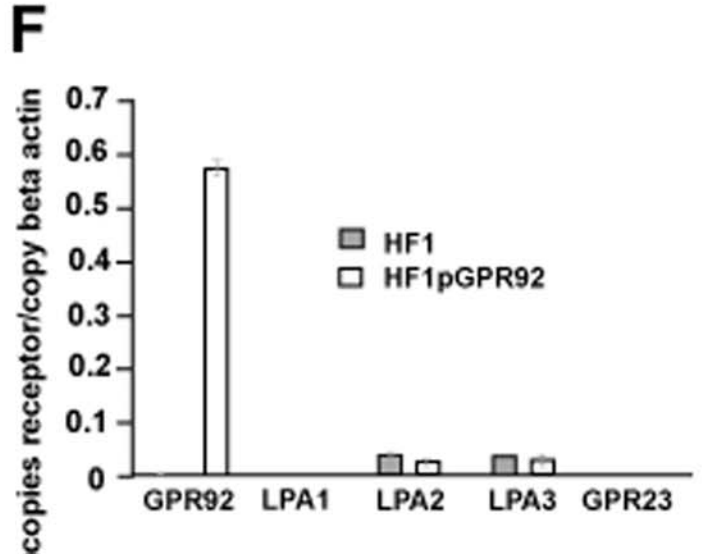


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Figure 3

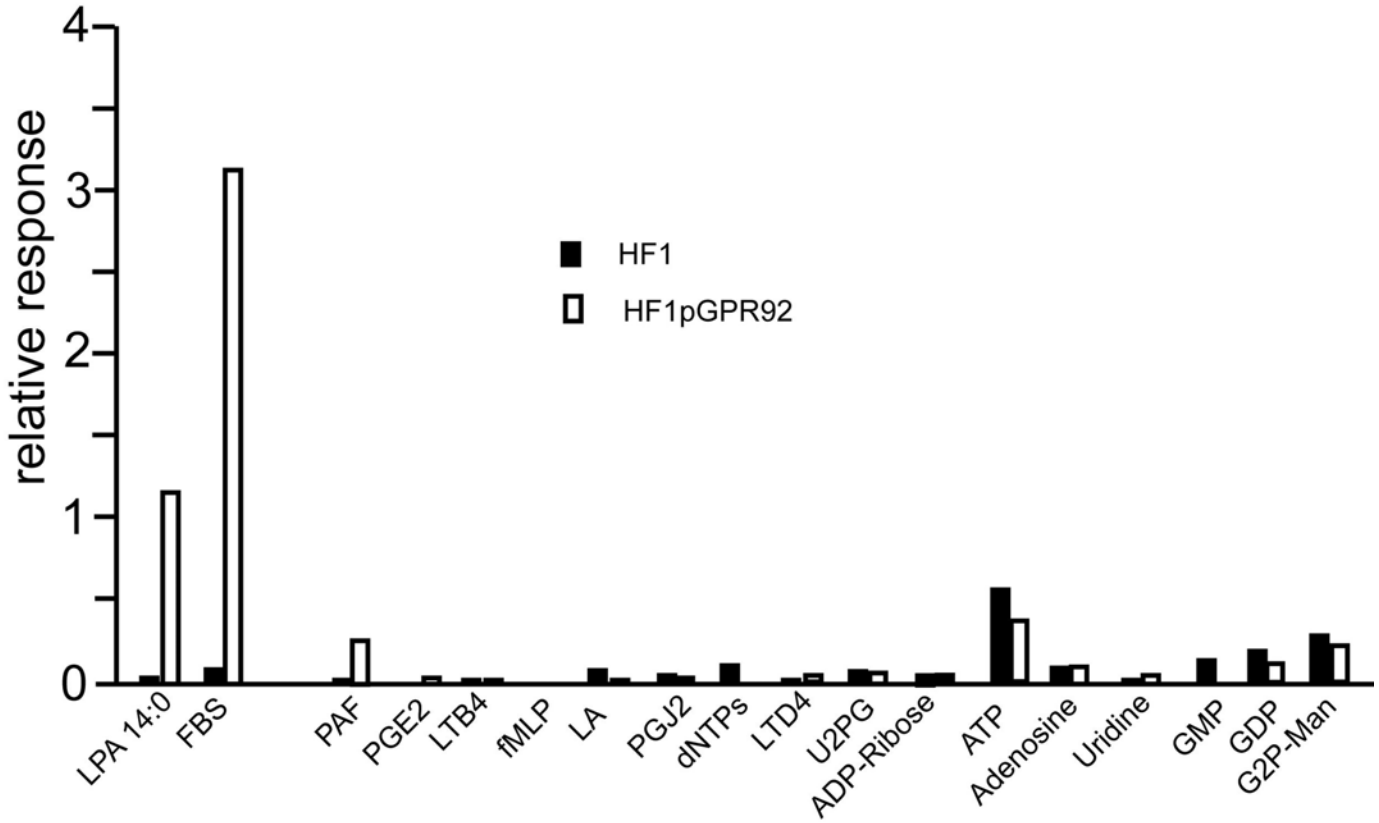

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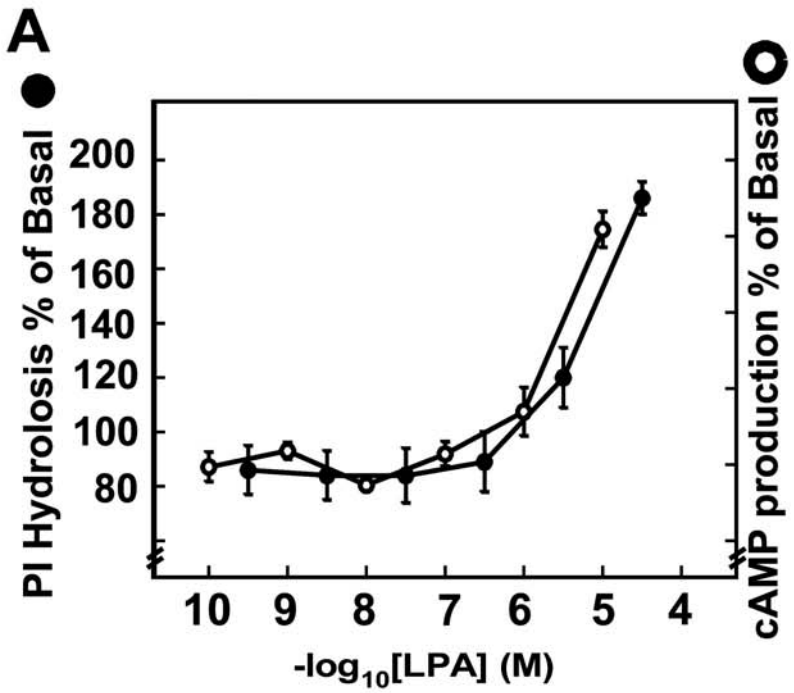
Figure 4



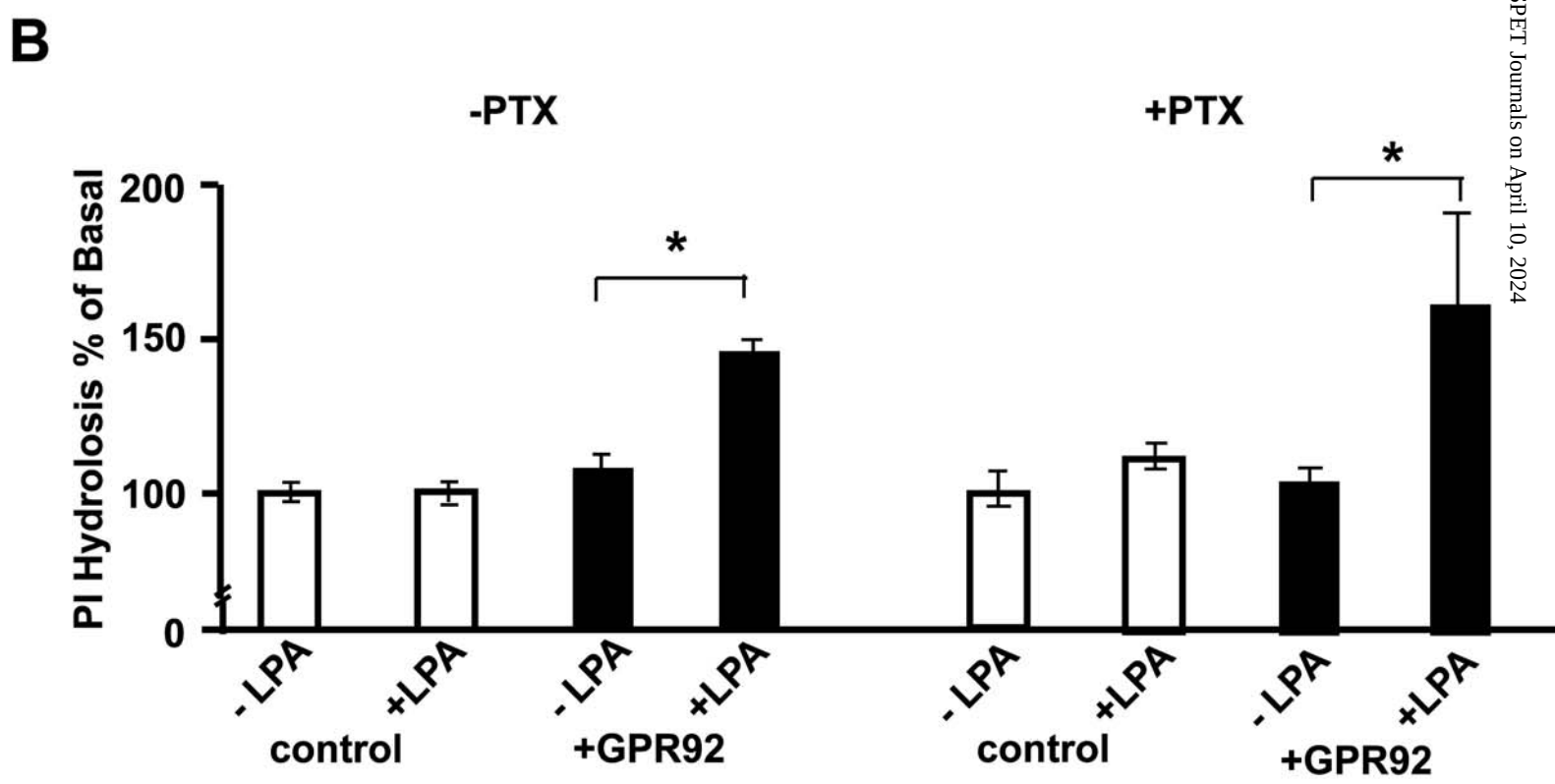


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Figure 5

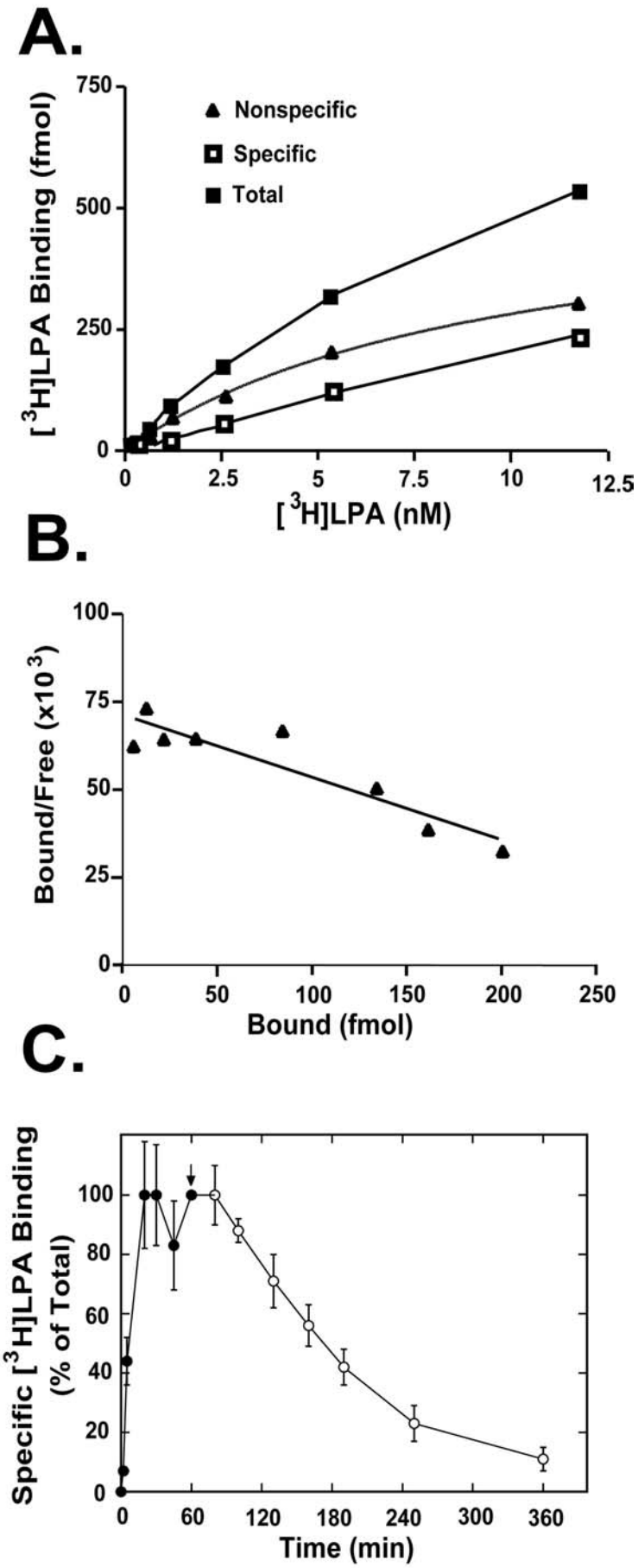

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Figure 6

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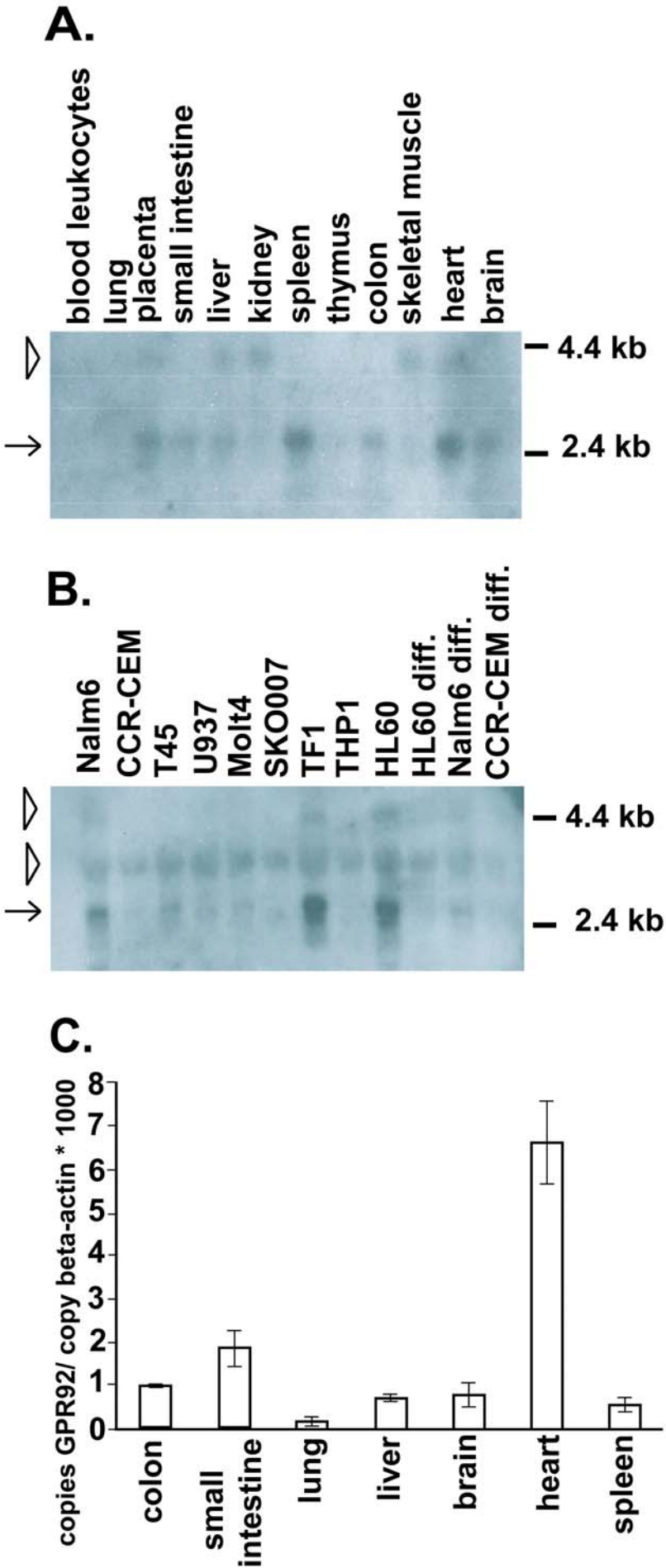

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Figure 7

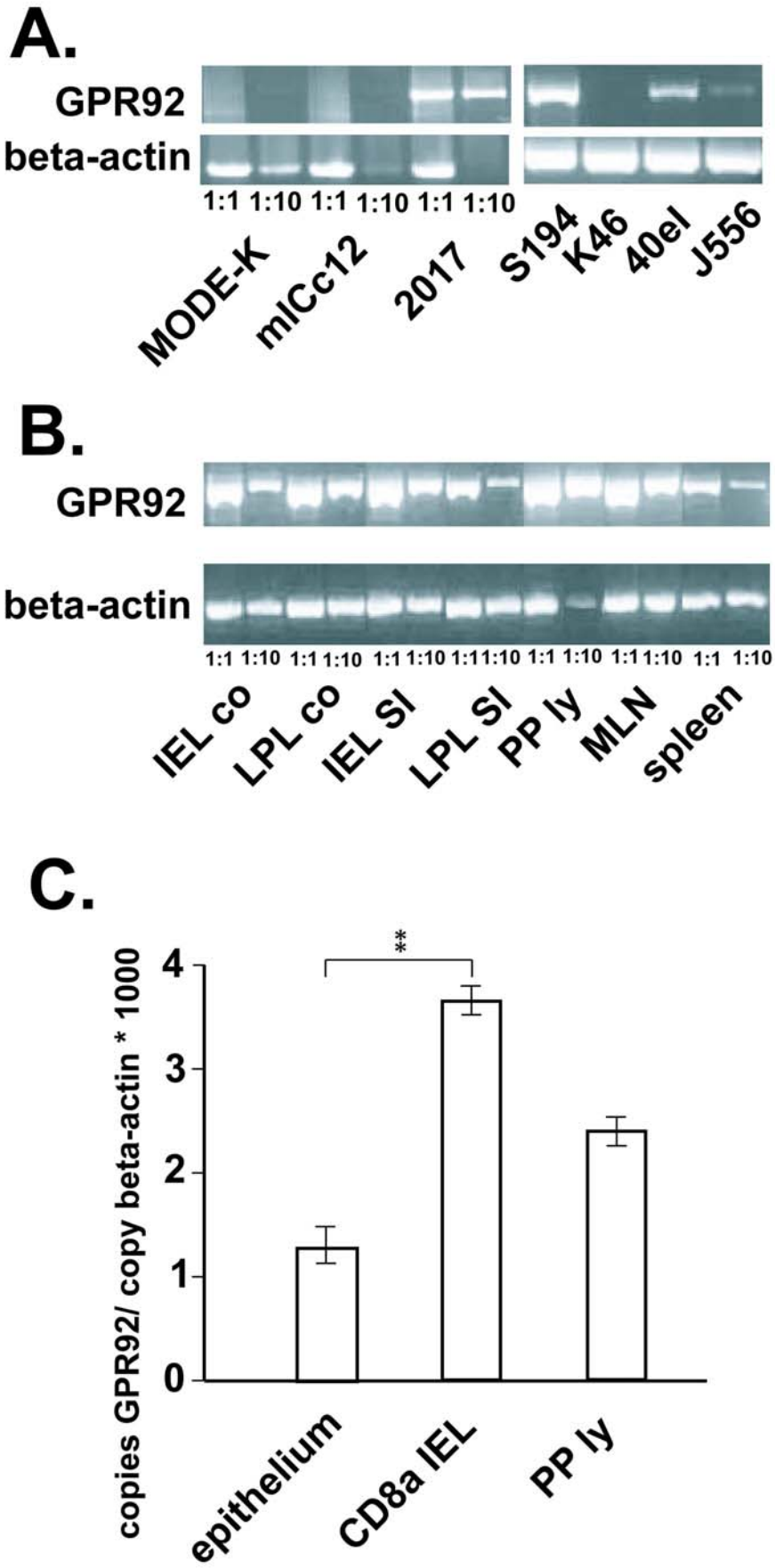

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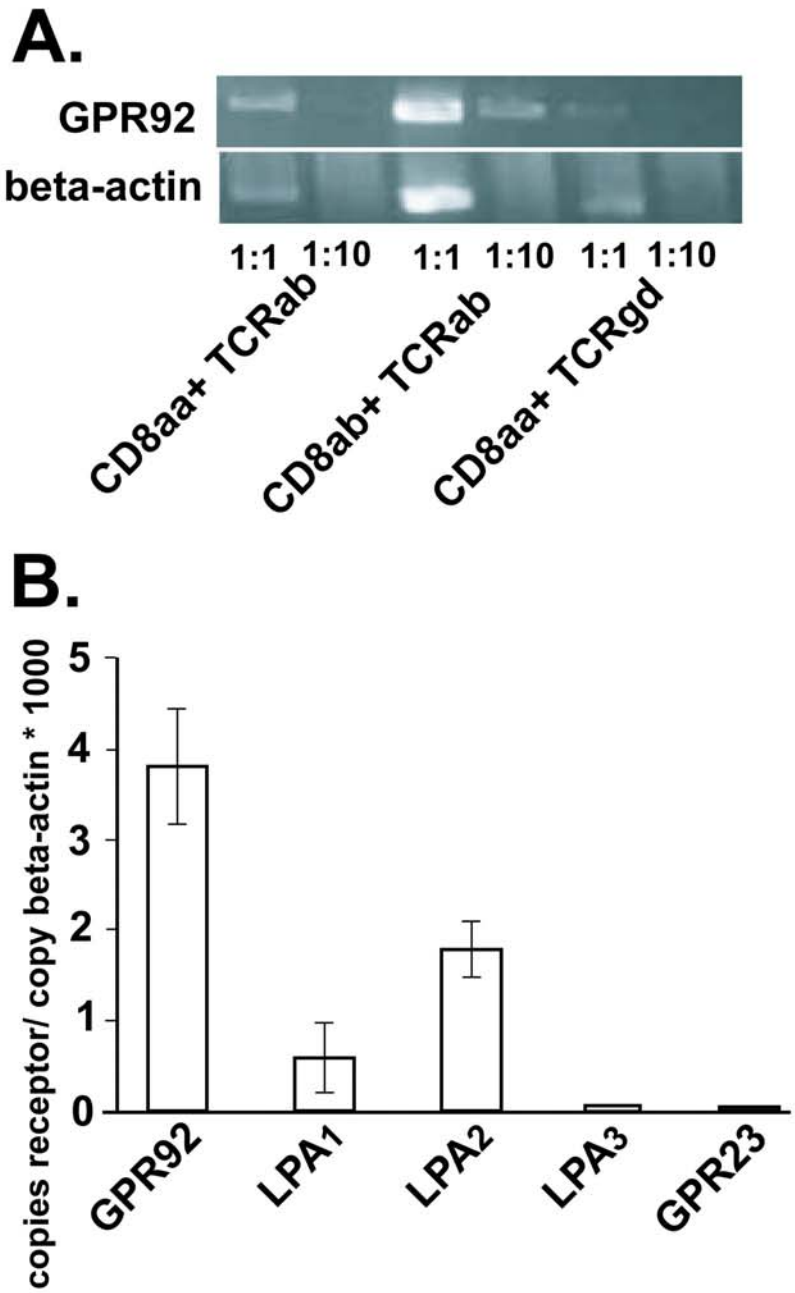
Figure 8



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