Migration to Apoptotic “Find-me” Signals Is Mediated via the Phagocyte Receptor G2A*

Received for publication, August 8, 2007, and in revised form, December 7, 2007. Published, JBC Papers in Press, December 18, 2007. DOI 10.1074/jbc.M706586200

Christoph Peter1,2, Michaela Waibel1,2, Caius G. Radu3, Li V. Yang4, Owen N. Witte5, Klaus Schulze-Osthoff6,7, Sebastian Wesselborg2,3,#, and Kirsten Lauber1,2

From the 1Department of Internal Medicine I, University of Tuebingen, 72076 Tuebingen, Germany, 2Department of Molecular and Medical Pharmacology and 3Department of Microbiology, Immunology, and Molecular Genetics, Howard Hughes Medical Institute, UCLA, Los Angeles, California 90095, and 4Institute of Molecular Medicine, University of Duesseldorf, 40225 Duesseldorf, Germany

Phagocytosis of apoptotic cells is fundamentally important throughout life, because non-cleared cells become secondarily necrotic and release intracellular contents, thus instigating inflammatory and autoimmune responses. Secreted “find-me” and exposed “eat-me” signals displayed by the dying cell in concert with the phagocyte receptors comprise the phagocytic synapse of apoptotic cell clearance. In this scenario, lysophospholipids (lysoPLs) are assumed to act as find-me signals for the attraction of phagocytes. However, both the identity of the lysoPLs released from apoptotic cells and the nature of the phagocyte receptor are largely unknown. By a detailed analysis of the structural requirements we show here that lysophosphatidylcholine (lysoPC), but none of the lysoPC metabolites or other lysoPLs, represents the essential apoptotic attraction signal able to trigger a phagocyte chemotactic response. Furthermore, using RNA interference and expression studies, we demonstrate that the G-protein-coupled receptor G2A, unlike its relative GPR4, is involved in the chemotaxis of monocytic cells. Thus, our study identifies lysoPC and G2A as the crucial receptor/ligand system for the attraction of phagocytes to apoptotic cells and the prevention of autoimmunity.

The elimination of apoptotic cells is a critical end point of apoptosis and of fundamental importance for multicellular organisms. Non-cleared apoptotic cells otherwise become secondarily necrotic and release intracellular contents into the surroundings, thus instigating inflammatory reactions. For the timely and efficient removal of apoptotic cells, a network of interactions between the dying cell and the phagocyte has evolved that are displayed at the phagocytic synapse. Secreted find-me and eat-me signals exposed by the dying cell, together with bridging proteins and phagocyte receptors, comprise the central elements for removal of apoptotic cells and prevention of secondary necrosis (1). Growing evidence suggests that the defective clearance of apoptotic cells favors the onset of autoimmune diseases (2).

Lysophospholipids (lysoPLs),4 like lysophosphatidylcholine (lysoPC), have been shown to function as eat-me signals recruiting complement proteins for recognition by the phagocyte (3). In addition, we could demonstrate that lysoPC also acts as a chemotactic find-me signal attracting the phagocyte to the apoptotic cell (4). During apoptosis lysoPC is generated by the calcium-independent phospholipase-A2, that is activated by a caspase-3-dependent cleavage mechanism. Once released, lysoPC is assumed to bind to a phagocyte receptor, thereby triggering chemotaxis.

The nature of the phagocyte receptor involved in the attraction to apoptotic cells is currently unknown. Receptors involved in phospholipid signaling often exhibit significant promiscuity with many receptors recognizing more than one ligand and vice versa. For lysoPC different receptors have been proposed, including the G-protein-coupled receptor G2A (5) and its structural relative GPR4 (6). However, due to the failure to reproduce the original receptor binding data, the role of G2A and GPR4 as high affinity lysoPC receptors remains controversial (6, 7). Their biological role is further complicated by the fact that both G24 and GPR4 have been suggested to bind other ligands such as oxidized fatty acids (oxFAs) as well as extracellular prostaglandins, which implicated a pH-sensing function of both receptors (8, 9). As the receptor for phagocyte recruitment remains unknown, it is also unclear whether lysoPC represents the sole lipid find-me signal or whether additional phospholipids secreted during apoptosis are involved in chemotaxis. Finally, it is unknown whether lysoPC mediates its chemotactic

4 The abbreviations used are: lysoPL, lysophospholipid; ACS, apoptotic culture supernatant; FA, fatty acid; oxFA, oxidized FA; GPR4, G-protein-coupled receptor 4; HODE, hydroxyocta-decadienoic acid; lysoPA, lysophosphatidic acid; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; lysoPS, lysophosphatidylserine; lysoSM, lysosphingomyelin; PAF, platelet-activating factor; PC, phosphatidylcholine; SDF-1 α, stromal cell-derived factor 1 α; siRNA, small interference RNA; qRT, quantitative reverse transcription; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

* This work was supported by the DFG We 1801/2-4 (to K. L. and S. W.), GRK1302 (to K. L. and S. W.), and SFB 685 (to K. L. and S. W.), the Interdisciplinary Center of Clinical Research Tübingen (IZKF; F6. 01KS9602) (to K. L. and S. W.), the Wilhelm Sander-Stiftung (1423-98101) (to S. W.), the Landesforschungsschwerpunktprogramm of the Ministry of Science, Research, and Arts of the Land Baden-Württemberg (1250-0-0) (to K. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and supplemental “Experimental Procedures.”

†1 Both authors contributed equally to this work as first authors.

‡2,3 These authors contributed equally to this work as senior authors.

§3 To whom correspondence should be addressed: Dept. of Internal Medicine I, Eberhard Karls University, Otterried-Müller-Str. 10, D-72076 Tuebingen, Germany. Tel.: 49-7071-29-84113; Fax: 49-7071-29–5865; E-mail: sebastian.wesselborg@uni-tuebingen.de.
Apoprotic “Find-me” Signals and G2A

effect via direct receptor binding or indirectly via another unknown pathway.

These open questions therefore stimulated us to further investigate the nature of lipid attraction signal and corresponding receptor involved in the macrophage recruitment to apoptotic cells. By a detailed characterization of the structural requirements of the lysoPL agonist, we report here that lysoPC, but no other lysoPLs or lysoPC derivatives, represents the major phospholipid find-me attraction signal released by apoptotic cells. Moreover, using RNA interference and overexpression studies, we identified the receptor G2A, in contrast to its relative GPR4, as the crucial phagocyte receptor mediating the chemotactic response. Thus, our study elucidates the essential ligand/receptor relationships involved in the clearance of apoptotic cells and prevention of autoimmunity.

EXPERIMENTAL PROCEDURES

Cell Lines—Cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin/ml, 0.1 mg of streptomycin/ml, and 10 mM HEPES (all from Invitrogen). U937 G2A and U937 cells were generated as described (10). MCF-7 casp3 cells were a kind gift from R. U. Jänicke and A. G. Porter (11). Cells were grown at 37 °C in a 5% CO2 atmosphere and maintained in log phase.

Lipids, siRNA Oligonucleotides, Antibodies, and Other Reagents—Synthetic lysoPLs (18:0 lysoPC, 18:1 lysoPS, 18:0 lysoPE, 18:0 lysoPA lysoSM, 18:0 lysoPAF), (18:0, 2:0) PAF, and Reagents

dissolved in RPMI 1640 medium supplemented with 0.1% fatty acid-free bovine serum albumin (Sigma-Aldrich) were placed into the lower chamber of 8-μm pore ChemoTX plates (Neuroprobe Inc., Gaithersburg, MD). The filter was adjusted, the stained cell suspension was added on top, and the assay was incubated for 120–180 min at 37 °C. Subsequently, the transmigrated cells were collected by centrifugation and lysed in 100 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100). Green fluorescence was analyzed, and transmigration was calculated in percent-age of total cells deployed (mean values ± S.D. from triplicate experiments).

Measurement of Cell Viability—Cell viability tests were performed in triplicate as described previously (4).

Transfection with siRNA Oligonucleotides—Transfection with siRNA oligonucleotides was carried out twice (at day 0 and day 3) with the Gene Pulser II + Capacity Extender II (Bio-Rad) and 0.4-cm gap cuvettes. 5 × 108 THP-1 cells were electroporated with 2 μM siRNA oligonucleotides in 500 μl of OptimEM™ medium (Invitrogen) by a single pulse (800 μF, 200 V, time constant 20–30 ms). The cells were cultured for 3 days before electroporation was repeated. All following experiments were carried out at day 6.

Preparation of Membrane Protein Extracts and Immunoblotting—Expression of G2A was detected by immunoblotting of membrane protein extracts. Therefore, 1 × 107 cells were collected by centrifugation, washed in cold phosphate-buffered saline, and resuspended in 1 ml of buffer A containing 20 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The cells were homogenized in a Dounce homogenizer, and the homogenates were centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was centrifuged at 50,000 × g for 2 h. The resulting membrane pellet was solubilized in 5% Triton X-100, 50 mM Tris-HCl, pH 7.6, and 150 mM NaCl containing 3 μg/ml aprotinin, 3 μg/ml leupeptin, 3 μg/ml pepstatin A, and 2 mM phenylmethylsulfonyl fluoride. Subsequently, 100–200 μg of membrane protein extracts were applied to reducing 8–15% SDS-PAGE and Western blot analysis as previously described (4).

qRT-PCR Analysis—The detection of G2A and GPR4 mRNA levels was performed by quantitative reverse transcription (qRT)-PCR analysis with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and qPCR Mastermix Plus (Eurogentec, Seraing, Belgium). The following probes and primers (MWG Biotech, Ebersberg, Germany) were used: G2A forward, 5'-TGT GCC CAA TGC TAC TGA AAA AC-3'; G2A reverse, 5'-CGA CCA GGA CTA TCC GTC TCT CT-3'; G2A probe FAM, 5'-TTA CAA TGG AAA CCG CAC CCC AGT GAC-3'-TAMRA; GPR4 forward, 5'-TTT TGC GAG GCC GTC GTC TTT-3'; GPR4 reverse, 5'-GTT CCG CCA CAC AGT TGA-3'; GPR4 probe FAM, 5'-CTG CAT ACC ACA GCT CAC CCT GGT CTT TCA C-3'-TAMRA; ALAS-1 forward, 5'-TCC ACT GCA GCA GTA CAC TAC CA; ALAS-1 reverse, 5'-ACG GAA GCT GTG TGC CAT CT-3'; ALAS-1 probe VIC, 5'-AAA GAA ACC CCT CCG GCC AGT GAG AA-3'-TAMRA.

Total RNA from 2 × 106 cells was extracted with the NucleoSpin® RNA II kit (Macherey & Nagel, Düren, Germany). 1 μg...
of total RNA was reverse transcribed with 200 units of SuperScript RT II™ reverse transcriptase (Invitrogen) in the presence 50 μM random hexamers (Amersham Biosciences), 400 μM dNTPs (Promega), and 1.6 units/μL RNasin™ (Invitrogen). 40–80 ng of the resulting cDNA were applied to the following qRT-PCR analyses (20-μl final volume) with 200 nM primers and 100 nM probe. For the study of siRNA knockdown effects, relative quantification was performed employing the standard curve method. The results were normalized on δ-aminolevulinate-synthase-1, and the cell population transfected with the control oligonucleotide was used as calibrator. The comparison of G2A and GPR4 expression in THP-1 cells was done with the \((1 + E)^{-1}\) method (efficiency of amplification = 0.96 (G2A) or 0.92 (GPR4)). All experiments were performed in duplicates and are presented as mean values.

RESULTS

We have previously found that apoptotic cells secrete find-me signals ensuring the recruitment of macrophages and their final engulfment (4). During this process, caspase-3-mediated activation of calcium-independent phospholipase-A₂ was shown to result in the release of the phospholipid lysoPC that is involved in the chemotactic response. Our previous study, however, could not exclude that lysoPC derivatives or additional lipid signals contribute to the attraction of macrophages. Furthermore, the identity of the corresponding phagocyte receptor remained elusive. To address these objectives, we therefore first performed a detailed characterization of the lysoPL agonist(s), which also allowed us to narrow down the nature of the involved lipid receptor.

Headgroup Specificity—The substrate and headgroup specificity of calcium-independent phospholipase-A₂ is relatively unknown, and first reports demonstrated little preference for PC (12). Thus, it was conceivable that apart from lysoPC other lysoPLs might be generated and involved in phagocyte attraction. To explore this possibility, we first investigated whether lysoPLs with different headgroups could compete with the chemotactic signal in apoptotic culture supernatants (ACS). To neutralize the chemotactic gradient, we added lysoPC, lyso-phosphatidylserine (lysoPS), and lysophosphatidylethanolamine (lysoPE) to THP-1 monocytes in the upper chamber of a transmigration plate and applied ACS of MCF-7_casp3 cells to the lower chamber (diagram in Fig. 1A). Subsequent measurement of cell migration revealed that only lysoPC could efficiently block chemoattraction of THP-1 cells by ACS, whereas lysoPS and lysoPE had little effect (Fig. 1A). To confirm that this was not due to a potential cytotoxicity of the phospholipids, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide assay showing that the viability of THP-1 cells was not measurably altered by any lysoPL (Fig. 1B).

Next, we performed the reverse assay and tested whether the lysoPLs could directly induce THP-1 cell migration. When the lysoPLs were added to the lower transmigration chamber (diagram Fig. 1C), only lysoPC was able to attract THP-1 cells. From these data we can conclude that (i) a phosphocholine headgroup in the lysoPL released by apoptotic cells is required to stimulate cell migration and that (ii) phosphoserine or phos-
Phosphoethanolamine headgroups only weakly antagonize the apoptotic find-me signal.

**Role of the Lipid Backbone**—It had been shown that, in addition to PC, calcium-independent phospholipase-A2 can also hydrolyze PAF to lysoPAF (12). We therefore investigated the structural requirement of the lipid backbone. Because of the similarity to lysoPC, we included PAF and lysosphingomyelin (lysoSM) in our experiments. With the same experimental design as in Fig. 1A, lysoPC, PAF, lysoPAF, and lysoSM were tested for their potential to compete with the lysoPL attraction signal in ACS. As can be seen in Fig. 2A, all lipids neutralized the apoptotic chemoattractant. Again, examination of THP-1 cell viability did not reveal any profound cytotoxic effect (Fig. 2B).

Because of their antagonistic effects, we next studied whether PAF, lysoPAF, and lysoSM also induce migration directly. Remarkably, when applied to the lower transmigration chamber only lysoPC, and to a lesser extent lysoSM, could stimulate THP-1 cell chemotaxis (Fig. 2C). From these results we presume that migration can be induced by glycerol or sphingosine lysoPLs, whereas antagonizing effects on lysoPL-induced migration can also be exerted by sn-1 etherlipids with a free sn-2 OH- or small acyl residue (see Fig. 2D).

**Role of LysoPC Metabolites**—Although lysoPC induced chemotaxis and neutralized the chemotactic activity in ACS, due to its inherent instability, we could not exclude that a lysoPC metabolite acts as the actual find-me signal. For lysoPC, different ways of metabolism are known (Fig. 3E). First, lysoPC can be reesterified to PC. Second, it can be hydrolyzed to lysophosphatidic acid (lysoPA) and a free choline residue by phospholipase D or to glycerophosphocholine and a free fatty acid.

**FIGURE 2.** Role of the lipid backbone: lysoPC, PAF, lysoPAF, and lysoSM have similar antagonizing effects on the chemoattractant in ACS but only lysoPC and lysoSM stimulate THP-1 cell migration. A, competitive effect of phospholipids with different backbones on the chemoattractant in ACS; B, viability of THP-1 cells treated with different phospholipids; C, stimulation of THP-1 cell migration by different phospholipids; D, effects on migration stimulated by ACS and viability as well as direct stimulation of migration were determined as in Fig. 1, A–C. Error bars represent S.D. of triplicates. D, schematic diagram of the antagonist/agonist profile of the phagocyte receptor, which mediates migration in response to ACS.
(FA) by lysophospholipase A1. Therefore, we tested the metabolites PC, lysoPA, and glycerophosphocholine in the competition assay with ACS but could not detect any neutralizing potential (Fig. 3A) or cytotoxic effect (Fig. 3B). Next, we examined the role of the free FA. We deliberately focused on oxFAs because FA oxidation occurs during apoptosis (13) and chemo-

FIGURE 3. LysoPC itself and no metabolic derivative is responsible for the induction of THP-1 cell migration. A, competition of different lysoPC derivatives with the chemoattractant in ACS (measured as in Fig. 1A). B, viability of THP-1 cells treated with different lysoPC derivatives (measured as in Fig. 1B). C, competition of different oxFAs with the chemoattractant in ACS (measured as in Fig. 1A). D, viability of THP-1 cells treated with different oxFAs (measured as in Fig. 1B). Error bars represent S.D. of triplicates. E, schematic diagram of putative metabolization steps for lysoPC.
taxis to oxFAs in contrast to non-oxFAs has been reported (14).
To this end, we tested (9)- and (13)-hydroxyoctadecadienoic acid (HODE) together with (9)- and (13)-hydroperoxyoctade-
cadienoic acid in the neutralization assay with ACS. As shown in Fig. 3C, no significant reduction of THP-1 cell migration could be detected. Again, cytotoxic effects were ruled out by a viability test (Fig. 3D). Moreover, (9)- or (13)-HODE and (9)- or (13)-hydroperoxyoctadecadienoic acid themselves did not stimulate THP-1 cell chemotaxis (data not shown). Thus, these results suggest that only lysoPC itself, and no metabolic deriv-
avatives, acts as the effective chemotacticant.

Characterization of the Phagocyte Receptor Mediating the Recruitment to Apoptotic Cells—Our established agonist/an-
tagonist profile of the chemotactic activity hinted at the possi-
bility that the G-protein-coupled receptors G2A or GPR4 might be involved in the recruitment of phagocytes to ACS. Both receptors have originally been reported to bind lysoPC and, with a weaker affinity, lysoSM (5, 6); however, their func-
tional role in mediating lysoPC biological effects has been obscured because of nonvalid receptor binding data (5–7). Fur-
thermore, whether these receptors are involved in the attrac-
tion of monocytes to apoptotic cells was completely unknown.

To investigate a functional role of G2A and GPR4, we com-
pared their expression levels in THP-1 cells by qRT-PCR. Fig. 4A shows that THP-1 cells expressed >5-fold more G2A than
GPR4 mRNA. Furthermore, we found that, in contrast to
THP-1 cells, the monocytic cell line U937 expressed consider-
ably less G2A transcripts (Fig. 4B). The difference of G2A
expression was even more evident on the protein level, as dem-
onstrated by Western blot analysis that revealed no anti-G2A
reactive band in U937 cells but a prominent expression in
THP-1 cells (Fig. 4C).

In view of this different expression pattern, we compared the
migration activity of THP-1 and “low level G2A” expressing
U937 cells toward ACS and lysoPC. Interestingly, as depicted in
Fig. 4D, both stimuli induced chemotaxis only in THP-1 cells
but not in U937 cells. To verify that U937 cells had no general
migration defect, supernatants of necrotic MCF-7casp3 cells and
SDF-1α/H9251 was not affected in G2A-silenced cells.

Disruption of G2A expression, because qRT-PCR revealed ~20% of residual
G2A mRNA (Fig. 5C). Also, in Western blot analysis G2A pro-
tein was markedly reduced but still detectable (Fig. 5D). Addi-
tionally, other lysoPC receptors such as GPR4 might be res-
ponsible for the residual migration observed in G2A-silenced
THP-1 cells. However, silencing of GPR4 expression with two
different GPR4-specific siRNA oligonucleotides had no signif-
cant impact on the transmigration to ACS (Fig. 5E), even though the knock down was as efficient as in the case of G2A
with ~20% of residual GPR4 mRNA (Fig. 5F). Thus, expression
of G2A, but not GPR4, appears to be required for migration to
apoptotic lysoPL attraction signals.

Expression of G2A Reconstitutes Migration to ACS and
LysoPC—To corroborate these findings and to investigate
whether G2A is sufficient for migration to lysoPL find-me
signals, we employed retroviral transduction experiments with
G2A in U937 cells that are unresponsive to ACS. Comparison of the
low level G2A U937 cells transduced with the retroviral
vector alone and U937 cells transduced with the G2A construct
revealed that expression of G2A strongly restored the migration
activity toward ACS and lysoPC (Fig. 6A). Again, expres-
sion of G2A did not affect the migration to purified SDF-1α.

In addition, we compared the two U937 cell populations for
migration toward different lysoPLs. Regardless of G2A expres-
sion, however, neither lysoPS nor lysoPE was able to trigger cell
migration (Fig. 6B). Interestingly, expression of G2A reconsti-
tuted the migration of U937 cells not only to lysoPC but also to
lysoSM (Fig. 6B). This observation is consistent with our previ-
sous finding that indeed both phospholipids might act as puta-
tive phagocyte receptor agonists (Fig. 2C).

To confirm our above established agonist/antagonist profile
(Fig. 2D), we incubated G2A-transduced U937 cells with the
different lysoPLs in a neutralization experiment. To this end,
lysoPC was added to the lower and the other lysoPLs were
added with the cells to the upper transmigration chamber. As
shown in Fig. 6C, only lysoPC itself, PAF, lysoPAF, and lysoSM
could neutralize lysoPC-stimulated migration of U937/G2A cells,
supporting the data obtained with THP-1 cells and ACS (Figs. 1
and 2).

Finally, we analyzed migration of mouse J774A.1 macro-
phages with different G2A expression levels. Again, RNA inter-
ference and retroviral overexpression were used to inhibit and
enhance G2A expression, respectively. As shown in supple-
mental Fig. S1, lysoPC could only induce chemotaxis in G2A
high expressing J774A.1 macrophages, whereas the G2A-si-
cenced cells showed no migration. Furthermore, the other
lysoPLs exerted only weak effects in G2A-expressing cells (supple-
mental Fig. S1). Taken together, these results suggest that in
both human and murine cells lysoPC and lysoSM are the only
lysoPLs inducing monocyte migration via G2A, an event that
can be antagonized by PAF and lysoPAF.

Discussion
The attraction of the phagocyte appears to be the initial step
in the clearance of apoptotic cells. The players involved in this
process, however, have been ill-defined. We showed previously
that during apoptosis caspase-3-mediated cleavage leads to the
activation of calcium-independent phospholipase-A2, thereby
generating a lipid attraction signal for the recruitment of
phagocytes (4). Whether lysoPC was the sole attraction signal or whether other lysoPLs or metabolites were involved in this process remained unknown. Neither was the corresponding receptor on phagocyte site identified. In this study, we show that among the putative lysoPLs generated by calcium-independent phospholipase-A₂ during apoptosis only lysoPC represents a major find-me signal that stimulates migration via the phagocyte receptor G2A both in human and murine cells.

G2A forms together with T cell death-associated gene 8 (TDAG8), ovarian cancer G-protein-coupled receptor 1 (OGR1), and GPR4 a subgroup of structurally related G-protein-coupled receptors that had been originally proposed to

---

**FIGURE 4.** U937 cells do not migrate to lysoPL find-me signals. 

A, relative quantitation of G2A and GPR4 mRNA levels in THP-1 cells. Total RNA of 2 × 10⁶ THP-1 cells was extracted and reverse transcribed, and 80 ng of the resulting cDNA were used for qRT-PCR. Relative quantitation was performed with the (1 + E)⁻¹ method, and G2A mRNA level was set as calibrator. The inset shows the primary amplification curves. B, relative quantitation of G2A mRNA levels in THP-1 and U937 cells. Total RNA of 2 × 10⁶ THP-1 or U937 cells was extracted and reverse transcribed, and 80 ng of the resulting cDNA were used for qRT-PCR as described under “Experimental Procedures.” Relative G2A mRNA level in THP-1 cells was used as calibrator. C, detection of G2A on protein level by immunoblot analysis of THP-1 and U937 membrane protein extracts. Membrane proteins of 1 × 10⁷ THP-1 or U937 cells were extracted as described under “Experimental Procedures,” and 130 μg of the extract were applied to 8–15% SDS-PAGE with consecutive anti-G2A immunoblot analysis. Detection of sodium-proton-exchanger-1 (NHE-1) protein was used as loading control. Asterisks indicate unspecific bands of the anti-G2A antibody. D, migration of THP-1 and U937 cells to supernatants of healthy, apoptotic, or necrotic cells and lysoPC or SDF-1 α. Supernatants of MCF-7_casp3 cells and lysoPC or SDF-1 α were applied to the lower transmigration chamber, and migration of THP-1 or U937 cells was assessed as described under “Experimental Procedures.” Error bars represent S.D. of triplicates.
bind proinflammatory lipids (5, 6, 15, 16). More recent studies have challenged the identification of lipid agonists for these receptors and have suggested that they function primarily as proton sensors (8, 15, 17). However, in contrast to OGR1, GPR4, and TDAG8, G2A lacks essential histidine residues that were previously shown to be important for pH sensing.

FIGURE 5. Knock down of G2A but not GPR4 expression results in decreased migration to ACS and lysoPC. A, influence of G2A knock down on migration to supernatants of healthy, apoptotic, and necrotic cells. Supernatants of MCF-7casp3 cells were prepared and applied to the lower transmigration chamber. Migration of THP-1 cells electroporated with a control oligonucleotide or two different G2A-specific siRNA oligonucleotides was assessed as described under “Experimental Procedures.” B, effect of G2A knock down on migration stimulated by lysoPC or SDF-1 α. 10 μM lysoPC or 200 ng/ml SDF-1 were added to the lower chamber of a double chamber plate, and migration of siRNA-treated THP-1 cells (as in Fig. 5A) was assessed. C, knock down of G2A mRNA expression by siRNA oligonucleotides. G2A mRNA level was measured in siRNA-treated THP-1 cells as depicted under “Experimental Procedures.” The G2A mRNA level in THP-1 cells treated with the control siRNA was set as 100% calibrator. D, knock down of G2A protein expression by siRNA oligonucleotides. G2A was detected by immunoblot analysis of siRNA-treated THP-1 membrane protein extracts. Membrane proteins of 1 × 10⁶ THP-1 cells were extracted as described under “Experimental Procedures,” and 100 μg of membrane protein were applied to 8–15% SDS-PAGE with consecutive anti-G2A immunoblot analysis. Detection of sodium-proton-exchanger-1 (NHE-1) protein was used as loading control. E, effect of GPR4 knock down on migration to supernatants of healthy and apoptotic cells. Migration of THP-1 cells electroporated with a control oligonucleotide or two different GPR4-specific siRNA oligonucleotides was assessed as in Fig. 5A. F, knock down of GPR4 mRNA expression by siRNA oligonucleotides. GPR4 mRNA level was measured in siRNA-treated THP-1 cells as in Fig. 5C. Error bars represent S.D. of triplicates.

FIGURE 6. Expression of G2A reconstitutes migration to ACS and lysoPC. A, migration of U937G2A or U937 cells to supernatants of healthy and apoptotic cells or 10 μM lysoPC or 200 ng/ml SDF-1 α, respectively, was measured as described under “Experimental Procedures.” B, stimulation of U937 cell migration by different lipids. LysoPC, lysoPS, lysoPE, and lysoSM were added at 5 μM to the lower transmigration chamber, and chemotaxis assay with U937G2A or U937 cells was performed as depicted under “Experimental Procedures.” C, neutralization of lysoPC induced U937G2A cell migration by different phospholipids. LysoPC was added at 5 μM to the lower transmigration chamber, 20 μM of lysoPC, lysoPS, lysoPE, PAF, lysoPAF, or lysoSM were added to the U937G2A cells in the upper transmigration chamber, and chemotaxis of U937G2A cells was assessed. Error bars represent S.D. of triplicates.
Apoptotic “Find-me” Signals and G2A

This and the observation that G2A-deficient thymocytes and splenocytes show no impaired response to extracellular protons (19) suggest that G2A exerts a distinct biological function.

Similar to the pH-sensing ability, the phospholipid binding function of the OGR1 family of receptors has been debated. The initial description of the lysoPC binding activity for both G2A and GPR4 has been retracted, mainly because of inconsistencies in these studies. Nevertheless, using RNA interference and retroviral overexpression, our experiments clearly show that G2A mediates the attraction of monocytes in response to lysoPC and ACS. Our data are strengthened by follow-up studies demonstrating a functional role of G2A in lysoPC-mediated chemotaxis of different cell types (10, 18).

In addition to lysoPC and lysoSM, G2A has recently been reported to bind oxFAs (9). In this study the authors showed intracellular calcium mobilization, AMP decline, and JNK activation induced by (9)-HODE and other oxFAs but found no detectable calcium flux by lysoPC in G2A-overexpressing cells (9), yet they failed to detect (9)-HODE-induced ERK phosphorylation, an event described to occur upon lysoPC-mediated G2A stimulation (20). Although the authors did not measure cell migration, in the present study we could not observe cell migration in response to (9)-HODE or other oxFAs (data not shown). Moreover, lysoPL-induced migration was not neutralized by oxFAs (Fig. 3C). From these data it can be concluded that oxFAs do not stimulate G2A-mediated monocyte migration but, rather, distinct signaling events. Ectopic expression studies with different Go subunits and partial sensitivity to pertussis toxin led Obinata et al. (9) to the conclusion that (9)-HODE-stimulated G2A signaling is Gq- and Gi-coupled. This, however, contrasts with other studies demonstrating a pertussis toxin insensitivity of lysoPC-induced and G2A-mediated migration. Nevertheless, it is conceivable that certain G2A agonists trigger different signaling cascades via dynamic G-protein interactions. For instance, oxFAs might induce calcium release and JNK activation via Gq or Gi interactions, whereas cell migration induced by lysoPC or lysoSM could be mediated by G11/G12/13 interaction and subsequent Rho and ERK activation.

So far, valid binding data are not available for either lysoPC or for oxFAs. It remains therefore unknown whether the interaction between G2A and its putative ligands is direct or indirect. Lipid mediators such as externalized phosphatidylserine and oxidized phospholipids as well as lysoPC play a role in the recognition of apoptotic cells. In the phagocytic synapse, these lipid signals do not act directly on the corresponding phagocyte receptors but indirectly via certain bridging proteins (1). In this context, complement recruitment has been described for lysoPC (3). Consequently, the interaction between soluble lysoPC and G2A could also be mediated via a bridging protein. Alternatively, lysoPC-mediated G2A activation might be controlled by a different mechanism. In this regard, Wang et al. (20) showed that intracellular sequestration and surface exposition of G2A control the signaling responses toward lysoPC.

In our experiments employing RNA interference-mediated knock down of G2A expression, we could not completely inhibit monocyte migration to ACS. Although this might be due to an incomplete suppression of G2A, it is also conceivable that other receptors additionally contribute to monocyte migration. We could exclude a role of GPR4, because knock down of GPR4 expression had no effect on migration toward lysoPL attraction signals. This fits to the suggestion of GPR4 acting as a proton sensor rather than a lysoPL receptor (15, 19), even although other biological effects of lysoPLs, for instance in endothelial cells, might be still mediated by GPR4 (21, 22). Other receptors capable of lysoPC binding include scavenger receptors or the complement receptors 3 and 4, which recognize complement-bound lysoPC (23). Finally, the calreticulin/CD91 system has also been described to bind to complement proteins and collectins (24, 25) and could thereby mediate lysoPC-induced migration. However, whether these receptor systems indeed mediate chemotaxis to lysoPC has not been studied so far.

The observation that G2A knock down did not completely abolish migration could also be explained by the existence of additional lysoPC-independent find-me signals in ACS. In view of the great variety of eat-me signals presented by the apoptotic cell, lysoPC might not be the only apoptotic find-me signal. In this context, S19 ribosomal protein, split human tyrosyl-tRNA synthetase, or thrombospondin-1 have been described (26–28) as chemotaxant signals. Certainly, the lysoPC/G2A receptor system apparently plays a predominant role in the clearance of apoptotic cells. It is now widely accepted that defects in the removal of apoptotic cells can lead to autoimmunity (2). Interestingly, G2A knock-out mice develop an autoimmune syndrome (29) similar to mice with a deficiency in certain eat-me signals, such as MFG-E8 or C1q (30, 31). This similar phenotype underscores the importance of both find-me and eat-me signals for the clearance of apoptotic cells.

Acknowledgments—We thank R. U. Jänicke and A. G. Porter for providing MCF-7casp3 cells.

REFERENCES
1. Lauber, K., Blumenthal, S. G., Waibel, M., and Wesselborg, S. (2004) Mol. Cell 14, 277–287
2. Gaipl, U. S., Franz, S., Voll, R. E., Sheriff, A., Kalden, J. R., and Herrmann, M. (2004) Curr. Rheumatol. Rep. 6, 401–407
3. Kim, S. J., Gershov, D., Ma, X., Brot, N., and Elkon, K. B. (2002) J. Exp. Med. 196, 655–665
4. Lauber, K., Bohn, E., Krober, S. M., Xiao, Y. J., Blumenthal, S. G., Lindemann, R. K., Marini, P., Wiedig, C., Zobywalski, A., Baksh, S., Xu, Y., Autenrieth, I. B., Schulze-Osthoff, K., Belka, C., Stuhler, G., and Wesselborg, S. (2003) Cell 113, 717–730
5. Kabarowski, J. H., Zhu, K., Le, L. Q., Witte, O. N., and Xu, Y. (2001) Science 293, 702–705
6. Zhu, K., Baudhuin, L. M., Hong, G., Williams, F. S., Cristina, K. L., Kabarowski, J. H., Witte, O. N., and Xu, Y. (2001) J. Biol. Chem. 276, 41325–41335 Retraction in J. Biol. Chem. 280, 43280
7. Witte, O. N., Kabarowski, J. H., Xu, Y., Le, L. Q., and Zhu, K. (2005) Science 307, 206
8. Murakami, N., Yokomizo, T., Okuno, T., and Shimizu, T. (2004) J. Biol. Chem. 279, 42484–42491
9. Obinata, H., Hattori, T., Nakane, S., Tatei, K., and Izumi, T. (2005) J. Biol. Chem. 280, 40676–40683
10. Yang, L. V., Radu, C. G., Wang, L., Riedinger, M., and Witte, O. N. (2005) Blood 105, 1127–1134
11. Jänické, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) J. Biol. Chem. 273, 9357–9360
12. Tang, J., Kriz, R. W., Wolfman, N., Shaffer, M., Seehra, J., and Jones, S. S. (1997) *J. Biol. Chem.* **272**, 8567–8575
13. Huber, I., Vales, A., Mitulovic, G., Blumer, M., Schmid, R., Witztum, J. L., Binder, B. R., and Leitinger, N. (2002) *Arterioscler. Thromb. Vasc. Biol.* **22**, 101–107
14. Henricks, P. A., Engels, F., van der Vliet, H., and Nijkamp, F. P. (1991) *Prostaglandins* **41**, 21–27
15. Ludwig, M. G., Vanek, M., Guerini, D., Jones, C. E., Junker, U., Hofstetter, H., Wolf, R. M., and Seuwen, K. (2003) *Nature* **425**, 93–98
16. Ishii, S., Kihara, Y., and Shimizu, T. (2005) *J. Biol. Chem.* **280**, 9083–9087
17. Wang, J. Q., Kon, J., Mogi, C., Tobo, M., Damirin, A., Sato, K., Komachi, M., Malchinkhuu, E., Murata, N., Kimura, T., Kuwabara, A., Wakamatsu, K., Koizumi, H., Uede, T., Tsujimoto, K., Kurose, H., Sato, T., Harada, A., Misawa, N., Tomura, H., and Okajima, F. (2004) *J. Biol. Chem.* **279**, 45626–45633
18. Radu, C. G., Yang, L. V., Riedinger, M., Au, M., and Witte, O. N. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 245–250
19. Radu, C. G., Nijagal, A., McLaughlin, J., Wang, L., and Witte, O. N. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 1632–1637
20. Wang, L., Radu, C. G., Yang, L. V., Bentolila, L. A., Riedinger, M., and Witte, O. N. (2005) *Mol. Biol. Cell* **16**, 2234–2247
21. Qiao, J., Huang, F., Naikawadi, R. P., Kim, K. S., Said, T., and Lum, H. (2006) *Am. J. Physiol.* **291**, L91–L101
22. Kim, K. S., Ren, J., Jiang, Y., Ebrahem, Q., Tipps, R., Cristina, K., Xiao, Y. I., Qiao, J., Taylor, K. L., Lum, H., Anand-Apte, R., and Xu, Y. (2005) *FASEB J.* **19**, 819–821
23. Gasque, P. (2004) *Mol. Immunol.* **41**, 1089–1098
24. Vandivier, R. W., Ogden, C. A., Fadok, V. A., Hoffmann, P. R., Brown, K. K., Botto, M., Walport, M. J., Fisher, J. H., Henson, P. M., and Greene, K. E. (2002) *J. Immunol.* **169**, 3978–3986
25. Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., Bratton, D. L., Oldenborg, P. A., Michalak, M., and Henson, P. M. (2005) *Cell* **123**, 321–334
26. Horino, K., Nishiura, H., Ohzako, T., Shibuya, Y., Hiraoka, T., Kitamura, N., and Yamamoto, T. (1998) *Lab. Invest.* **78**, 603–617
27. Moodley, Y., Rigby, P., Bundell, C., Bunt, S., Hayashi, H., Misso, N., McAnulty, R., Laurent, G., Scaffidi, A., Thompson, P., and Knight, D. (2003) *Am. J. Pathol.* **162**, 771–779
28. Wakasugi, K., and Schimmel, P. (1999) *J. Biol. Chem.* **274**, 23155–23159
29. Le, L. Q., Kabarowski, J. H., Weng, Z., Satterthwaite, A. B., Harvill, E. T., Jensen, E. R., Miller, J. F., and Witte, O. N. (2001) *Immunity* **14**, 561–571
30. Hanayama, R., Tanaka, M., Miyasaka, K., Aozasa, K., Koike, M., Uchiyama, Y., and Nagata, S. (2004) *Science* **304**, 1147–1150
31. Botto, M. (1998) *Exp. Clin. Immunogenet.* **15**, 231–234