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Exploring G protein-coupled receptor signaling networks using SILAC-based phosphoproteomics

Grace R. Williams [a], Jennifer R. Bethard [a], Mary N. Berkaw [a], Alexis K. Nagel [a,b], Louis M. Luttrell [c,d], Lauren E. Ball [a,b,*]

[a] Department of Molecular and Cellular Pharmacology, Medical University of South Carolina, Charleston, SC 29425, USA
[b] Department of Oral Health Sciences, Medical University of South Carolina, Charleston, SC 29425, USA
[c] Department of Medicine, Medical University of South Carolina, Charleston, SC 29425, USA
[d] Research Service of the Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29401, USA

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ABSTRACT

The type 1 parathyroid hormone receptor (PTH_1R) is a key regulator of calcium homeostasis and bone turnover. Here, we employed SILAC-based quantitative mass spectrometry and bioinformatic pathways analysis to examine global changes in protein phosphorylation following short-term stimulation of endogenously expressed PTH_1R in osteoblastic cells *in vitro*. Following 5 min exposure to the conventional agonist, PTH(1-34), we detected significant changes in the phosphorylation of 224 distinct proteins. Kinase substrate motif enrichment demonstrated that consensus motifs for PKA and CAMK2 were the most heavily upregulated within the phosphoproteome, while consensus motifs for mitogen-activated protein kinases were strongly downregulated. Signaling pathways analysis identified ERK1/2 and AKT as important nodal kinases in the downstream network and revealed strong regulation of small GTPases involved in cytoskeletal rearrangement, cell motility, and focal adhesion complex signaling. Our data illustrate the utility of quantitative mass spectrometry in measuring dynamic changes in protein phosphorylation following GPCR activation.

1. Introduction

As our appreciation of the "pluridimensionality" of G protein-coupled receptor (GPCR) efficacy [1] has grown, it has become increasingly important to consider ligand action in the context of signaling networks. At physiological levels of expression, individual GPCRs may engage multiple downstream signaling pathways simultaneously, using both heterotrimeric G proteins and non-G protein effectors, e.g. arrestins, as conduits [2,3]. As these initial signals propagate, the potential for crosstalk expands exponentially, rapidly exceeding the capacity of simple pathway-focused approaches, e.g. protein immunoblots, to capture the complexity. Adding to the problem is the knowledge that ligand structure can "bias" the efficiency with which a GPCR activates its downstream effectors relative to the native ligand [4,5]. "Biasing" GPCR signaling by varying ligand structure holds the promise of novel therapeutics, but at the same time introduces the specter of unexpected "on-target" effects arising from activating GPCRs in "unnatural" ways [6]. Yet another concern is the impact of system factors, e.g. cell background-specific variations in receptor density and expression of downstream effectors, which can introduce wide variations in the observed response [5]. Given these challenges, an "ideal" approach to understanding GPCR signal transduction would involve studying GPCRs in a native context using methods capable of both capturing high-dimensionality data and deconvoluting it to generate information about the net effect on biological pathways and processes.

Abbreviations: CID, collision induced dissociation; ETD, electron transfer dissociation; FTMS, Fourier transform mass spectrometry; GO, Gene Ontology; GPCR, G protein-coupled receptor; GSEA, geneset enrichment analysis; HpH RPLC, high pH reversed phase liquid chromatography; IPA, Ingenuity® Systems Pathways Analysis; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MS, mass spectrometry; OGE, off gel electrophoresis; PTH, parathyroid hormone; PTH_1R, type 1 parathyroid hormone receptor; RANKL, receptor activator of nuclear factor kappa-B ligand; SCX, strong cation exchange chromatography; SILAC, stable isotopic labeling by amino acids in cell culture; S/T/Y, serine, threonine, tyrosine; TiO_2, titanium dioxide; TMT, tandem mass tags; Runx, Runt-related transcription factor.

* Corresponding author at: Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 173 Ashley Avenue, BSB 358, MSC 509, Charleston, SC 29425 USA.
E-mail address: balllle@musc.edu (L.E. Ball).

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Quantitative phosphoproteomic analysis provides an avenue for elucidating transient phosphorylation-mediated signaling events following receptor–ligand interactions [7,8]. Dynamic post-translational modifications impact signal transduction by affecting protein interactions, subcellular localization, enzyme activity, and protein half-life. Recent in-depth phosphoproteomic studies indicate that seventy-five percent of proteins are phosphorylated, the majority of which (85%) are multiply phosphorylated [9], and phosphorylation is a key post-translational mediator of cellular signaling necessary for growth, proliferation, motility, differentiation, survival, and the response to hormones and therapeutics. While subject to its own limitations, mass spectrometric approaches provide the capacity to capture a global "snapshot" of stimulus-induced changes in the phosphoproteome that reflect the first few minutes of receptor activation. Combining this information with bioinformatic approaches that translate phosphorylation sites into kinase substrate consensus motifs, link kinases to potential substrates, and identify signaling pathways and regulated biological processes, can produce a holistic picture of signaling at the network level, and generate testable hypotheses about the links between proximal signaling events and downstream phenotypic responses. Indeed, proteomic studies investigating the phosphorylation regulated by stimulation of GPCRs are revealing unanticipated signaling intermediates, finding non-canonical signaling events, and enabling the prediction of activated kinases [10–15].

Here, we apply quantitative stable isotopic labeling by amino acids in cell culture (SILAC)-based mass spectrometry to probe the protein phosphorylation networks regulated by short term exposure to hPTH(1-34), a conventional agonist of the type 1 parathyroid hormone (PTH_1R). PTH_1R signaling is complex, resulting from activation of diverse heterotrimeric G protein species, i.e. G_s, G_i, and $G_{q/11}$, as well as non-canonical arrestin-mediated signaling events [16–18], so to reduce artifacts related to receptor overexpression our experiments were performed in cultured differentiating osteoblastic cells expressing native levels of receptor. In addition to detecting the expected contributions of G_s-cAMP and $G_{q/11}$-calcium signaling, our results demonstrate a robust phosphorylation network regulating small GTPases and cytoskeletal rearrangement, and provide insights into PTH_1R-mediated signaling in a physiological context.

2. Materials and methods

2.1. General considerations

Continued advances in methodology, instrumentation, and computational proteomics are enabling the identification and functional assessment of differentially regulated post-translational modifications [19]. For *in vitro* studies, SILAC is considered the gold standard [7,20]. Utilizing SILAC, changes in protein expression and phosphorylation can be measured, providing a comprehensive view of the dynamic phosphorylation events occurring following ligand–receptor stimulation. As compared to isobaric tagging and label free approaches, SILAC-labeled proteins are combined in the first step of the sample preparation workflow thereby reducing the effect of technical errors introduced when preparing multiple samples in parallel. This yields a higher sensitivity to measure smaller changes in the extent of phosphorylation.

On the other hand, SILAC requires metabolic incorporation of amino acids into proteins prior to cell stimulation, so that individual peptide tandem mass spectra can be assigned to their sample of origin, e.g. stimulated versus non-stimulated, when the mixed samples are analyzed. Thus the approach is not amenable to analysis of proteins prepared directly from tissue. Further, comparison of the phosphoproteomes of multiple murine tissues has revealed the occurrence of tissue-specific sites of protein phosphorylation necessitating analysis in the cell type of interest [21]. To circumvent these limitations, we chose to metabolically label MC3T3-E1 pre-osteoblast cells, an immortalized cell line derived from newborn mouse calvaria, to establish a system to investigate the receptor-proximal effects of PTH_1R activation in a near native cell background.

2.2. Cell culture and stable isotope labeling with amino acids

MC3T3-E1 subclone 4 pre-osteoblast cells (CRL-2593, ATCC) were used as a model of osteoblast differentiation and function. When grown in osteogenic media containing ascorbic acid and β-glycerophosphate, the cells differentiate, express markers reflecting different stages of osteoblast differentiation, and secrete a mineralized hydroxyapatite matrix [22–25]. The presence of the PTH_1R in these cells has been confirmed and the cAMP response to receptor stimulation is readily detectable after 4 days in culture [26–28]. This response is maintained after 10 days of culture in osteogenic media.

Reagents for cell culture and SILAC labeling of MC3T3-E1 cells:

(1) Stable isotope labeled amino acids:

Light Arg: Arg0 L-Arginine-HCl (Fisher Scientific: PI-89989)Light Lys: Lys0 L-Lysine-2HCl (Fisher Scientific: PI-89987)
Medium Arg: Arg6 ($^{13}C_6$ L-Arginine-HCl) (Fisher Scientific: PI-88210)
Medium Lys: Lys4 (4,4,5,5-D4 L-Lysine-2HCl) (Cambridge Isotopes: DLM-2640–0.5)
Heavy Arg: Arg10 ($^{13}C_6$ $^{15}N_4$ L-Arginine-HCl) (Fisher Scientific: PI-89990)
Heavy Lys: Lys8 ($^{13}C_6$ $^{15}N_2$ L-Lysine-2HCl) (Cambridge Isotopes: CNLM-291-0.1)

(2) SILAC Media: For "light", "medium", or "heavy" SILAC media add labeled amino acids to custom MEMα media lacking arginine and lysine (Gibco®). For a final concentration of 0.5 mM arginine add 0.015 ml of 10 mg/ml arginine per ml media. For a final concentration of 0.4 mM lysine add 0.0073 ml of 10 mg/ml lysine per ml of media.

(3) SILAC Growth Media: For growth media add 10% dialyzed fetal calf serum (FCS) (Fisher Scientific) and 1% penicillin/streptomycin to SILAC media. The FCS is dialyzed to eliminate a source of unlabeled lysine or arginine.

(4) SILAC Osteogenic Media: For osteogenic differentiation media (MEMα, 10% FCS, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) add 1.53 mg beta glycerol phosphate per ml of media and 1 μl of 1000× (5 mg of L-ascorbic acid per ml of media) to SILAC growth media.

Protocol for cell culture and SILAC labeling of MC3T3-E1 cells:

1. To enable full incorporation of isotopically labeled arginine and lysine into the cellular protein pool, grow pre-osteoblastic MC3T3-E1 cells subconfluently at 37 °C and 5% CO_2 for at least 5 doublings in SILAC growth media light ([$^{12}C_6{}^{14}N_2$] Lys, [$^{12}C_6{}^{14}N_4$] Arg), medium ([$^2H_4$] Lys, [$^{13}C_6$] Arg), or heavy ([$^{13}C_6{}^{15}N_2$] Lys, [$^{13}C_6{}^{15}N_4$] Arg) amino acids.
2. Efficient metabolic labeling of cellular proteins is confirmed by LC–MS/MS [29]. Once the labels are fully incorporated (⩾95%), cells can be seeded or aliquots stored in liquid nitrogen for subsequent experiments.
3. Seed the SILAC-labeled cells at 1×10^5 cells per cm^2 and grow to confluence in growth media. To obtain sufficient starting material for TiO_2 enrichment of phosphopeptides from