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Chemical Proteomic Profiling of Lysophosphatidic Acid-Binding Proteins

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Abstract

Lysophosphatidic acid (LPA) is an endogenous cell signaling molecule, and dysregulation of LPA signaling pathways is accompanied by several types of cancer. Herein, we developed a chemical proteomic method for the proteome-wide identification of LPA-binding proteins. The method involves the synthesis of a desthiobiotin-conjugated LPA acyl phosphate probe for the covalent labeling, enrichment, and subsequent LC-MS/MS identification of LPA-binding proteins at the proteome-wide level. By conducting labeling reactions at two different probe concentrations (10 and 100 μM) in conjunction with an SILAC (stable isotope labeling by amino acids in cell culture)-based workflow, we characterized the LPA-binding capabilities of these proteins at the entire proteome scale, which led to the identification of 86 candidate LPA-binding proteins in HEK293T cells. Moreover, we validated that two of these proteins, annexin A5 and phosphoglycerate kinase 1, can bind directly with LPA. Together, we developed a novel LPA probe for the identification and characterizations of LPA-binding proteins from the entire human proteome. The method should be adaptable for the identification of other lipid-binding proteins.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b04850.

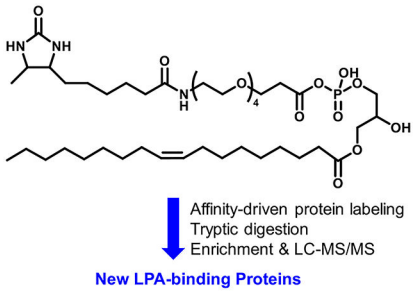
Table S1, identification data for all peptides and proteins from desthiobiotin-PEG4-LPA-affinity probes reacted with cell lysates (XLSX)

Table S2, identification data for all peptides and proteins from desthiobiotin-C3-LPA-affinity probes reacted with cell lysates (XLSX)

Table S3, A list of all quantified proteins under the 100 μM:10 μM LPA-affinity probe labeling conditions and a list of proteins with $R_{LPA10/1}$ ratios being smaller than 3 (XLSX)

Detailed experimental procedures, chemical structures for the desthiobiotin-LPA probes, MS and MS/MS data for the synthesized probes, MS/MS for probe-labeled peptides, and SDS-PAGE for monitoring the purifications of recombinant proteins (PDF)

The authors declare no competing financial interest.



Lysophosphatidic acid (LPA) is a ubiquitous phospholipid that exists in all eukaryotic tissues and plays critical roles in various cellular processes.[1] The impact on these cellular processes is due in part to the heterogeneity of LPA-binding protein subtypes, expression patterns, and effector pathways.[1] LPA can bind to and activate some G protein-coupled receptors (GPCRs); thus, it acts as an extracellular signaling molecule.[2] It was also reported that aberrant LPA signaling is associated with many types of cancer.[3–6] The ability of LPA signaling to influence such a wide array of important pathways renders LPA receptors attractive targets for drug discovery.

To date, six cell-surface LPA receptors (i.e., LPAR1–LPAR6) have been discovered.[7–12] Additional putative LPA receptors, i.e., GPR87 and P2Y10,[13–15] were proposed, though they await further validation. Much, however, remains to be learned about LPA signaling, where the functions of LPA remain incompletely understood, and some of its known functions are not attributable to known LPA receptors.[16,17] Hence, there are likely yet-identified LPA-binding proteins, and identification and quantification of LPA-binding proteins at the entire proteome scale are important for investigating biological events underlying the intricate signaling network comprised of LPA receptors and their downstream effector proteins.

Recent advances in mass spectrometry (MS) have greatly facilitated protein identification and quantification in complex sample matrices;[18] however, proteomic studies of specific families of proteins by MS remain a challenge. Here, we utilize a chemical proteomic method, which has been widely used for studying functional subgroups of proteins,[19,20] to selectively label, identify, and characterize potential LPA-binding proteins in the entire human proteome.

Our experimental design is based on prior knowledge about lipid-binding proteins. In particular, structural studies showed that many lipid-binding proteins frequently carry one or more lysine residues at their ligand-binding sites. For instance, Lys34 in sphingosine 1-phosphate receptor 1 is very close to the sphingolipid-binding pocket,[21] and Lys61 in phosphatidylinositol transfer protein α (PITPα) assumes an essential role in binding with phosphatidylinositol.[22] These lysine residues, similar to those situated in the ATP binding pocket of nucleotide-binding proteins,[23,24] may react with an acyl phosphate group via a nucleophilic substitution reaction to form a covalently modified protein. Hence, we developed an LPA analog, which bears an acyl phosphate moiety to target the lysine residue in the LPA-binding site (Figure 1).

The LPA-affinity probe has three components: LPA, which targets the LPA-binding pocket of cellular proteins; an enrichment moiety, i.e., desthiobiotin, which facilitates downstream purification; a linker (Figure 1). In this vein, we chose to employ desthiobiotin as an enrichment handle because it binds less tightly to avidin agarose than biotin,[25] which facilitates a better recovery of the modified peptides or proteins during the enrichment. When the LPA-affinity probe interacts with LPA-binding proteins, the carbonyl carbon in the acyl phosphate reacts with the lysine residue at the phospholipid-binding pocket to yield a stable amide bond, thereby modifying the proteins with a desthiobiotin tag (Figure 1b).

We synthesized two different probes, with the linker being a γ-aminobutyric acid or polyethylene glycol (PEG_4) (Figure S1). The probes were synthesized following similar procedures as described previously for desthiobiotin-ATP acyl phosphate probes (Supporting Materials and Methods and Figure S2 show the MS and MS/MS characterizations of the probes).[26,27]

After removal of small molecules (including phospholipids) with a NAP-5 size-exclusion column, we incubated the whole-cell protein lysate with the probe, digested the resulting protein mixture with trypsin, enriched desthiobiotin-labeled peptides from the mixture using streptavidin beads, and analyzed the affinity-purified desthiobiotin-conjugated peptides by LC–MS/MS in the data-dependent acquisition mode. Our initial experiment with the use of the desthiobiotin-C3-affinity probe and 1 mg of whole-cell protein lysate led to the identification of a modest number of desthiobiotin-labeled peptides. We reasoned that this might be attributed to the poor aqueous solubility of the probe. Thus, we replaced the C3 linker with a PEG_4 linker to improve the aqueous solubility of the probe.

We next employed the desthiobiotin-PEG_4-LPA probe and further optimized the reaction conditions. In this respect, we incubated 1 mg of protein lysate with 10, 25, 50, 100, or 200 μM of the desthiobiotin-PEG_4-LPA-affinity probe under various binding conditions. We identified the largest number of putative LPA-binding proteins from the whole cell lysate when using 200 μM probe in the presence of Mg^{2+}, Ca^{2+}, and Cu^{2+}. As we reduced the LPA probe concentration to 100 μM, the total number of identified LPA-binding proteins decreased to about one-fifth of the number when compared with the use of 200 μM LPA probe; however, the relatively high probe concentration may lead to diminished specificity, which could result in the identification of proteins arising from nonspecific reactions. Hence, we decided to employ 100 μM probe for the subsequent experiments.

With the use of the desthiobiotin-PEG_4-LPA probe and the protein lysates derived from two different cell lines, i.e., HEK293T and HeLa, we were able to identify 3485 labeled peptides from 939 proteins (Figure S2c,d and Table S1). Figure 2a shows the representative MS/MS for a desthiobiotin-labeled peptide derived from phosphoglycerate kinase 1 (PGK1). In contrast, the use of the desthiobiotin-C3-LPA probe under otherwise identical conditions only led to the identification of 789 unique desthiobiotin-conjugated peptides from 317 distinct proteins (Table S2), again underscoring the better aqueous solubility of the probe introduced by the PEG_4 linker.

This initial analysis, however, did not lead to the identification of any known LPA receptors. We reason that this could be attributed to the relatively low expression levels of LPA receptors in these cell lines. Indeed, upon ectopic expression of LPA receptor 1 (LPAR1) in HEK293T cells, we were able to identify successfully the desthiobiotin-PEG_4-modified peptide from LPAR1 (Figure 2b).

Due to the relatively high reactivity of the acyl phosphate probe, lysine residues not at the LPA-binding sites may also be modified through nonspecific electrostatic interactions. To further investigate the specific LPA-binding proteins at the entire proteome scale, we devised an affinity profiling strategy using a SILAC (stable isotope labeling by amino acids in cell culture)-based workflow (Figure 3). In this vein, as described in previous studies about the proteome-wide discovery of reactive cysteine-containing proteins and nucleotide-binding proteins,[26,28,29] binding of a protein with a specific reactive group of the probe greatly augments the rate for the coupling reaction between the acyl phosphate moiety and the lysine residue at the binding site. Therefore, different probe concentrations will confer distinct labeling behaviors for lysine residues, which reflects the binding specificities between LPA and target proteins.

We performed our SILAC experiment by allowing low (10 μM) and high (100 μM) concentrations of the LPA probe to react respectively with an equal amount of light- and heavy-labeled cell lysates (forward experiment). We subsequently combined the two protein samples, digested the ensuing mixture with trypsin, and enriched desthiobiotin-labeled peptides from the peptide mixture with streptavidin beads. We also performed a reverse labeling experiment to minimize the bias introduced by the labeling process. The affinity-purified desthiobiotin-labeled peptides were again analyzed by LC-MS/MS (Figure 3). We used peak intensity ratios to obtain quantifi cation results of the light- and heavy-desthiobiotin-labeled peptides from the two experimental states to derive the LPA-binding affinity ratio, $R_{\mathrm{LPA10/1}}$, which reflects the relative binding affinities of LPA toward specific lysine residues in individual proteins.

We assumed an $R_{\mathrm{LPA10/1}}$ of ~ 1.0 reflects specific LPA-binding to lysine since, even at a relatively low probe concentration, the lysine residue at the LPA-binding site still possesses hyper-reactivity and is completely labeled. In contrast, lysines not involved with LPA binding will only be partially labeled because the limited amount of labeling reagent reacts preferentially with lysine at the LPA-binding site, which will display a concentration-dependent increase in labeling efficiency, thereby resulting in an $R_{\mathrm{LPA10/1}} \gg 1$. We attempted to include all the putative LPA-binding proteins in our analysis with the use of a lenient criterion: $R_{\mathrm{LPA10/1}} < 3$. With this criterion, we found that, among the more than 400 proteins that were quantified in at least two separate SILAC labeling experiments, 86 are candidate LPA-binding proteins, which included 142 desthiobiotin-modified lysine residues (Figure 4a and Table S3). Figures 4b–e and S3 show the representative MS and MS/MS for the light- and heavy-labeled desthiobiotin-conjugated peptides derived from annexin A5 (ANXA5) and creatine kinase B. On the basis of gene ontology (GO) analysis of these proteins using DAVID,[30,31] we observed many lipid-binding proteins exhibiting $R_{\mathrm{LPA10/1}} < 3$, while most ATP-binding proteins (e.g., creatine kinase B-type) had $R_{\mathrm{LPA10/1}} \gg 1$. This comparison indicates that specific labeling of lysine residues occurred mainly on lipid-

binding proteins and nonspecific labeling occurred more frequently on ATP-binding proteins.

On the basis of GO analysis using DAVID, more than 50 targets predicted to be LPA-binding proteins from our quantitative affinity profiling results were not previously documented as LPA-binding proteins. Among them, ANXA5 and PGK1 exhibited high specificity with $R_{LPA10/1}$ values of 1.93 (based on the average value of five quantified peptides from this protein) and 1.80 (based on the average value of seven quantified peptides), respectively (Table S3). In addition, ANXA5 has been widely used to detect apoptotic cells owing to its high affinity for phosphatidylserine,[32] and it was also reported that ANXA5 can bind to lipopolysaccharides.[33] Therefore, LPA is likely a binding partner of ANXA5.

We next utilized isothermal titration calorimetry (ITC) to assess the direct interactions between LPA and recombinant ANXA5 and PGK1 (Figure S4). Our results showed that PGK1 and ANXA5 bind to LPA with K_d values of 80.6 and 23.4 μM respectively (Figure 5), supporting the direct binding of these two proteins to LPA.

In summary, we report here a strategy using an LPA-affinity chemical probe to enrich and identify putative LPA-binding proteins in the human proteome. We successfully synthesized the desthiobiotin-tagged LPA-affinity probe and used it to react with the whole-cell protein lysate, which followed by tryptic digestion, affinity enrichment of the ensuing desthiobiotin-conjugated peptides, and LC-MS/MS analysis, allowed for the proteome-wide identification of LPA-binding proteins. A total of 939 putative LPA-binding proteins were identified from two different cell lines with the use of ~1 mg of protein lysate. By combining this strategy with SILAC, we were able to characterize LPA–protein interactions at the entire proteome, which resulted in the discovery of 86 proteins exhibiting highly selective interactions with LPA at the entire proteome level. Moreover, we validated that ANXA5 and PGK1 can bind directly with LPA.

There are several advantages of this chemical proteomic approach. First, the method provides facile enrichment, identification, and quantification of LPA-binding proteins. Second, the method allows for the identification of the specific lysine residues involved in binding with LPA, which may provide important knowledge for developing small molecule inhibitors for LPA-binding proteins and/or for designing mutant proteins for interrogating the biological functions of these proteins. A limitation of the current probe is that metabolic labeling is required for the quantitative analysis of LPA-binding proteins. Nevertheless, this can be overcome by incorporating stable isotope-labeled linker into the probe, as described previously for the ATP acyl phosphate probes.[26] Moreover, in light of the fact that many proteins exhibit tissue-specific expression, future work with the use of cell lines derived from different human tissue origins will also expand the coverage of LPA-binding proteins, as described previously for proteome-wide studies of protein kinases and small GTPases.[27,34–36]

It can be envisaged that the method can also be adapted for studying other lipid-binding proteins at the entire proteome scale. In particular, a similar acyl phosphate probe can be

designed for the enrichment and subsequent identification of interaction proteins for those phospholipids carrying a terminal phosphate group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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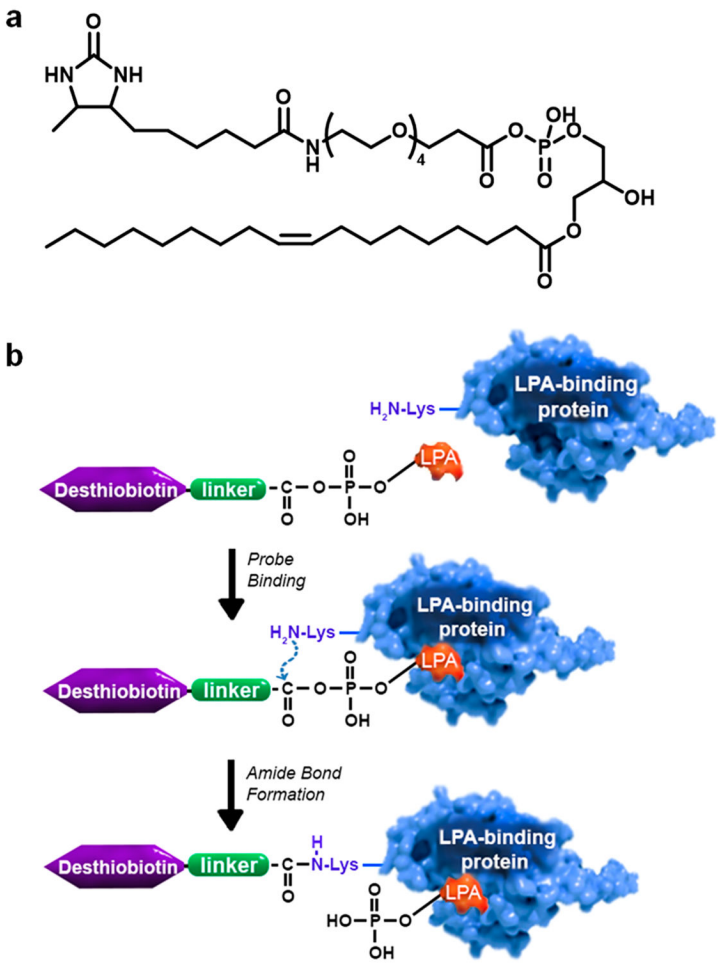

Figure 1.

(a) Chemical structure of the desthiobiotin-PEG$_4$-LPA probe. (b) A schematic diagram showing the reaction between the LPA-affinity probe with an LPA-binding protein.

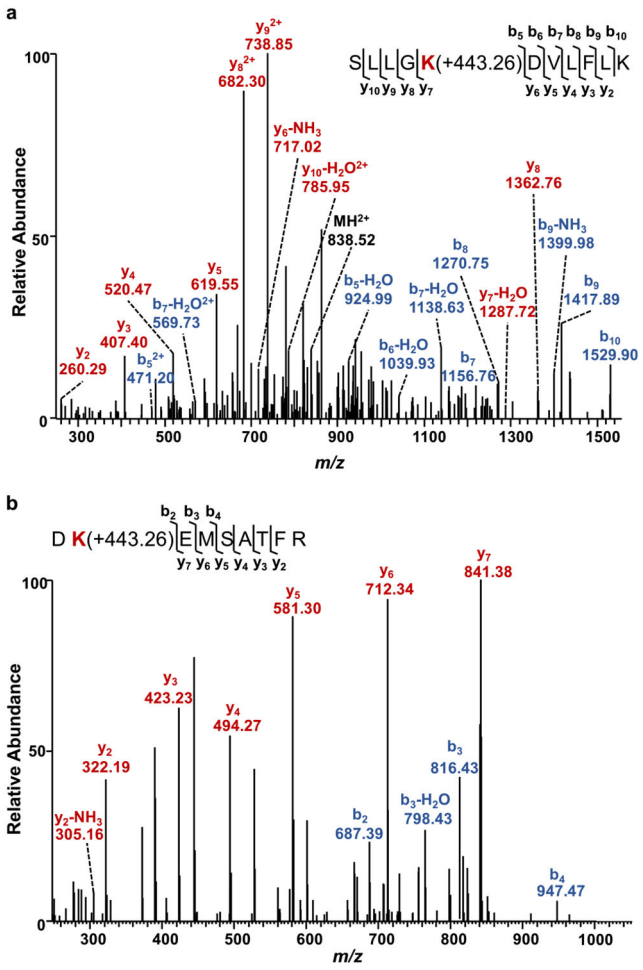

Figure 2.

MS/MS of the $[M + 2H]^{2+}$ ion of the desthiobiotin-labeled peptide of (a) SLLGK#DVLFLK from PGK1 identified from HEK293T cells; (b) DK#EMSATFR from LPAR1. The peptide shown in (b) was identified from HEK293T cells with ectopic expression of LPAR1. “K#” indicates the desthiobiotin-labeled lysine.

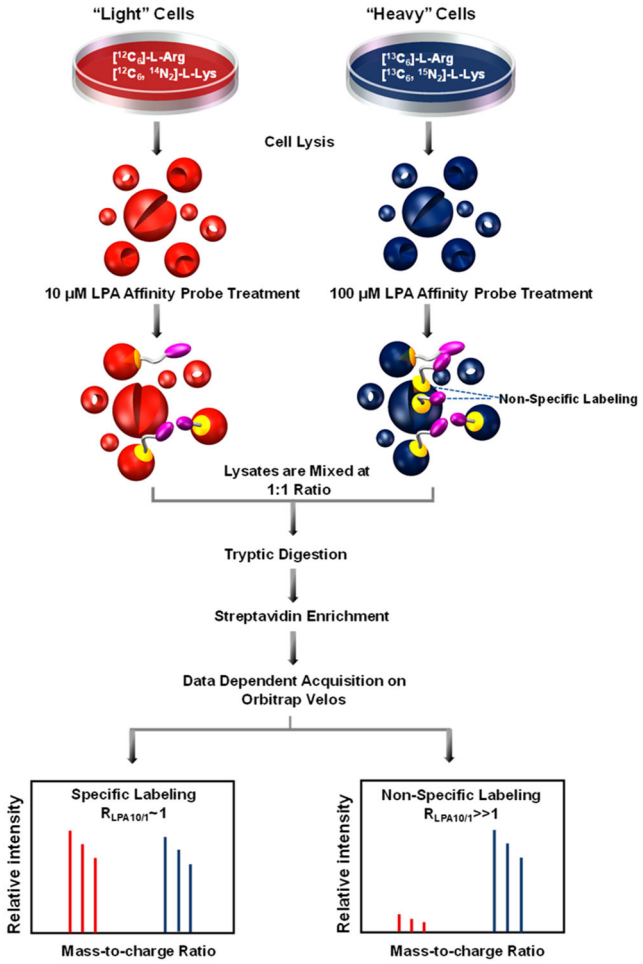

Figure 3.

A SILAC-based strategy for quantitative LPA-affinity profiling of the global proteome.

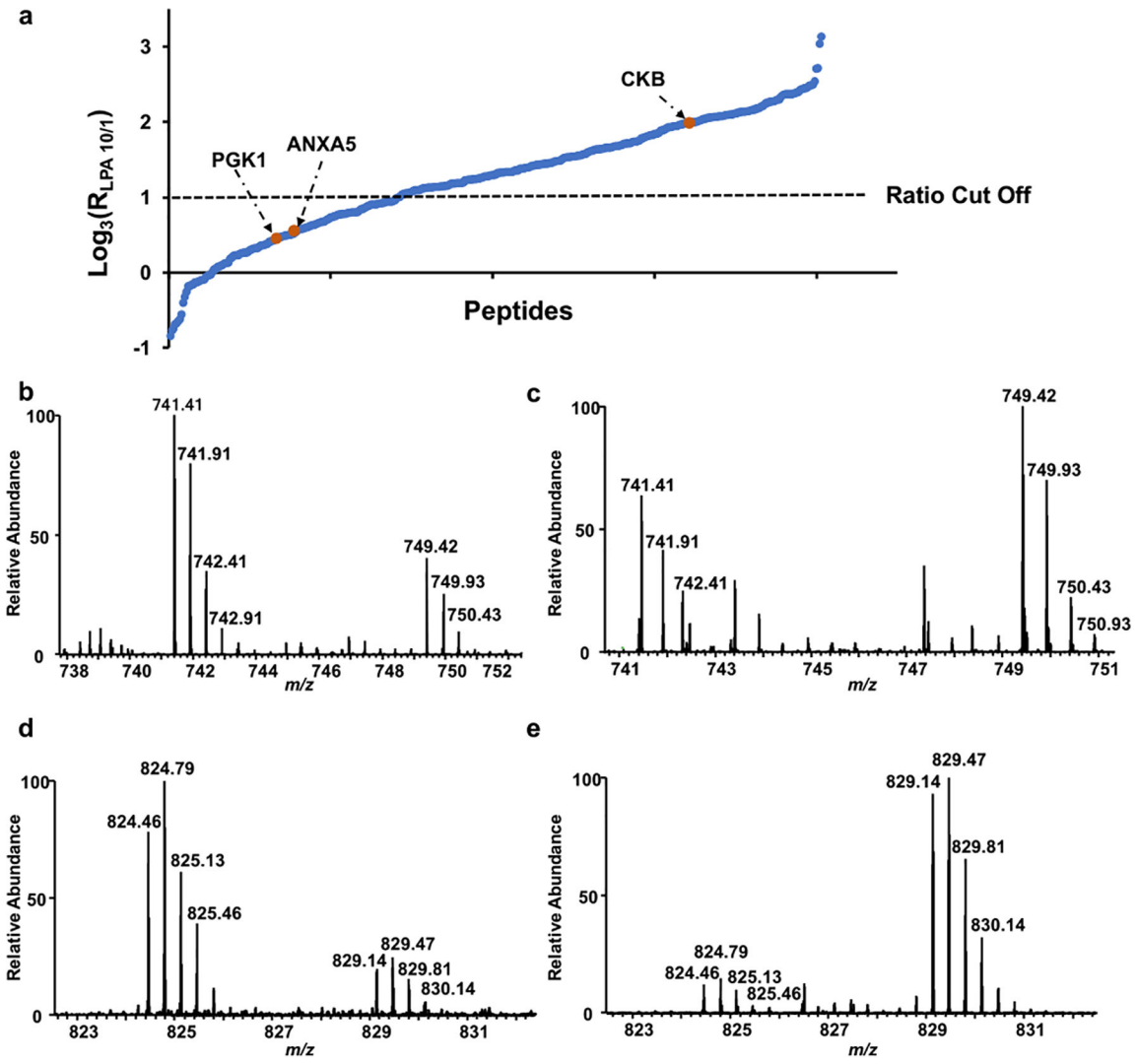

Figure 4.

(a) Measured $R_{LPA10/1}$ ratio of peptides from HEK293T SILAC cell lysates with low (10 μM) and high (100 μM) concentrations of LPA-affinity probe. (b) Light- and heavy-labeled peptides from forward- and reverse-SILAC-based affinity profiling experiments. (b, c) Peptide SELTGK#FEK with a low $R_{LPA10/1}$ ratio from annexin A5. (d, e) Peptide AIEK# LAVEALSSLDGDLAGR with a high $R_{LPA10/1}$ ratio from creatine kinase B-type. "K#" designates the desthiobiotin-PEG_4 labeled lysine.

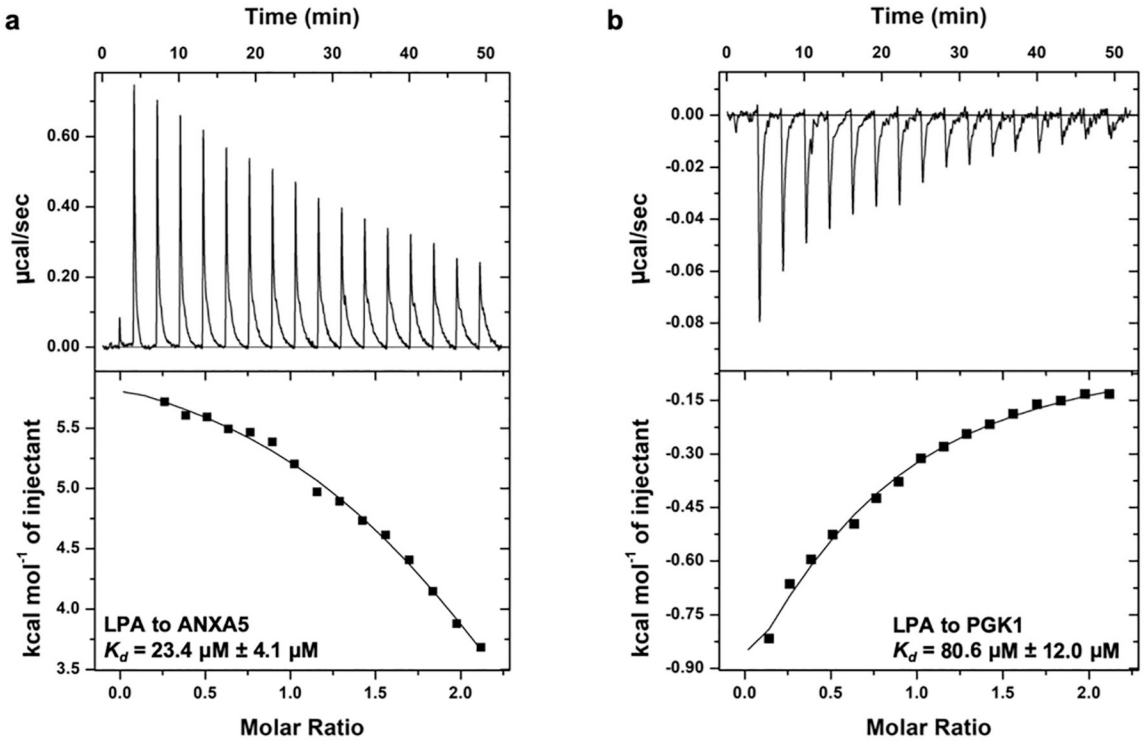

Figure 5.

ITC for assessing the binding affinity between LPA and ANXA5 (a) or PGK1 (b). LPA (1 mM) was titrated over 0.1 mM ANXA5 or PGK1 in a buffer containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5% glycerol at 25 °C.