Glycoprotein Capture and Quantitative Phosphoproteomics Indicate Coordinated Regulation of Cell Migration upon Lysophosphatic Acid Stimulation

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The lipid mediator lysophosphatidic acid (LPA) is a serum component that regulates cellular functions such as proliferation, migration, and survival via specific G protein-coupled receptors. The underlying signaling mechanisms are still incompletely understood, including those that operate at the plasma membrane to modulate cell-cell and cell-matrix interactions in LPA-promoted cell migration. To explore LPA-evoked phosphorylation with a focus on cell surface proteins, we combined glycoproteome enrichment by immobilized lectins with SILAC-based quantitative phosphoproteomics. We performed biological replicate analyses in SCC-9 squamous cell carcinoma cells and repeatedly quantified the effect of 1.5- and 5-min LPA treatment on more than 700 distinct phosphorylations in lectin-purified proteins. We identified many regulated phosphorylation events on various types of plasma membrane proteins such as cell adhesion molecules constituting adherens junctions, desmosomes, and hemidesmosomes. Several of these LPA-regulated phosphorylation sites have been characterized in a biological context other than G protein-coupled receptor signaling, and the transfer of this functional information suggests coordinated and multifactorial cell adhesion control in LPA-induced cell migration. Additionally, we identified LPA-mediated activation loop phosphorylation of the serine/threonine kinase Wnk1 and verified a role of Wnk1 for LPA-induced cell migration in knock-down experiments. In conclusion, the glycoproteome phosphoproteomics strategy described here sheds light on incompletely understood mechanisms in LPA-induced cell migratory behavior. Molecular & Cellular Proteomics 9.2337–2353, 2010.

The plasma membrane separates the interior of a mammalian cell from the environment. To respond to external signals such as growth factors, cells possess various types of plasma membrane-spanning receptors that communicate to the intracellular signaling machinery in a ligand-regulated manner. G protein-coupled receptors (GPCRs), which are integral membrane proteins with seven transmembrane helices, constitute the largest superfamily of cell surface receptors. GPCRs mediate intracellular activation of heterotrimeric G proteins in response to extracellular ligand binding. A plethora of different factors are known to act on GPCRs, including peptide ligands, proteases, nucleotides as well as bioactive lipid molecules such as lysophosphatidic acid (LPA). LPA induces various biological responses including proliferation and migration in a wide range of mammalian cell types and has been implicated in the progression of several human cancers (1, 2). Upon LPA binding to its cognate receptors, heterotrimeric G proteins from the Gα, Gβ, and G12/13 families are activated by guanine nucleotide exchange factors resulting in their dissociation into activated Gα and Gβγ subunits. Activated G protein subunits interact with various effector proteins including phospholipase C and adenylate cyclase isozymes as well as guanine nucleotide exchange factors for Rho family GTPases, which either directly or via second messenger production communicate to cellular kinase signaling. GPCR activation by LPA is also known to trigger the proteolytic activity of ADAM transmembrane metalloproteases, such as ADAM17, which processes epidermal growth factor receptor (EGFR) ligand precursors on the extracellular side to release mature growth factors triggering EGFR activation (3–8). The molecular mechanisms involved in the control of ADAM metalloprotease activity are not clear yet. The resulting trans-activation of the EGFR tyrosine kinase provides a link to

1 The abbreviations used are: GPCR, G protein-coupled receptor; ADAM17, a disintegrin and metalloproteinase domain-containing protein 17; BisTris, bis[2-hydroxyethyl]aminotris(hydroxymethyl)methane; DHB, dihydroxybenzoic acid; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; FDR, false discovery rate; GO, gene ontology; LPA, lysophosphatidic acid; NHE1, natrium/hydrogen exchanger 1; RTK, receptor tyrosine kinase; SILAC, stable isotope labeling by amino acids in cell culture; SOTA, self-organizing tree algorithm; WGA, wheat germ agglutinin; ACW, acetonitrile.
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signaling modules such as mitogen-activated protein kinase cascades and has been implicated in the control of cell proliferation and migration upon LPA treatment (9). Regarding the induction of cell motility upon LPA, previous studies have reported several signaling elements in addition to EGFR transactivation that contribute to this complex cellular behavior. In particular, RhoGTPase-dependent signals that activate downstream effectors such as Rho kinase and focal adhesion kinase are involved in the control of cytoskeletal organization and cell attachment to the surrounding extracellular matrix (ECM) (10, 11). The coordinated regulation of such integrin-mediated interactions is required to enable cell movement and occurs in dedicated macromolecular assemblies such as focal adhesion complexes and hemidesmosomes (12, 13). Despite the key role of integrins, the molecular mechanisms that underlie their functional modulation upon GPCR activation are poorly understood. Moreover, cell-cell contacts such as adherens junctions and desmosomes have to dissociate prior to cell migration. Likewise, it is unclear how the components of these structures, such as members of the cadherin family, might be regulated by GPCR-mediated signaling pathways. Both LPA levels and LPA$_{1-3}$ receptor expression are often elevated in cancer patients, and the bioactive lipid acts as a potent inducer of cell migration and invasion in vitro. Due to the key role of protein phosphorylation in LPA-induced signal transmission, comprehensive phosphorylation analysis of regulated proteins might generate new insights into pro-migratory signaling mechanisms in cancer cells. Mass spectrometry (MS)-based analysis has emerged as the key method for unbiased protein phosphorylation studies due to various technological advances in recent years (14, 15). Because of the substoichiometric nature of many site-specific phosphorylation events, phosphopeptides constitute only a small fraction in total peptide samples. Therefore, they need to be efficiently enriched prior to MS analysis, which has become routinely possible by phosphate group-selective purification strategies employing capture reagents such as immobilized metal ion affinity chromatography or titanium dioxide beads (16). Moreover, phosphopeptide analysis has benefited enormously from the availability of hybrid mass spectrometers that combine the sensitivity and speed of linear ion traps with the high resolution and accuracy of orbitrap mass analyzers (17). These advances together with quantitative approaches such as stable isotope labeling by amino acids in cell cultures (SILAC) (18, 19) and substantial progress in computational proteomics (20) now allow for concomitant identification and quantification of several thousand phosphorylation sites from single cellular extracts (21–23).

We previously analyzed cell signaling responses in A498 kidney carcinoma cells upon LPA and heparin-binding EGF-like growth factor treatment by monitoring phosphorylation changes in total cell lysate and protein kinase-enriched fractions (24). In a complementary approach, we now aimed for a systematic survey of LPA-induced phosphorylation changes on plasma membrane proteins and their interaction partners. Furthermore, we were interested in time-resolved analysis of LPA-induced phosphorylation changes on ADAM17 and the EGFR to gain further insights into possible mechanisms underlying the still enigmatic EGFR transactivation process. In our present study, we therefore analyzed SCC-9 squamous carcinoma cells due to their pronounced EGFR transactivation response upon LPA. As plasma membrane proteins usually contain covalently attached carbohydrate structures, we performed lectin affinity enrichment of glycosylated proteins prior to SILAC-based quantitative phosphoproteomics (25). This experimental strategy enabled us to acquire in-depth data about LPA regulation of diverse glycoproteins and revealed coordinated phosphoregulation of cell adhesion proteins as likely mechanism underlying cell migratory behavior.

**EXPERIMENTAL PROCEDURES**

**Antibodies—** Antibodies against Wnk1, EGFR, and the EGFR Tyr(P)-1173 were obtained from Cell Signaling Technology; tubulin-specific antibody was from Sigma. Anti-phosphotyrosine-specific 4G10 antibody was prepared as described (26). Endogenous EGFR was precipitated with mouse monoclonal antibody 108.1 (27).

**Cell Culture and LPA Stimulation—** SCC-9 cells were cultured in a 1:1 mixture of F12 Ham’s (Invitrogen) and Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with l-glutamine (2 mM; PAA Laboratories), sodium pyruvate (1 mM; Invitrogen), hydrogencortisone (0.4 mg/liter; Sigma), 1% penicillin/streptomycin (PAA Laboratories), and 10% fetal bovine serum (FBS; Invitrogen). Cell culture conditions were 37 °C and 7% CO$_2$ in a humidified atmosphere. For SILAC experiments the cells were cultured in medium containing either unlabeled l-arginine (Arg-0) at 42 mg/liter and l-lysine (Lys-0) at 71 mg/liter or equimolar amounts of the isotopic variants l-[U-$13C_6,15N_4$]arginine and l-[U-$^{12}$H$_4$]lysine (Arg-6, Lys-4), or l-[U-$^{15}$C$_6$,$^{15}$N$_4$]arginine and l-[U-$^{13}$C$_6$,$^{15}$N$_2$]lysine (Arg-10, Lys-8) (Cambridge Isotope Laboratories or Sigma) for at least six cell doublings. Prior to stimulation with 10 μM LPA (Sigma) for 1.5 and 5 min, cells were serum-starved for 48 h with an additional exchange of medium without FBS 16 h before lysis.

**Cell Lysis and Lectin Affinity Pulldowns—** Cells were grown on 20-cm dishes for each labeling condition. After LPA stimulation, cells were lysed for 15 min with 300 μl of ice-cold lysis buffer/dish (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mM sodium fluoride, 2.5 mM sodium orthovanadate, 50 mg/ml calyculin A, 0.5 μM bathymatin, 1:100 phosphatase inhibitor cocktails 1 and 2 from Sigma). Protein concentrations were determined with the BCA protein assay (Pierce). Equal protein amounts of the differentially SILAC-encoded lysates were adjusted to the same volume and then subjected to preclearing with Sepharose CL-4B beads (Sigma) for 2.5 h at 4 °C on a rotating wheel. Precleared lysates were used for affinity purification with lectin beads. Therefore, concanavalin A, wheat germ agglutinin (WGA) and lentil agglutose beads (all from Sigma) were mixed at a ratio of 1:1:2 and then washed with lysis buffer and metal ion-containing buffer (1 mM NaCl, 5 mM MgCl$_2$, 5 mM MnCl$_2$, 5 mM CaCl$_2$). For each labeled lysate, six affinity pulldowns were carried out in 1.5-ml Eppendorf tubes, with each containing 4 mg (4.7 mg) of...
lysat incubated with 100 μl of mixed lectin beads in the first (second) biological replicate experiment on a rotating wheel at 4 °C overnight. Afterward, lectin beads were washed once with lysis buffer and twice with detergent-free buffer. Bound proteins were eluted with LDS buffer (Invitrogen) for 10 min at 70 °C. Subsequently, all elution fractions from all incubations with the different SILAC lysates were combined.

In-gel Digestion and Phosphopeptide Enrichment—In each biological replicate experiment 80% of the combined eluate was separated by electrophoresis on a 10% NuPAGE Novex BisTris gel (Invitrogen) followed by cutting into 16 slices that covered the resolved molecular weight range. Subsequent in-gel digestion was performed as described elsewhere (28). 10% of the peptides extracted from each slice was desalted on home-made C_{18} StageTips (29). 90% of the extracted peptides from two neighboring slices were combined, and the resulting eight samples were subjected to phosphopeptide enrichment with TiO_{2} beads (GL Sciences) (30, 31). For phosphopeptide purification, 5 mg of TiO_{2} beads were washed once with elution buffer (NH_{4}H_{2}O water in 20% ACN, pH 10.5) and equilibrated with washing buffer (50% ACN, 0.1% trifluoroacetic acid). TiO_{2} beads were loaded with dihydroxybenzoic acid (DHB) by incubation with loading buffer (5 g/liter DHB in 15% ACN). Samples were adjusted to 5 g/liter DHB and incubated with 5 mg of TiO_{2} beads for 45 min at room temperature on a rotating wheel. Beads were then washed four times with washing buffer, and bound phosphopeptides were eluted twice with 60 μl of elution buffer for 10 min at 25 °C. Eluates were cleared by filtration through C_{18} StageTips. To release peptides that might have been retained by C_{18} StageTips, filters were washed further with 30 μl of 80% ACN, 0.5% acetic acid, and the resulting flow-through fraction was combined with the filtered sample. ACN was removed by vacuum concentration, samples were then mixed with an equal volume of 5% ACN, 0.1% trifluoroacetic acid and analyzed by LC-MS.

In-solution Digestion—20% of the combined eluate obtained from lectin affinity purification was precipitated according to the method described by Wessel and Flügge (32). Precipitated protein was resolved in urea buffer (7 M urea, 2 M thiourea, 50 mM Hepes, pH 7.5, 1% n-octyl glucoside), reduced, alkylated, and sequentially digested with Lys-C (Wako) and modified trypsin (sequencing grade; Promega) at enzyme/substrate ratios of 1:100 as described (21). Subsequently, phosphopeptide enrichment was performed by three consecutive incubations with 2.5 mg of TiO_{2} beads with the previous supernatant fractions contacted with fresh beads in each round of incubation.

Mass Spectrometric Analysis—Peptide samples were analyzed by online C_{18} reversed-phase nanoscale LC-MS. Using an Agilent 1100 nanoflow system (Agilent Technologies), samples were injected onto a 15-cm reversed-phase, fused silica capillary column (75-μm inner diameter, packed in-house with 3-μm ReproSil-AQ Pür C_{18} beads; Dr. Maisch GmbH). The nano-HPLC system was connected to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) via a nano-electrospray ion source (Proxeon Biosystems). Loaded peptides were separated in 140-min runs with a gradient from 5 to 40% ACN in 0.5% acetic acid. Xcalibur software 2.0 was used in the positive ion mode for data-dependent acquisition on the LTQ-Orbitrap as described (33). The instrument was recalibrated in real time by co-injection of an internal standard from ambient air into the C-trap ("lock mass option") (17). Full scan MS spectra were recorded in the Orbitrap mass analyzer with resolution r = 60,000 at m/z = 400 after accumulation to a target value of 1,000,000 charges in the linear ion trap. In parallel, up to five of the most intense multiply charged ions per cycle were isolated and fragmented in the LTQ part of the instrument. Multistage activation was enabled to activate phosphopeptide-derived neutral loss species at 97.97, 48.99, or 32.66 m/z below the precursor ion for 30 ms during fragmentation (pseudo-MS^{3}) (34). All raw files acquired in this study were uploaded to the Tranche file-sharing database (ProteomeCommons.org, hash: 7d39WtqA32Q1Io9x9oDuBTv4A6-bi6sfH4AEZ0lWYysSFQ7oUyBmk1m6ibWWh9zOLq0JDFJSpysqT-J1CTrinRrkr8AAAAAAbkQ= —).
control siRNA #1 (Ambion), respectively. The transfection was carried out with the transfection reagent Lipofectamine™ 2000 (Invitrogen) and performed as recommended by the manufacturer. Briefly, 600 pmol of siRNA in a volume of 30 μl and 30 μl of transfection reagent were incubated separately with 720 μl of OptiMEM (Invitrogen) for 5 min at 25 °C, then combined and incubated for a further 30 min at 25 °C. Afterward, the transfection solution was added to the cells to a final volume of 6 ml of OptiMEM. Cells were washed with PBS after 4 h, and medium was replaced by normal F12 Ham’s/DMEM for SCC-9 cell culture. After 24 h, cells were trypsinized and seeded into 12 wells at either 100% confluence for migration scratch assays or at 25% confluence for proliferation assays. On the following day, cells were starved with medium lacking FBS for another 24 h. Scratches were done with 200-μl pipette tips, and cells were then washed four times with PBS to remove loose or dead cells. Cells were then incubated with starvation medium either with or without 10 μM LPA. Pictures were taken immediately after scratching and 22 h later. The distances between the boundaries of the scratches were measured with Adobe Photoshop CS, version 8.0.1. To check for proliferation in parallel to the scratch assay, replicate dishes of transfected cells were counted at the time of the scratch or treated with LPA for 22 h as in the scratch assay and then counted. At the same time the knockdown efficiency for Wnk1 was monitored by immunoblot analysis. Moreover, LPA-induced tyrosine phosphorylation of EGFR was analyzed in Wnk1 knock-down and control-transfected cells that were transfected as described above and serum-starved as in the preparative SILAC experiments.

Bioinformatics Analysis—Analyses for overrepresented gene ontology (GO) annotation terms were performed with the functional annotation tool DAVID (36, 37). IPI identifiers were converted to Ensemble accession numbers prior to submitting the resulting gene lists for foreground and background data to DAVID. Specifically, proteins with reproducibly and significantly regulated phosphopeptides upon LPA treatment were compared with all quantified phosphoproteins for the identification of significantly overrepresented GO biological process terms, as defined by a maximal DAVID EASE score of 0.05 for categories represented by at least two proteins.

For the extraction of phosphorylation site motifs, all reproducibly and significantly LPA-induced class I serine and threonine phosphorylation sites were combined and analyzed with the Motif-X algorithm (38). Sequences were uploaded with the phosphorylated residue at the central position and six amino acids on each side. The background dataset consisted of all remaining phosphorylated serines and threonines with confident site assignments. The minimum number of motif occurrences was set to 5, with a p < 10^{-4} required to be rated as significant.

Time-dependent phosphorylation profiles of reproducibly and significantly regulated class I phosphosites were clustered with the self-organizing tree algorithm (SOTA) of the MeV software (version 4.1) (39). The average ratios of regulated phosphosites from experiments 1 and 2 were calculated before clustering, which was done using Euclidean distance as matrix with a maximal number of four cycles. Apart from that, default settings were used. The list of regulated phosphosites contained the ratios of the 1.5 and 5-min LPA time points and a ratio set to 1 for the time point 0 min in unstimulated cells. Prior to clustering, the MeV software performed log_{10} transformation and normalization of all values.

All quantified proteins harboring phosphopeptides were uploaded to the STRING protein-protein interaction database, version 8.2 (40). The interaction network was generated exclusively on the basis of experimental and database knowledge. Only interactions of high confidence (score ≥0.7) were considered, and the resulting network was visualized with Cytoscape, version 2.7.0.

RESULTS AND DISCUSSION

Experimental Strategy for Quantitative Glycoproteome Analysis—Proteins located at the plasma membrane fulfill many functions that range from the initial recognition of environmental signals to the final execution of cell signaling information to modulate interactions with surrounding cells or extracellular matrix components. To analyze the phospho-regulation of plasma membrane proteins in response to the serum-derived growth factor LPA, we devised a proteomics approach based on glycoprotein capture and SILAC quantification (Fig. 1A and supplemental Table 1). We metabolically labeled SCC-9 cell populations with either normal arginine and lysine (Arg-0/Lys-0) or combinations of heavier isotopic variants of the two amino acids (Arg-6/Lys-4 and Arg-10/Lys-8) until full incorporation into SCC-9 cell proteomes was achieved. Subsequently, the differentially labeled cell populations were serum-starved for 48 h to reduce intracellular signaling to a basal level prior to short term LPA incubations. In a first SILAC experiment, we left Arg-0/Lys-0-labeled cells untreated, whereas the Arg-6/Lys-4- and Arg-10/Lys-8-encoded SCC-9 cells were stimulated for 1.5 and 5 min with LPA, respectively (Fig. 1A). We then prepared total cell lysates from the differently treated cell populations. To enrich for glycosylated proteins such as plasma membrane proteins, cell lysates were subjected to parallel in vitro associations with lectin affinity resins. For high coverage of the cell surface glycoproteome, we combined three lectin resins (lentil, WGA, and concanavalin A-agarose) with different affinities for sugar modifications (41). The elution fractions from the three lectin pulldowns were pooled prior to further MS sample preparation. We resolved 80% of the lectin-purified material by gel electrophoresis and then performed trypsin digestions in gel slices followed by phosphopeptide affinity enrichment with TiO_{2} beads. In addition, we prepared total peptide samples from small aliquots of these in-gel digests for protein identification and quantification. The remaining 20% of lectin-enriched material was digested in-solution with trypsin. We subjected the resulting peptide sample to three consecutive enrichments with TiO_{2} microspheres, in which the initial phosphopeptide amount was in excess of the binding capacity of the affinity reagent. In this fractionation strategy, phosphopeptides with high affinity are preferentially bound in the first incubation step, whereas those with lower affinity for TiO_{2} remain in the supernatant and are retained in the subsequent purifications with fresh beads. In our study, we were interested in the protein phosphorylation events in the glycoproteome and not in the glycosylation sites themselves. Therefore, we lectin-purified intact proteins instead of proteolytically derived glycopeptides as typically done in most previous glycoproteome studies (42–45).

Overall, our fractionation strategy resulted in 27 peptide samples that were measured by high resolution LC-MS on a linear ion trap/orbitrap hybrid mass spectrometer (LTQ-Orbi-
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A

Exp. 1: 20 dishes
          ArgO / Lys0
          Arg6 / Lys4
          Arg10 / Lys8
          / 1.5 min LPA
          1.5 min LPA 5 min LPA
          Lysate Lysate Lysate
          Pull down with lectins
          Pooled eluates
          20% 80%
          In-solution digestion
          Eluate TiO₂
          TiO₂ Supernatant
          Eluate TiO₂
          TiO₂ Supernatant
          Eluate
          LC-MS
          Identification & quantification
          Analysis & bioinformatics

B

Exp. 1
Unstimulated (0 min)
1.5 min LPA (644)
5 min LPA (1085)
IP: α-EGFR
Blot-Ab: α-Tyr(P)
170 kDa
Blot-Ab: α-EGFR

Exp. 2
1.5 min LPA (644)
5 min LPA (1085)
Unstimulated (0 min)
IP: α-EGFR
Blot-Ab: α-Tyr(P)
170 kDa
Blot-Ab: α-EGFR

C

Exp. 1, EGFR, GSTAENAEpYL
5 min LPA
Unstimulated
Relative abundance
1.5 min LPA
645.77
648.78
650.76
644 646 648 650 652 m/z

Exp. 2, EGFR, GSTAENAEpYL
5 min LPA
Unstimulated
Relative abundance
1.5 min LPA
645.77
648.78
650.76
644 646 648 650 652 m/z

D

Exp. 1, EGFR, ESDGDVIISGNK
Unstimulated 1.5 min LPA 5 min LPA
673.84 675.85 677.85
674 676 678 680 m/z

Exp. 2, EGFR, ESDGDVIISGNK
1.5 min LPA 5 min LPA Unstimulated
673.85 675.85 677.85
674 676 678 680 m/z
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Quantitative phosphoproteomics approach to identify phosphorylation changes in lectin-purified proteins upon LPA stimulation. A, experimental workflow showing SILAC labeling and stimulation schemes in biological replicate experiments and the protein enrichment and MS sample preparation strategy ($\text{TiO}_2$, phosphopeptide purification with $\text{TiO}_2$ beads; $C_{18}$, peptide desalting by $C_{18}$ StageTips) followed by MS data acquisition and analysis. The numbers of arrows indicate how many samples were analyzed by LC-MS per biological replicate analysis. B, SILAC-encoded and serum-starved SCC-9 cells stimulated with LPA before lysis as indicated. EGFR was immunoprecipitated and analyzed by immunoblotting with anti-phosphotyrosine and EGFR-specific antibodies. C, MS spectra of the unmodified EGFR peptide EISDGDVIISGNK from SILAC experiments 1 and 2. D, MS spectra of the EGFR phosphopeptide harboring LPA-induced Tyr(P)-1197 from SILAC experiments 1 and 2.

Overall, our analysis of lectin-enriched SCC-9 cell fractions resulted in the identification and quantification of more than 1,000 distinct proteins (supplemental Tables 4 and 5) and nearly 1,700 distinct phosphopeptides (supplemental Tables 5 and 6). In both cases these represented highly confident assignments due to data filtering for protein and peptide FDRs of less than 1%. Moreover, we could pinpoint more than 1,600 distinct site-specific phosphorylation events on quantified peptides with localization probabilities of at least 0.75 by post-translational modification scoring (class I sites, supplemental Table 7). Compared with our previous work on LPA signaling, 1,135 of the 1,685 class I sites reported in our present study represented new ones. Only 550 phosphosites were shared between the two datasets (24). This indicated that the glycoproteome fractionation approach of this study provided highly complementary results and thereby considerably expanded our knowledge about LPA signaling in cancer cell lines. In our present study, the overlap between the two biological replicate analyses was 67% on the protein, 51% on the phosphopeptide, and 45% on the phosphorylation site level (Fig. 2A). Serine, threonine, and tyrosine modifications accounted for 84.1%, 13.5%, and 2.4% of all quantified phosphorylation sites, respectively.

We first analyzed to which extent LPA affected protein abundance in lectin-purified fractions. To identify significant changes over inherent interexperimental variability, we divided protein ratios determined in the first by those measured in the second experiment and then analyzed the distribution characteristics of the resulting ratios-of-ratios (supplemental Fig. 2A). Threshold values of significant regulation were set to 2.5 of the ratios-of-ratios distribution, and this criterion had to be met in both replicate analyses for at least one LPA time point to identify a protein as regulated. Of 677 proteins quantified in both experiments, only glycogen phosphorylase and calpain-2 catalytic subunit precursor exhibited significant and reproducible increase in abundance in lectin-purified fractions. Interestingly, calpain-2 activity is known to be required for deadhesion processes in cell migration (46). Our finding might reflect cellular relocalization of this protease. Moreover, six proteins were found in reduced levels upon LPA treatment (supplemental Fig. 2B). As protein expression changes are unlikely to occur within the time frame of our experiments, observed protein differences might be due to, for example, LPA-regulated protein-protein interactions or protein distribution to lysis buffer-insoluble compartments. However, as only about 1% of all reproducibly quantified changes are unlikely to occur within the time frame of our experiments, observed protein differences might be due to, for example, LPA-regulated protein-protein interactions or protein distribution to lysis buffer-insoluble compartments.
proteins changed significantly, we conclude that LPA stimulation did not affect protein abundance for the vast majority of lectin-enriched proteins.

For comprehensive analysis of phosphoregulation, we quantified LPA-mediated phosphorylation changes both on the level of phosphopeptides and confidently assigned class I phosphorylation sites. Phosphopeptides were specified by the amino acid sequence and number of phosphate groups, and phosphosite ratios were computed by MaxQuant based on all quantified phosphopeptides bearing an identified site. As LPA treatment affected only two phosphoproteins in their abundance in lectin-enriched fractions, measured phosphopeptide and phosphosite ratios were highly similar to the corresponding ratios normalized for protein abundance (measured by quantification of non-phosphorylated peptide species from phosphoproteins; supplemental Fig. 3) (33). We therefore considered phosphopeptide and phosphosite ratios without normalization for further analysis as this strategy ensured consistent analysis of phosphorylation changes irrespective of whether protein abundance ratios were measured. However, in all cases where normalization for protein abundance was pos-

Fig. 2. Overview of quantification results and comparison of replicate SILAC experiments. A, comparison of protein groups, phosphopeptides, and class I phosphosites quantified in two biological replicate analyses. B, comparison of log₂-transformed LPA versus control phosphopeptide ratios from experiments 1 and 2 for 1.5- and 5-min treatment. The squares indicate significant and reproducible up- and down-regulation, corresponding to ratios of greater than 1.77 and 1.65 or ratios of less than 0.57 and 0.61 for the 1.5- and 5-min time points, respectively. C, numbers of repeatedly quantified and reproducibly regulated phosphopeptides and phosphosites.
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A

| Pathway                                                                 | Fold Enrichment |
|------------------------------------------------------------------------|-----------------|
| Regulation of ion transport                                           | 0.57 (0.047)    |
| Regulation of metal ion transport                                     | 0.57 (0.047)    |
| G protein-coupled receptor protein signaling pathway                   | 3.01 (0.002)    |
| Positive regulation of protein kinase cascade                         | 2.76 (0.035)    |
| Positive regulation of transport                                      | 2.57 (0.026)    |
| Positive regulation of cell proliferation                             | 2.53 (0.002)    |
| Regulation of cell migration                                           | 2.26 (0.033)    |
| Positive regulation of cell communication                             | 2.25 (0.008)    |
| Positive regulation of signal transduction                            | 2.16 (0.017)    |
| Cell surface receptor linked signal transduction                      | 1.98 (2.15E-05) |
| Enzyme linked receptor protein signaling pathway                      | 1.92 (0.021)    |

B

Network diagram showing relationships between different pathways such as nuclear transport, cell-cell adhesion, ion transport, cell-matrix adhesion, plasma membrane receptors, and RNA processing.
sible, these ratio values are provided in the supplemental Tables 6 and 7. To assess the reproducibility of these datasets, we compared phosphopeptide and phosphosite ratios that were quantified in both biological replicate analyses. As visualized in x-y scatter plot comparisons of the two independent SILAC experiments, we observed a high concordance of measured ratios for both the 1.5- and 5-min LPA stimulation times (Fig. 2B).

To define thresholds for significantly regulated peptides, we used the same strategy as described for protein quantification and analyzed the ratios-of-ratios distribution of peptides quantified in both replicate experiments. The $\log_2$-transformed values followed a Gaussian distribution with the mean close to 0 and standard deviations ($\sigma$) of around 0.3 for both time points in the two experiments (supplemental Fig. 4). We considered changes of more than 2.5 $\sigma$ as significant, corresponding to ratios of more than 1.77 and 1.65 or ratios of less than 0.57 and 0.61 for the 1.5-min and 5-min LPA time points, respectively. Additionally, we required significantly regulated phosphopeptide or phosphosite ratios to be consistently above or below these values in both experiments (Fig. 2B and supplemental Fig. 5). According to these stringent criteria, about one-fifth of the more than 700 repeatedly quantified phosphopeptides and phosphosites were reproducibly regulated after LPA treatment at one or both time points (Fig. 2C). Notably, the number of regulated sites and peptides only slightly increased from the 1.5-min to the 5-min time point of LPA stimulation, indicating that rapid induction of phosphorylation changes that were found in the lectin-purified subproteome. We detected up-regulation in case of more than 70% of the LPA-triggered phosphorylation changes. The remaining regulated phosphopeptides were found in reduced abundance, which suggests a considerable number of rapid dephosphorylation events on the analyzed proteins.

**Bioinformatics Analysis of LPA-induced Phosphoregulation**—We then focused on reproducibly and significantly up-regulated phosphosites found in lectin-enriched proteins to extract phosphorylation motifs reflecting preferential substrate requirements of LPA-activated protein kinases. Due to the small number of regulated phosphorylation events on tyrosine residues, we only considered confidently assigned (class I) serine and threonine sites in the context of their 12 surrounding residues. These up-regulated sites after 1.5- and/or 5-min LPA treatment were analyzed with the Motif-X software against the background of all other localized phosphorylation events (38). We sought to identify combinations of amino acids that are significantly overrepresented in proximity of LPA-induced phosphoaocceptor sites. Besides two motifs with hydrophobic residues in the +1 position, this iterative analysis extracted RXXpS/pT (where X denotes any amino acid) as most prominent motif found in the subset of LPA-stimulated phosphorylation sites (supplemental Fig. 6). This matches a known kinase substrate motif shared by protein kinase A, protein kinase C, and calmodulin-dependent protein kinase (48), with the latter two representing prominent signal transducers upon LPA stimulation (1, 49).

To explore which biological processes in the glycoproteome may be preferably phosphoregulated upon short term LPA treatment, we used the GO annotations to identify overrepresented GO biological process terms for proteins with reproducibly regulated phosphorylation events (Fig. 3A and supplemental Table 8). Compared with all phosphoproteins quantified in both experiments, GO analysis not only revealed the expected categories relating to functions in signal transduction, but we also found significant enrichment for cell migration and ion transport across the plasma membrane, with the latter exhibiting the highest ratio among all overrepresented GO terms. We then investigated the protein interaction properties of all quantified phosphoproteins. We submitted our data to the STRING database to retrieve functional and physical interactions of high confidence (40). Interestingly, STRING analysis revealed that parts of the network were highly enriched for proteins with known plasma membrane localization (Fig. 3B and supplemental Table 9). These phosphoproteins formed subnetworks containing, for example, cell adhesion molecules and plasma membrane receptors together with some associated cytosolic partners. In particular, we found two prominent cell adhesion modules, one composed of hemidesmosomal and the other of adherence junction and desmosomal proteins. In Fig. 3B, we indicated which nodes were found with reproducible phosphorylation changes in both experiments. Additional candidates for LPA regulation due to quantifications in only one of the two experiments are also marked, as well as those proteins that were either unregulated or not consistently regulated according to the aforementioned ratio thresholds. Although more than half of all plasma membrane phosphoproteins were LPA-regulated in one or both experiments, fewer than one-third of all other network components exhibited phosphorylation changes.

**Fig. 3. Bioinformatics analysis of LPA-regulated phosphoproteins.** A, GO analysis of LPA-induced phosphoproteins. Proteins identified with phosphopeptides reproducibly regulated upon LPA were compared with all phosphoproteins quantified in lectin-enriched fractions. For significantly overrepresented GO biological process terms, fold enrichment is shown. The DAVID EASE score, which was required to be $<0.05$, is shown in parentheses for all biological process terms. B, phosphoprotein interaction network based on all quantified phosphoproteins in lectin-enriched fractions. The network was generated by querying the STRING database for high confidence interactions. Proteins detected with reproducible and significant phosphorylation changes in both experiments are highlighted in red, additional candidates for LPA regulation based on phosphopeptide quantification in only one of the two experiments are depicted in orange, and phosphoproteins that were either not regulated or not consistently regulated are shown in blue. Square-shaped nodes indicate proteins localizing to the plasma membrane.
Notably, many of those constituted additional modules that related to functions such as nuclear transport and RNA processing. Although we cannot exclude that their presence is due to physical interactions with highly glycosylated membrane proteins, these cytosolic modules might be enriched due to O-GlcNAc modification of some of their components on serine and/or threonine residues. O-GlcNAc was reported to exhibit weak affinity for the lectin WGA, which was one of the ligands we used for affinity purification (47).

Finally, to categorize the observed phosphorylation changes, we used SOTA to cluster the temporal profiles of reproducibly LPA-regulated class I serine, threonine, and tyrosine sites. Phosphorylation profiles were assigned to five clusters that were representative of the distinct phosphorylation kinetics observed in our study (Fig. 4 and supplemental Table 7). Clusters 1–3 contained up-regulated, whereas clusters 4 and 5 comprised down-regulated phosphorylation sites. Notably, almost half of all regulated phosphorylation sites were rapidly induced and constituted cluster 1 (Fig. 4).

**Evidence of Signaling Cross-talk within the GPCR Super-family**—Interestingly, we found several rapid modifications on non-LPA GPCRs that were assigned to cluster 1. These included phosphorylations on β₂-adrenergic receptor, prostaglandin E₂ receptor, proteinase-activated receptor 2, and nic-
otinic acid receptor 1 (Table I). Moreover, GPR39A exhibited a rapid phosphorylation decrease at one site assigned to cluster 4. Although the functional consequences of these rapid, site-specific phosphorylation changes are not yet known, they point to fairly extensive interreceptor communication among distinct members of the GPCR superfamily.

**Table I**

Selected LPA-stimulated phosphorylation sites

| Protein IPI Position Sequence Ratio 1.5-min LPA/control | Ratio 5-min LPA/control |
|---------------------------------------------------------|------------------------|
| **G protein-coupled receptors**                        |                        |
| β-Adrenergic receptor IPI00465066 246 FHVNLSQVEODG      | 18.57                  |
| Nicotinic acid receptor 1 IPI00157746 326 EPDNRSTSVELLT | 2.11                   |
| Nicotinic acid receptor 1 IPI00157746 328 DNNRSTVELTG   | 3.01                   |
| Prostaglandin E2 receptor EP2 subtype IPI00016314 240 CGPSLGSQRGPGP | 5.90                   |
| Proteinase-activated receptor 2 precursor IPI00296446 373 VKQMOVSLTSKKK | 7.47                   |
| **Cell adhesion molecules**                            |                        |
| Catenin β1 IPI00017292 552 LTQRRTSMMGTQQ                 | 2.46                   |
| Catenin β1 IPI00017292 556 RTSMGGQQQFVE                  | 2.76                   |
| Catenin α1 IPI00182540 349 ERGSLASELDSLRK                | 1.53                   |
| Catenin α1 IPI00182540 352 SLASLDKRGKP                  | 2.89                   |
| CD44 antigen precursor IPI00017292 556 RTSMGGQQQFVE      | 2.76                   |
| Catenin β1 IPI00017292 552 LTQRRTSMMGTQQ                 | 2.46                   |
| Catenin β1 IPI00017292 556 RTSMGGQQQFVE                  | 2.76                   |
| Catenin α1 IPI00182540 349 ERGSLASELDSLRK                | 1.53                   |
| Catenin α1 IPI00182540 352 SLASLDKRGKP                  | 2.89                   |
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| Catenin α1 IPI00182540 349 ERGSLASELDSLRK                | 1.53                   |
| Catenin α1 IPI00182540 352 SLASLDKRGKP                  | 2.89                   |
| CD44 antigen precursor IPI00017292 556 RTSMGGQQQFVE      | 2.76                   |
LPA-triggered Phosphoregulation of the EGFR and ADAM17—Our glycoproteome fraction strategy enabled the sensitive analysis of LPA-induced phosphorylation changes on the EGFR and ADAM17. In SCC-9 cells, this metalloprotease mediates the transactivation process through shedding of the EGFR ligand amphiregulin (5) (Table I and supplemental Fig. 7A). Although metalloproteases have been implicated in the GPCR-mediated EGFR transactivation more than a decade ago, the underlying molecular mechanisms are still enigmatic (4). In this context, phosphorylation of ADAM17 as a candidate mechanism to modulate its catalytic activity is of particular interest. We detected significant and reproducible phosphorylation changes at Ser-785, Thr-735, and Thr-791 of ADAM17. Phosphorylation of Thr-735, which we found induced upon LPA, was shown to positively regulate metalloprotease activity (50). This might be further enhanced by LPA-triggered down-regulation of Thr(P)-791 due to the reported negative role of this site-specific phosphorylation on ADAM17 activity (51). However, both sites were more slowly modulated than EGFR phosphorylation at Tyr-1172 and Tyr-1197 (supplemental Fig. 7B), which are major autoposphorylation sites induced upon extracellular ligand binding (52, 53). This argues against a functional role of the two ADAM17 phosphorylation sites in early EGFR transactivation, albeit these LPA-induced signaling events might prolong the active state of ADAM17. Interestingly, in one of the two biological replicate experiments, we identified and quantified the previously unknown phosphorylation site Thr-761, which was as rapidly LPA-induced as EGFR tyrosine phosphorylation (supplemental Fig. 7, B and C). Although biological replicate quantification was not achieved for Thr(P)-761, an about 4-fold induction after 1.5-min LPA treatment was reproducibly found in three independent quantifications of differently methionine-oxidized peptide species in our second experiment (supplemental Fig. 7D). Due to such a temporal profile, Thr(P)-761 might be involved in rapid ADAM17 metalloprotease activation preceding EGFR ligand shedding in the transactivation process.

We also monitored LPA-induced Ser/Thr phosphorylation sites on the EGFR, with Ser(P)-1166 being highly up-regulated after 1.5-min LPA treatment, and Thr(P)-693 and Ser(P)-1042 exhibiting slower induction (Fig. 4 and supplemental Fig. 7B). Notably, EGFR phosphorylation at Thr-693 is implicated in receptor desensitization, based on studies showing that mutational inactivation of this site results in impaired EGFR endocytosis (54). Collectively, our results about ADAM17 and EGFR phosphoregulation reveal potential mechanisms involved in LPA-triggered EGFR transactivation and desensitization and therefore validate lectin-based prefractonation as a strategy for sensitive analysis of these central signal transducers. Moreover, in addition to the EGFR, LPA-regulated Ser/Thr phosphorylation was found on a variety of other RTKs pointing to interreceptor communication as a rather prominent aspect in early LPA signaling (Fig. 3B and Table I).

Phosphoregulation of Ion Cotransporters and Exchangers—Besides RTKs and GPCRs, ion transporters constitute a major class of integral membrane proteins enriched in lectin-purified SCC-9 fractions. Therefore, they were amenable to sensitive phosphorylation site analysis in our study (supplemental Fig. 1). We found LPA-regulated site-specific phosphorylations on several proteins with ion transmembrane transporter activity (Table I) (55). Upon LPA treatment, the natrium/hydrogen exchanger 1 (NHE1) was regulated on the phosphorylation sites Ser-703 and Ser-796 assigned to clusters 2 and 1, respectively (Fig. 4). Phosphorylation at Ser-703 is mediated by p90 RSKs and known to enhance NHE1 exchange activity upon serum stimulation, which results in proton efflux coupled to the influx of sodium ions (56). Our data recapitulate this regulatory mechanism in response to a specific ligand, the bioactive lipid LPA. As both Ser-703 and Ser-796 reside within a shared RXRSDP motif, the latter residue might also be targeted by members of the RSK family, the described Ser-703-modifying protein kinases. Thus, our analysis revealed LPA-regulated phosphorylation events that might be involved in the molecular control of NHE1 functions, which, in addition to ion transport, include the recruitment of signaling proteins and regulation of cytoskeletal dynamics (57). Moreover, a major determinant of osmotic homeostasis is the potassium/chloride cotransporter KCC3, which was recently described to be activated in hypotonic conditions due to cooperative dephosphorylation of Thr(P)-991 and Thr(P)-1048 (58). We did not detect these two sites in our analysis, but found LPA-induced phosphorylation of Ser-45 in KCC3, an unreported site located in the N-terminal domain of the cotransporter. Although the functional consequences of this LPA-regulated modification and additional ones detected on multidrug resistance-associated protein 1 (ABCC1) and sodium-coupled neutral amino acid transporter 1 (SLC38A1) remain to be investigated, these findings demonstrate the identification of GPCR-regulated phosphorylation events on diverse ion transporters as a basis for further functional studies (Table I).

Regulation of Cell Adhesion Proteins—The bioactive lipid LPA potently induces cell migration in SCC-9 and other cancer cells, which requires the coordinated modulation of integrin-mediated interactions with the ECM. Integrins are composed of α and β subunits to form heterodimeric transmembrane proteins with bidirectional signaling properties, in a way that either intracellular signals can regulate binding to ECM proteins such as fibronectin, laminin, or collagen, or vice versa, ECM proteins act as extracellular ligands to evoke integrin-mediated signals on the cytoplasmic side. Integrins are glycosylated, and we therefore detected as many as 12 different subunits upon lectin-based enrichment from SCC-9 extracts. Notably, we monitored LPA-induced phosphorylation changes on several of these cell adhesion molecules. LPA induced rapid and sustained phosphorylation of Ser-1042 in integrin α3 and Ser-1103 in integrin α6 (Table I). These phos-
Phosphorylation changes were both assigned to cluster 1 (Fig. 4) due to their kinetics and occurred within a shared QPpSXXE motif. Interestingly, phosphorylation of that particular motif was implicated in integrin-mediated regulation of cytoskeletal organization (59). As these two subunits are engaged in heterodimers with distinct functions, namely the α6/β1 and α6/β4 integrins, our data indicate coordinated regulation of different cell adhesion complexes by an LPA-induced kinase targeting a shared substrate motif on the α subunits. Interestingly, the QPpSXXE motif is evolutionary conserved across vertebrate species from frog to man, further highlighting the functional importance of the corresponding serine phosphorylation site (59).

Although both sites were regulated with a similar temporal profile, up-regulation of Ser(P)-1103 on the α6 integrin subunit was more pronounced with a 7- and 10-fold induction measured after 1.5- and 5-min LPA treatment, respectively. This strong increase preceded LPA-induced phosphorylation of Ser-1364 on the associated β4 integrin subunit assigned to cluster 2 (Fig. 4). Notably, phosphorylation of this site is known to disrupt intracellular binding to plectin 1, which connects β4 integrin to keratin intermediate filaments (60). The heterodimeric α6/β4 integrin is a key component of hemidesmosomes, which link cytoskeletal structures in epithelial cells with ECM components of the basement membrane. Thus, our data point to a molecular mechanism by which LPA-induced signals dissolve hemidesmosomes through phosphorylation-dependent dissociation of plectin 1-integrin complexes. In contrast to increased phosphorylation of Ser-1364, we detected rapid LPA-induced down-regulation on a number of sites located in the connective segment of integrin β4. Furthermore, LPA-induced reciprocal regulation was also detected on the aforementioned integrin β4 interactor plectin 1 (Table I). Although the functional roles of these site-specific phosphorylations are presently unclear, our targeted phosphoproteome analysis suggests coordinated phosphoregulation of hemidesmosomal structures as a previously unappreciated element in LPA signaling.

Our analyses also revealed LPA-induced phosphoregulation of many proteins involved in the formation of cell-cell contacts. These included δ-catenin and β-catenin associated with adherence functions as well as desmoglein and plakoglycan.
philins, which are involved in desmosome assembly. Interestingly, the N-terminal domain of δ-catenin contains regulatory sequences implicated in adherens junction disassembly upon dephosphorylation of this protein region (61). Although LPA was reported to reduce the overall phosphorylation level in the N-terminal domain of δ-catenin (62), the corresponding phosphorylation sites have not been mapped in the context of GPCR signaling. We recorded rapid loss of phosphorylation on Ser-252, which was decreased by 4-fold within only 1.5-min LPA treatment and assigned to cluster 4 due its temporal profile (Fig. 4). Moreover, δ-catenin was reciprocally regulated on the phosphorylation level, as evident from two up-regulated phosphorylation sites located at Ser-349 and Ser-352, both of which exhibited fast and sustained induction upon LPA stimulation (Fig. 4, cluster 1). Interestingly, we also observed phosphorylation of the δ-catenin-interacting protein β-catenin on Ser-552 upon LPA treatment (Table I). This β-catenin site is a known Akt kinase substrate site. Its phosphorylation releases β-catenin from the cell-cell contacts, which is a prerequisite for the protein to translocate into the nucleus for transcriptional control (63). Collectively, our identification of concerted phosphorylation/dephosphorylation reactions on functionally relevant sites indicates the interplay of mechanisms by which LPA-triggered signals modulate δ-catenin and β-catenin to destabilize adherence junctions and facilitate cell migration. We not only reproducibly detected significant phosphoregulation on several adherens junction proteins, but also on two components of desmosomes: desmoglein 2 and plakophilin 3. Both adherens junctions and desmosomes form stable extracellular cell-cell contacts, but interact with distinct cytoskeletal structures (actin filaments and cytokeratin filaments, respectively) on the intracellular side of the plasma membrane. LPA treatment resulted in the gradual down-regulation on Thr(P)-922 of desmoglein 2 and the reciprocal regulation of the plakophilin 3 phosphorylation sites Ser-123 and Ser-314. These results identify desmosomes as additional cellular components targeted by LPA-induced signaling, which might control desmosomal architecture and adhesive properties through site-specific phosphoregulation.

LPA-dependent Regulation of Wnk1 and Its Role in Cell Migration—We detected the serine/threonine protein kinase Wnk1 as a prominent phosphoprotein in lectin-enriched fractions. This suggests that Wnk1 either associates with transmembrane glycoproteins or, alternatively, bound to immobilized lectin molecules due to O-linked GlcNAc modification (47). We identified seven phosphorylation sites that were quantified in both replicate experiments and could be confidently assigned to specific residues (Fig. 5A). Four of these sites were significantly up-regulated and exhibited more than 2-fold induction already after 1.5-min LPA treatment (Fig. 5B). These included the functionally characterized site Ser(P)-382, which is a known autophosphorylation site of Wnk1. Ser(P)-382 stabilizes the active kinase state due to its location in the activation loop region of the Wnk1 kinase domain (64, 65). Because of this biochemical evidence for rapid induction of cellular Wnk1 activity, we established a knock-down protocol based on RNA interference with duplex siRNAs to explore Wnk1 function in LPA signaling further. As phosphorylation changes on Wnk1 followed a similar kinetics as EGFR autophosphorylation on tyrosine residues we tested whether Wnk1 acts upstream of the EGFR in the transactivation process. However, despite efficient down-regulation of Wnk1 protein in SCC-9 cells, LPA-induced autophosphorylation of EGFR was rather similar in Wnk1-depleted cells compared with control-transfected cells (supplemental Fig. 8A). We therefore conclude that Wnk1 does not act as an intermediate signal transducer in LPA-evoked EGFR transactivation. As the
induction of cell migration and cell proliferation represent major biological outcomes in LPA-treated SCC-9 cancer cells, we used the RNAi knock-down approach to test for possible Wnk1 functions in these biological processes. To examine the migratory behavior we performed wound closure assays in SCC-9 cells. In accordance with earlier reports, LPA treatment induced a pronounced migratory response in control-transfected SCC-9 cells (66). Notably, RNAi-mediated Wnk1 ablation resulted in strong impairment of LPA-induced migration in SCC-9 cells, which was consistently observed with two distinct Wnk1 siRNAs (Fig. 5C). These data indicate a pro-migratory effect that might be due to LPA-induced up-regulation of Wnk1 signaling activity. In contrast, Wnk1 protein knock-down had only a minor effect on SCC-9 cell proliferation measured in parallel to migration (supplemental Fig. 8B).

Together, our results provide the first evidence for Wnk1 regulation and its functional relevance in GPCR-mediated signaling. In the light of earlier data showing Wnk1 involvement in both cell migration and proliferation in EGF-treated neural progenitor cells (67), it appears that the overall repertoire of Wnk1 functions might differ in a cell context- or growth factor-dependent manner. Moreover, previous studies reported Wnk1 activation upon cell treatments such as osmotic stress as well as membrane depolarization (64, 65, 68–71). Thus, our data further support the notion that rather diverse external stimuli converge on Wnk1 to regulate cellular responses such as ion transport regulation, cell migration, and others.

**CONCLUSIONS**

In our present study, we have used SILAC-based phosphoproteomics and lectin-based affinity enrichment to investigate the glycosylated SCC-9 cell subproteome. Our principal goal was to identify phosphorylation changes on plasma membrane components upon short term LPA treatment. The straightforward affinity purification strategy reported here allowed for sensitive analysis of diverse integral plasma membrane proteins such as GPCRs, RTKs, ion transporters, and cell adhesion molecules (Fig. 6). Regarding the last category, we found regulation for a considerable number of phosphorylation sites with reported functions in cell adhesion and migration but no previously described connection to GPCR signaling. Thus, our results provide links to existing knowledge and thereby illuminate the underlying molecular mechanisms by which LPA signaling controls these biological processes. We found short time LPA treatment to affect three types of adhesion molecule structures—hemidesmosomes, adherens junctions, and desmosomes—for each of which phosphorylation was detected on various protein constituents. Thus, our phosphoproteomics data indicate coordinated control mechanisms that affect multiple components within several subcellular structures to generate a complex biological response. With respect to the numerous LPA-regulated phosphorylation events on cell adhesion molecules, it will be interesting to identify the protein kinases mediating these site-specific modifications and to explore possible roles of Wnk1, which we identified as an LPA-regulated signal transducer involved in cell migration. In addition to LPA-regulated phosphorylation sites with reported functions in different biological situations, our analyses unveil many uncharacterized phosphorylation events, including, for example, LPA-induced phosphorylations on the metalloprotease ADAM17 as well as on various GPCRs. Thus, our data reveal new starting points for mechanistic studies on poorly understood aspects such as LPA-induced shedding of cell surface proteins or possible regulatory mechanisms within expressed GPCR superfamily members. Finally, we expect other applications for the straightforward experimental approach described in this study. Similar experimental strategy should be useful to study cell surface proteins and their dynamic regulation upon a wide range of environmental signals and promote our molecular understanding of membrane-proximal signal processing.

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