Tumor and Stem Cell Biology

LPA Receptor Heterodimerizes with CD97 to Amplify LPA-Initiated RHO-Dependent Signaling and Invasion in Prostate Cancer Cells

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Abstract

CD97, an adhesion-linked G-protein–coupled receptor (GPCR), is induced in multiple epithelial cancer lineages. We address here the signaling properties and the functional significance of CD97 expression in prostate cancer. Our findings show that CD97 signals through Gα12/13 to increase RHO-GTP levels. CD97 functioned to mediate invasion in prostate cancer cells, at least in part, by associating with lysophosphatidic acid receptor 1 (LPAR1), leading to enhanced LPA-dependent RHO and extracellular signal–regulated kinase activation. Consistent with its role in invasion, depletion of CD97 in PC3 cells resulted in decreased bone metastasis without affecting subcutaneous tumor growth. Furthermore, CD97 heterodimerized and functionally synergized with LPAR1, a GPCR implicated in cancer progression. We also found that CD97 and LPAR expression were significantly correlated in clinical prostate cancer specimens. Taken together, these findings support the investigation of CD97 as a potential therapeutic cancer target. Cancer Res; 71(23); 1-12. ©2011 AACR.

Introduction

Expression of the G-protein–coupled receptor (GPCR) CD97 is induced in a variety of epithelial cancers. CD97 is normally expressed on cells of hematopoietic origin and smooth muscle but is expressed at very low levels or is absent in normal epithelial cells (1, 2). Carcinomas derived from various epithelial tissues consistently show increased CD97 expression compared with normal adjacent tissue, and in some cases, the level of CD97 expression positively correlates with tumor grade and poor prognosis. CD97 induction has been described in thyroid (3), colorectal (4), gastric, pancreatic, esophageal (3), oral squamous cell (5), and prostate carcinomas (6). It has been unclear as to whether CD97 is a marker of epithelial transformation or alternatively might play a functional role. Of interest is the description of CD97 at the invasion front of carcinomas, hinting at a potential function in progressing cells (3). However, the existence of CD97-dependent signaling associated with tumors has not been established. Here, we have investigated whether CD97 plays a functional signaling role in prostate cancer.

CD97 is a member of the adhesion-linked GPCRs, a family of membrane-bound proteins characterized by a large extracellular subunit containing one to a few adhesion motifs noncovalently associated with a conserved GPCR 7-transmembrane domain (TM7; ref. 7). Most adhesion-linked GPCRs are translated as a single polypeptide that is proteolytically processed intracellularly to produce the 2 associated subunits. CD97 belongs to a subclass of adhesion GPCRs that contain variable numbers of epidermal growth factor (EGF)-like repeats in the extracellular adhesion domain (1). Although the adhesion GPCR family members are thought to have the dual potential of cellular adhesion and signaling, a mechanistic understanding of adhesion-coupled signaling has not been established (2). Heterotrimeric Gα12/13-dependent signaling has been shown for one family member, GPR56, but signaling has not as yet been linked to binding of the known ligand, transglutaminase 2 (8, 9). In addition, 2 receptors, BAI1 and LTR, have known ligand-dependent functions, which require the transmembrane regions, but it has not been established functionally whether heterotrimeric G protein signaling is needed (10, 11). Specific CD97 isoforms have been shown to interact with integrins α5β1 and αvβ3 (12), the cell surface complement regulatory protein CD55 (13), and the extracellular matrix component chondroitin sulfate (14), although ligand-dependent signaling has not been shown.

In the present study, we show that CD97 complexes in cis with and positively regulates lysophosphatidic acid receptor (LPA) 1, a cognate receptor for LPA, resulting in increased LPA-dependent RHO signaling. LPA is a bioactive phospholipid that acts as a high-affinity ligand for LPARs to mediate various

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-11-2381
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cellular functions that contribute to transformation including proliferation, survival, cytoskeletal restructuring, and production of secreted factors such as cytokines and metalloproteases (15, 16). Bioactive LPA is produced in the extracellular matrix when complex lysocepholipids, predominantly lysophosphati-dylcholine, are hydrolyzed by the secreted enzyme, autotoxin (ATX). Elevated LPA production (17) and increased expression of cell surface LPARs 1, 2, and 3 have been reported for various human carcinomas, including prostate cancer. Using a transgenic mouse model, Liu and colleagues (20) showed directly that ATX and LPARs 1, 2, and 3 contribute to the initiation and progression of breast cancer. We describe here in prostate cancer a functional association between CD97 and LPAR1 that modifies LPA-initiated signaling, a finding that lends mechanistic understanding to the potential importance of CD97 induction in carcinomas.

Materials and Methods

The following technical information is provided in the supplementary data: "Antibodies and reagents," "Expression constructs," "Immunohistochemistry," "SRE-luciferase reporter assay," "FACS analysis and cell sorting," "CD97 silencing," and "In situ proximity ligation assay."

Cell culture

DU145, PC3, and LNCaP prostate cancer cells were obtained from American Type Culture Collection. PC3 cells permanently expressing a luciferase reporter were generated and cultured as reported (21). All culture media and reagents were from Invitrogen.

Immunofluorescence and confocal microscopy

Staining and image acquisition by confocal microscopy were carried out as previously described (22). Mouse monoclonal antibodies directed against hemagglutinin (HA) and vinculin were used at a 1:1,000 dilution.

Preparation of cell blocks

Prostate cells were made into cell blocks using the fibrin clot method as described (23). Blocks were fixed in 10% formalin and paraffin embedded. Sections were prepared and stained using the immunohistochemical procedure.

Immunoprecipitation and Western blot analysis

Relative levels of myc-tagged CD97-3EGF and myc-tagged CD97-β were determined by transfecting COS 7 cells with varying amounts of DNA using Lipofectamine Plus. Lysates were prepared and immunoprecipitations and Western blot analyses were conducted as described (24). Coimmunoprecipitation assays were conducted as described (25). The amount of GTP-bound RHOA was determined using the RHO Activation Assay Kit (Upstate Cell Signalling Solutions).

In vitro invasion and migration

Invasion chambers were prepared as described previously (12) except that growth factor-reduced Matrigel (BD Bioscience) was used. DU145 cell invasion was carried out as described (25). For Rho inhibition, cells were resuspended in medium containing CT04 (2 μg/mL). PC3 and LNCaP transmigration were set up as described above except that bovine serum albumin was not added to the serum-free media and cells were allowed to migrate for 24 hours. Migration was carried out the same way as in vitro invasion except that the membrane was coated with gelatin instead of Matrigel.

PC3 metastasis assay

Experimental metastasis assays and bioluminescent imaging were done as described previously (21).

Results

CD97 expression is induced in prostate adenocarcinomas and is expressed in some prostate cancer cell lines

Immunohistochemical analysis of a prostate cancer tissue array indicated that CD97 was expressed in the membrane and cytoplasm of adenocarcinoma cells, whereas it was undetectable in normal adjacent prostate epithelium (Fig. 1A). For the majority of prostate cancer samples, the CD97 staining intensity was relatively uniform, whereas the percentage of positive cells varied (Table 1). Fifty-nine percent of the adenocarcinomas were positive for CD97 with the majority (44.1%) showing positive CD97 staining in 50% to 80% of adenocarcinoma cells. In the prostate tissue array, 68% of adenocarcinomas with Gleason score 6–7 and 53% of adenocarcinomas with Gleason score 8–10 were stained positively for CD97 expression. The specificity of the rabbit polyclonal anti-CD97 antibody used to stain the prostate tissue array was verified by staining paraffin-embedded prostate epithelial cells with known CD97 expression patterns (Supplementary Fig. S1).

Fluorescence-activated cell-sorting (FACS) analysis of various prostate cell lines stained for CD97 revealed differential cell surface expression. Nontransformed, primary prostate cells (RWpE-1) were negative, whereas we observed positive expression for PC3 and DU145 (22) and negative (22Rv1 and LNCaP) prostate adenocarcinoma–derived cells lines (Fig. 1B). Western blot analysis produced similar results except that 22Rv1 cells seemed to express very low levels of CD97 not detected by the FACS analysis (Fig. 1C). The expression patterns of CD97 in the prostate cancer tissue array and in different prostate cell lines confirm an earlier report that CD97 is induced in prostate adenocarcinomas (6).

Ectopic overexpression of CD97 stimulates Gα12/13-dependent RHOA activation

Although CD97-mediated signaling has not yet been elucidated, the conserved GPCR transmembrane structure of CD97 suggests that its function is G protein coupled. There are a number of examples of highly expressed GPCRs that induce signal activation in a ligand-independent manner (26). Here, we combined ectopic CD97 overexpression with assays for various signal transduction pathways. We observed CD97-dependent induction of activity for the transcriptional reporter SRE-luciferase, an indicator of RHOA activity that is commonly used to assay Gα12/13 activation (27). Transient transfection of
COS 7 cells with constitutively activated forms of RHOA and Ga12 but not Gaq (Fig. 2A) resulted in significantly elevated levels of normalized SRE-dependent luciferase activity, verifying the expected specificity of the assay. A variety of CD97 receptor configurations were assayed, including (i) full-length CD97 containing either 3 or 5 EGF-like repeats, (ii) the GPCR portion of CD97 (denoted CD97-b), and (iii) a GPCR-deficient control (denoted CD97-TM1) that consists of the extracellular region and only the first transmembrane segment. Full-length CD97 stimulated about a 4-fold increase in activity, whereas GPCR-deficient CD97 was inactive. It is of interest that the CD97 GPCR β-subunit alone stimulated a 50-fold increase in SRE-luciferase activity, suggesting higher constitutive activity of the β-subunit in the absence of the interacting extracellular domain (note the scale difference between the left and right panels of Fig. 2A). To normalize SRE activity relative to the level of CD97 GPCR subunit expression, the amount of DNA encoding C-terminal epitope-tagged full-length or β-only CD97 was titrated in transfections and the transfected cells were subsequently assayed for protein amount and luciferase activity. A comparison of similar levels of GPCR subunit expression (Fig. 2C) showed for SRE-luciferase activity a 5-fold increase for full-length CD97 and a 19-fold increase for the β-construct.

To determine whether CD97-stimulated SRE activity was dependent upon Ga12/13, an expression plasmid encoding the RGS domain (residues 1–246) of p115 Rho-GEF was cotransfected with CD97. As expected, RGS-p115 showed Ga12/13-specific inhibition but did not decrease downstream signals such as constitutively active RHOA-dependent SRE activity. As shown in Fig. 2A, RGS-p115 almost entirely inhibited full-length CD97-dependent SRE activity and inhibited about 80% of CD97-β-dependent SRE activity. To investigate whether SRE activation by CD97 was RHO dependent, an expression construct for C3 exoenzyme, which specifically inhibits RHO as a result of ADP ribosylation, was cotransfected with CD97 or

Figure 1. CD97 is expressed in human prostate cancer and in cell lines derived from prostate carcinomas. A, CD97 expression in human prostate adenocarcinoma and in matched normal adjacent tissue in a prostate cancer tissue array (US Biomax, Inc.). The tissue microarray was stained for CD97 using a rabbit polyclonal antibody. B, surface expression of CD97 as determined by FACS analysis in immortalized normal prostate epithelial cells and prostate cancer cell lines. C, Western blot analysis depicting total CD97 expression in the cell lines shown in (B).
control plasmids into COS 7 cells. The C3 exoenzyme blocked RHOA-dependent SRE activity, as expected. In addition, G\text{\alpha}_{12/13}-dependent SRE activity was substantially inhibited, indicating that the G\text{\alpha}_{12/13}-stimulated activity was mediated predominantly through RHO activation. All CD97-stimulated SRE-luciferase activity was inhibited by C3 exoenzyme showing strong RHO dependence (Fig. 2B). No effect of C3 exoenzyme on cotransfected HSV-1K promoter-driven Renilla expression was observed.

To further analyze the effects of sustained CD97 signaling, we generated clones of Chinese hamster ovary (CHO) cells expressing moderate levels of CD97-\beta. These clones displayed a flattened morphology with pronounced stress fibers and uniformly distributed focal adhesions compared with clones expressing empty vector (Fig. 2D). Treatment of CHO cells expressing CD97-\beta with a cell-permeable specific inhibitor of RHO (CT04) caused a reduction in stress fibers and focal adhesions and resulted in complete reversion of the flattened phenotype back to that of cells expressing empty vector (Fig. 2D). Likewise, transient expression of wild-type RHOA in CHO cells resulted in the same flattened morphology, which was readily reversed by treatment with CT04 (Fig. 2E), showing that this cytoskeletal change is characteristic of RHO activation in CHO cells.

**Depletion of CD97 in DU145 leads to inhibition of RHO-dependent, LPA-initiated downstream signaling and invasion**

To investigate potential physiologic functions associated with CD97 expression in prostate cancer, endogenous CD97 expression was depleted using ectopic short hairpin RNA (shRNA) in 2 prostate cancer cell lines, DU145 and PC3 (Figs. 3A and 4A). Depletion of CD97 expression in DU145 cells resulted in substantial inhibition of RHOA-GTP levels (Fig. 3A), suggesting that CD97 influences constitutive signaling in DU145. In addition, directed cell migration (Supplementary Fig. S2) and invasion (Fig. 3B) stimulated by 10% FBS were significantly decreased following CD97 knockdown. Inhibition of RHO activity and in vitro invasion were readily rescued by introduction of an shRNA-insensitive mutant of CD97-3EGF (Fig. 3A and B), indicating that the observed effects were specific to CD97. Treatment of DU145 cells with CT04 suppressed invasion of both control and CD97-depleted cells to the same baseline level, showing that the majority of CD97-dependent migration was RHOA dependent (Fig. 3B). We also investigated the impact of CD97 depletion on potential G\text{\alpha}_{12/13}-mediated downstream mediators that are known to play a role in cellular migration and invasion. Figure 3C shows that CD97 depletion led to decreased phospho-extracellular signal-regulated kinase (ERK) and phospho-AKT levels that were restored to parental DU145 levels by CD97 reexpression. We did not observe an effect on cell growth in vitro.

Because CD97-depleted DU145 cells exhibited reduced invasion to FBS in vitro, we investigated chemotactic constituents of serum and their ability to stimulate CD97-dependent cell invasion. A major chemotactic found in serum is LPA. A dominant pathway downstream of LPAR is G\text{\alpha}_{12/13}-mediated activation of RHOA and ERK (28). Another component of serum that is known to stimulate prostate cell invasion is hepatoctye growth factor (HGF), a ligand for the tyrosine kinase receptor, MET. HGF signals through phosphoinositide 3-kinase (PI3K) and PAK4 (29) as well as ERK and AKT (30, 31).

LPA-initiated signaling was assayed in parental and CD97-depleted cells, and we observed reductions in RHOA-GTP, phospho-ERK, and phospho-AKT levels, similar to the pattern previously seen in CD97-depleted cells grown in serum-containing medium. In contrast, for HGF-stimulated cells, CD97 depletion reduced the relatively weak RHOA-GTP signal but had minimal effects upon phospho-ERK and phospho-AKT levels (Fig. 3D), suggesting that CD97 does not have an impact on the dominant signaling downstream of HGF. Consistent with the signaling data, CD97 depletion had the most dramatic inhibitory effect upon RHO-dependent, LPA-stimulated invasion (Fig. 3E). In contrast, for PI3K-dependent HGF-stimulated chemotaxis, CD97 levels had a minimal effect. Our results indicate that the consequences of depleting CD97 in DU145

<table>
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<tr>
<th>Scoring</th>
<th>LPAR1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD97&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 15)</td>
<td>13.3%</td>
<td>100%</td>
</tr>
<tr>
<td>Adenocarcinoma Gleason 6–7 (n = 28)</td>
<td>28.6%</td>
<td>32.1%</td>
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<tr>
<td>Adenocarcinoma Gleason 8–10 (n = 40)</td>
<td>15.0%</td>
<td>47.5%</td>
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<td>Total adenocarcinoma (N = 68)</td>
<td>20.8%</td>
<td>41.2%</td>
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<sup>a</sup>LPAR1 staining was scored on the basis of intensity, which varied between cores. When staining was positive, most cells expressed LPAR1 (80%–100%). ++, intense staining; +, moderate staining; ±, weak staining; –, no staining. Representative degrees of LPAR1 staining used to score the prostate tissue array are shown in Supplementary Fig. S4. In adenocarcinomas, there was a significant correlation between the presence of CD97 and LPAR1 expression (P < 0.01; Fisher exact test).

<sup>b</sup>Staining of CD97 was scored on the basis of the number of cells expressing CD97. The intensity of staining was uniform and localized to cell membranes, cytoplasm, and nuclei. Representative CD97 staining in the prostate tissue array is depicted in Fig. 1. ++, CD97 expressed in most or all cells (80%–100%); +, CD97 expressed in a significant number of cells (50%–80%); ±, CD97 expressed in a minority of the cells (<50%); –, CD97 absent.
cells were strongest for LPA-stimulated RHO-dependent invasion and signaling, although there was also a weak, mostly RHO-dependent effect on HGF-initiated invasion and signaling. This weak effect may be a result of the cross-talk between the LPA and c-MET receptors or their downstream components (32, 33).

CD97 depletion in PC3 cells reduces experimental bone metastasis but not subcutaneous growth

To address the role of CD97 in tumorigenesis and experimental metastasis, we used the PC3 prostate cancer cell line transfected with a luciferase expression construct (PC3-luc). Figure 4A shows that shRNA-mediated knockdown of CD97 in PC3 cells resulted in suppression of GTP-bound RHO and ERK activity, consistent with our findings for DU145 cells. However, phosphorylation of AKT was not affected by CD97 expression in PC3 cells. Similarly to DU145, invasion of PC3 cells to LPA was significantly reduced following CD97 knockdown or addition of CT04, indicating that the invasion was predominantly CD97 and RHO dependent (Fig. 4B). There was no apparent effect of CD97 depletion upon cell growth in vitro.
The impact of suppressing CD97 expression in PC3 cells was determined in vivo by subcutaneous growth and experimental metastasis. Following inoculation of cells into the flanks of immunocompromised mice, subcutaneous growth of PC3-luc cells was first observed after 7 days and was monitored through day 27. Loss of CD97 did not seem to inhibit the subcutaneous growth of PC3-luc cells. For the CD97-deficient population, 14 of 14 inoculations resulted in tumors with an average tumor volume of 118 ± 70 mm³ at day 27. For the control population, 12 of 14 inoculations resulted in tumors with an average tumor volume of 92 ± 71 mm³. Metastases of PC3-luc cells were analyzed after inoculation via the left cardiac ventricle (Table 2). Mice were imaged for bioluminescence after inoculation and then once per week starting at week 3 until morbidity. PC3-luciferase cell populations displayed tropism to bone including jaw, long bones, and vertebral bodies, and the distribution of metastatic sites was approximately the same in the experimental and control groups. Table 2 shows the relative degree of metastasis in mice injected with parental PC3 cells and CD97-depleted cells. Of the mice injected with parental PC3 cells, approximately 93% (14 of 15) developed metastatic lesions, whereas 55% (12 of 22) of mice inoculated with CD97-depleted PC3 cells developed bone metastasis. In addition, metastasis formation was significantly decreased from 2.07 tumors per mouse injected with...
the parental population to 0.77 tumors per mouse in those injected with the CD97 knockdown population. There was not a statistically significant difference between the growth rates of established tumors as determined by their mean bioluminescence over time. Metastases were confirmed by necropsy, and portions of lesions were subjected to histologic analysis. No histologic differences between CD97-deficient and parental populations were apparent (Fig. 4C). Taken together, these data suggest that CD97 expression has a significant effect upon the ability of PC3 cells to initiate metastasis, consistent with effects upon migration, invasion, and/or colonization.

**CD97 heterodimerizes with LPAR1 resulting in enhanced invasion**

Mechanistic studies were pursued to investigate the significant effect of CD97 depletion on LPA-initiated invasion and signaling. There is increasing evidence that GPCRs can exist as selective heterodimers with specific ligand responses. We hypothesized that CD97 regulates RHO activity in prostate cancer cells, at least in part by interacting with LPARs. LPAR1, which is expressed in PC3 and DU145 but not in LNCaP, has been linked to LPA-initiated migration (18, 19). Techniques to assay an LPAR1–CD97 interaction are limited by a lack of sufficiently sensitive precipitating antibodies for endogenous levels of LPAR1 protein. Therefore, we generated constructs coding for tagged proteins to first analyze the CD97–LPAR1 interaction (Fig. 5A). CHO cells were transiently transfected with HA-tagged LPAR1 and myc-tagged CD97-3EGF and antibodies directed to either myc or to HA coimmunoprecipitated the other GPCR, indicating an interaction between LPAR1 and CD97 (Fig. 5B).
We used the same strategy to look at LPAR1–CD97 heterodimer formation in LNCaP prostate cancer cells that are known to lack LPAR1 (18) and CD97 (Fig. 1B and C). LPAR1-HA, CXCR4-HA, and CD97-myc were permanently transfected into LNCaP cells in the appropriate combinations and assayed for cell surface expression (Supplementary Fig. S3). In coimmunoprecipitation studies, an LPAR1–CD97 interaction was readily detectable (Fig. 5C), whereas CXCR4 coimmunoprecipitated far less CD97. To independently confirm LPAR1–CD97 interactions, we used the In Situ Proximity Ligation Assay (Duolink) to detect heterodimer formation in permanently transfected LNCaP cells (34). The white dots on the cell membranes shown in Fig. 5D indicate the localization of interacting proteins LNCaP cells (34). The white dots on the cell membranes shown to detect heterodimer formation in permanently transfected actions, we used the far less CD97. To independently con

CD97 expression positively correlates with LPAR1 in a prostate tissue array

For LPAR1–CD97 heterodimerization to be relevant for clinical prostate cancer, both receptors would be expected to co-occur in individual specimens of adenocarcinomas. The frequency of LPAR1 expression in prostate adenocarcinomas was investigated in tissue array samples that had been previously characterized for CD97 staining. Histologic staining showed that LPAR1 was expressed in a majority of epithelial cells in most samples of normal prostate (Supplementary Fig. S4) unlike CD97 (Fig 1A). In addition, about 80% of adenocarcinomas showed staining for LPAR1 with a shift toward more intense staining, relative to normal prostate (Table 1; Supplementary Fig. S4). Interestingly, statistical analysis (the Fisher exact test) revealed a significant association between the presence of CD97 and LPAR1 expression (P ≤ 0.01) in adenocarcinomas.

Discussion

The abnormal expression of CD97 in carcinomas has been described for various tissue origins. Here, we show that CD97 expression in clinical samples of prostate adenocarcinoma occurred at a high frequency (approximately 60%) across Gleason scores, whereas there was no detectable expression in normal prostate. An earlier study using a different antibody reported enhanced CD97 expression in prostatic intraepithelial neoplasia and localized prostate cancer relative to low levels of expression in normal prostate (6). Taken together, these data suggest that CD97 induction occurs early in prostate cancer progression. The potential significance of induced CD97 expression in prostate adenocarcinoma was investigated by analyzing the signaling properties of CD97 and the functional contribution of CD97 to prostate cancer invasion and experimental metastasis.

We show here that ectopic CD97 induces Goα12/13-dependent RHOA activation, showing intact heterotrimeric G protein–dependent signaling for the CD97-β subunit. Depletion of endogenous CD97 in DU145 and PC3 cell lines inhibited LPA-stimulated RHOA and ERK pathway activation. Consistent with decreased signaling, CD97 depletion inhibited LPA-stimulated invasion. In contrast, CD97 depletion had a
significantly smaller effect upon HGF-initiated signaling and invasion. Thus, CD97 action seems to be linked to LPA-initiated functions. A complementary approach using reconstitution was carried out in LNCaP cells, which do not normally express CD97 or LPAR1. In this case, CD97 increased LPA-initiated LPAR1-dependent invasion. These \textit{in vitro} data were extended \textit{in vivo} for PC3 cells to show that CD97 depletion reduced metastatic colonization to the bone by more than half.

Figure 5. CD97 interacts with LPAR to potentiate LPA-initiated RHO-dependent invasion \textit{in vitro}. A, schematic of myc-tagged CD97-3EGF and HA-tagged LPAR1. B, communoprecipitation of LPAR1 and CD97 in CHO cells transiently transfected with CD97 and/or LPAR1. The antibodies used for immunoprecipitation (IP) and Western blotting are indicated on the top and side of each panel, respectively. Asterisk indicates a background band. C, communoprecipitation of CD97 with LPAR1 or CXCR4 in permanently transduced LNCaP cells. Antibodies used for Western blotting are indicated on the left. D, stable LNCaP cell lines expressing the indicated proteins were subjected to \textit{in situ} proximity ligation assays (PLA). The detected heterodimers are depicted by white dots. Nuclei stained with DAPI are gray. Representative microscopic fields are shown and scale bars represent 10 \( \mu \)m. E, quantification of PLA results was carried out using the DuoLink Image Tool Software. F, \textit{in situ} PLA was carried out for parental and CD97-depleted DU145 cells permanently transduced with empty vector or LPAR1. The relative expression levels of CD97 and LPAR1 are shown. G, LNCaP cells permanently expressing the indicated proteins were assayed for their invasive activity to LPA. Invading cells in 5 microscopic fields (>200) were counted. To show that invasion was RHO dependent, cells were treated with 1 \( \mu \)g/mL CT04 before being loaded into the Transwell chamber. H, human SDF-1\( \alpha \) (50 ng/mL) was substituted for LPA in the invasion assay described above. Results for (E–H) are representative of 4 independent experiments. Error bars represent \( \pm \) SEM. *** \( P < 0.0001; ** \( P < 0.001; \) NS, nonsignificant difference at a 95% confidence interval. DAPI, 4',6-diamidino-2-phenylindole.
suggested an effect upon early tissue invasion and/or the establishment of micrometastases. Interestingly, in breast cancer, the LPA/LPAR pathway has been associated with bone metastases. In a xenograft model, LPAR signaling promoted both the establishment and subsequent expansion of osteolytic bone metastases (35).

We investigated the possibility that CD97–LPAR heterodimerization could mechanistically explain the positive effect of CD97 expression on LPA-initiated signaling. Using both ectopic expression and shRNA-mediated depletion, it was possible to show physical complex formation between epitope-tagged LPAR1 and either tagged or endogenously expressed CD97. It is possible that there exist additional interactions between CD97 and other GPCRs. However, one measure of specificity for the LPAR–CD97 interaction was indicated by the lack of physical and functional interaction between CD97 and CXCR4, a GPCR that has been suggested to play a role in prostate cancer invasion (36). Immunohistologic analysis of clinical prostate cancer samples revealed that LPAR1 is expressed in normal prostate and in about 80% of adenocarcinomas, with relatively increased levels compared with normal epithelium. There was a significant association of CD97 and LPAR1 expression in adenocarcinomas, confirming the potential for these receptors to synergize in prostate cancers. The role of the LPA–LPAR axis in tumorigenesis and metastasis has been well established (15). Thus, the data presented here provide a mechanistic rationale for induced CD97 expression on LPA-initiated signaling. Further analysis to test for the generality of the LPAR–CD97 association across tissue types will be of interest.

We describe the positive regulation by CD97 of ligand-dependent LPAR1 signaling. We have found no indication for ligand-dependent CD97 function in the context investigated here. For serum-stimulated invasion of prostate cancer cells, there were no effects of blocking antibodies directed to previously identified CD97 cell surface ligands including integrins αvβ1 and αvβ3 as well as CD55. Of course, we cannot exclude the possibility that other unidentified CD97 ligands are required. Thus, preliminarily, CD97 and LPAR1 heterodimerization seems to exemplify ligand-independent (CD97) regulation of ligand-dependent (LPAR) GPCR function. Several mechanisms have been identified for other heterodimerized GPCRs that account for positive regulation by a ligand-independent partner (37). Because both CD97 and LPAR1 couple to Gα12/13, one potential mechanism of synergy is the reinforcement or amplification of Rho-dependent signaling (15).

The data presented here have practical implications. LPARs are considered promising therapeutic targets for cancer treatment, and drugs that inhibit the LPA–LPAR axis are being developed (38). Increased expression of CD97 has been observed in a number of carcinomas, and induced expression of the closely related receptor, EMR2, has been described in breast cancer (39). The presence of CD97 (and possibly EMR2) should be included in the analysis of efficacy for LPAR-directed treatment. In addition, modulating CD97 expression with therapeutic antibodies or small-molecule inhibitors directed to the 7TM pocket are potential approaches to modifying LPAR responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank our many generous colleagues for sharing cell lines and reagents; Barbara J. Taylor and Subhadra Banerjee of the CCR FACS Core Facility and Theresa Davies-Hill for her excellent technical assistance.

Grant Support

The study was supported by the Intramural Research Program, Center for Cancer Research, National Cancer Institute.

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Received July 15, 2011; revised September 1, 2011; accepted September 23, 2011; published OnlineFirst October 6, 2011.

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Cancer Res  Published OnlineFirst October 6, 2011.