Quantitative measurement of epidermal growth factor receptor–mitogen-activated protein kinase signal transduction using a nine-plex, peptide-based immunoassay

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Abstract

Aberrant epidermal growth factor receptor (EGFR, ErbB1) signaling is implicated in cell transformation, motility, and invasion in a variety of cell types, and EGFR is the target of several anticancer drugs. However, the kinetics of EGFR signaling and the individual contributions of site-specific phosphorylation events remain largely unknown. A peptide-based, multiplex immunoassay approach was developed to simultaneously measure both total and phosphorylated protein in a single sample. The approach involves the proteolytic digestion of proteins prior to the isolation and quantitation of site-specific phosphorylation events within an individual protein. Quantitation of phosphorylated and total proteins, in picomolar to nanomolar concentrations, were interpolated from standard curves generated with synthetic peptides that correspond to the peptide targets used in the immunoassays. In this study, a bead-based, nine-plex immunoassay measuring total and phosphorylated protein was constructed to measure temporal, site-specific phosphorylation of key members of the EGFR pathway (ErbB1 receptor, MEK1, MEK2, ERK1, and ERK2) in A431 cells stimulated with epidermal growth factor. The effect of MEK inhibition on this pathway was determined using a known MEK kinase inhibitor, SL327. The results reported herein are the first quantitative measurements of site-specific phosphorylation events and total proteins in a single sample, at the same time representing a new paradigm for standardized protein and phosphorylation analysis using multiplexed, peptide-based, sandwich immunoassays.

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Recognition that dysregulation in intracellular signaling is widely involved in human diseases [1] has led to the large number of kinase inhibitors that currently are in various stages of clinical development or on the market as therapeutics [1–3]. The high failure rate of target-based approaches has prompted the need for more comprehensive systems approaches to drug discovery and development at the protein level. In response, phosphoproteomics is moving to the forefront of signal transduction research.

DNA microarrays for profiling messenger RNA (mRNA) expression have been firmly established as basic tools for studying biological systems as indirect measure-
ments of protein expression [4]. Protein levels do not always correlate with levels predicted by mRNA expression due to differences in translational efficiency, protein regulation, and stability [5,6]. More importantly, gene transcript profiling is not informative about critical post-translational modifications that regulate protein function. At the protein level, analysis of complex cellular signaling is constrained by limited throughput methods for generating the required data density and diversity. Protein-based methods that quantitatively measure protein abundance, post-translational modifications, and activity in multiplex formats are needed. Antibody array-based technologies promise to address some of these issues [7–10]. For antibody arrays to have a significant impact, several technical challenges must be overcome, including (i) the lack of suitable antibodies with the degree of specificity required for multiplex analysis, (ii) the difficulty in obtaining and qualifying protein standards needed for quantitative analyses, and (iii) the lack of “standardized” assay conditions for proteins with inherently different properties. For example, Sevecka and MacBeath [11] screened 61 antibodies for a reverse-phase array format to perform signaling state-based screens of a small molecule library. Half of the antibodies yielded single dominant bands on Western blots, but only 12 antibodies produced useful data (12/61 = 19%) in the reverse-phase array format.

Proteins are chemically heterogeneous, have complex structures, and participate in extensive protein–protein interactions in vivo. Protein complex formation and protein conformational changes can mask or change epitopes, making antibody binding difficult to predict. In addition, the binding epitopes of most antibodies are not well defined, making it challenging to develop specific immunoassays in a multiplex format. Sandwich immunoassays increase specificity due to the requirement for two independent antibody recognition events on a protein but still have issues in simultaneously measuring several complex proteins and cannot quantitatively measure total protein and post-translational modifications for a target protein in the same assay. A solution that overcomes these limitations is the peptide-based sandwich immunoassay. Although there is a precedent for the production of anti-peptide antibodies, their ability to subsequently recognize native protein structure has been complicated by the inaccessibility of the peptide epitopes in the context of the native protein structure [12].

In contrast to traditional protein-based immunoassay approaches, a novel sample processing and measurement technique that reduces inherent protein conformational issues was developed to allow analysis of biological samples at the peptide level. Beginning with a bioinformatics platform, in silico analysis of the human proteome is used to identify continuous linear sequences, or “epitope tags,” in proteins that are unique within the proteome. The epitope tags were used to design synthetic peptide immunogens and to develop specific antibodies that recognize and bind these predefined linear peptide sequences. Selected peptide sequences are made accessible to the antibodies by digesting proteins in a sample with a specific proteolytic enzyme prior to analysis.

By designing antibody pairs using epitope tags that reside on the same proteolytically cleaved peptide fragment, targets can be captured and detected in sandwich immunoassays with increased specificity. Sandwich methods yield more consistent measurements in cells and cellular extracts, as compared with single antibody detection, and are suitable for high-throughput analyses [13]. This approach generates high-affinity antibodies with preselected epitopes, allowing standardized conditions for assaying peptides instead of whole proteins in multiplex. For total protein measurements, antibodies are generated to two unique epitope tags on the same peptide fragment of a protein to form a “sandwich”. For site-specific, posttranslational modification (PTM) detection, the capture antibodies that are generated bind unique epitope tags flanking the PTM sites on the same fragment and combined with a motif or PTM-specific detection antibody. Quantification is achieved by interpolating protein concentration from standard curves using synthetic peptide and phosphopeptide standards. This enables quantitative and site-specific detection of total protein and multiple post-translational modifications on the same protein within the same sample simultaneously, a capability that is not possible with other technologies. This novel approach was used to quantify time-dependent changes in total and phosphorylated epidermal growth factor receptor (EGFR), MEK1, MEK2, ERK1, and ERK2 in multiplex in epidermal growth factor (EGF)-stimulated A431 human cancer cells.

Materials and methods

Reagents

Unless otherwise noted, all experiments were carried out at room temperature (~25 °C) with Milli-Q water deionized to 18 mΩ·cm resistance.

Epitope tag selection

Computational algorithms were used to screen the Ensembl human proteome (release 35, November 2004) for linear amino acid sequences that were 8 to 12 amino acids in length and unique across the proteome. These epitope tags are specific identifiers of a given protein in the human proteome. Analysis of proteolytic digestion sites in silico identified peptide fragments containing unique epitope tags. Epitope tags were selected based on predicted ease of synthesis, solubility, potential antigenicity, and spacing. To avoid epitope tag-based antibody cross-reactivity, a nearest neighbor calculation was performed for each N-mer set containing a nonredundant list of every N-mer present in the proteome. Each query epitope tag is compared against each sequence in the data-
base and ranked by similarity according to a substitution matrix. Two or more epitope tags that map to a single proteolytic fragment are selected for sandwich immunoassay development. Other protein features that are used to prioritize informative fragments include domain structures, post-translational modifications, and intracellular localization.

**Epitope tag antibody generation**

Peptide immunogens were synthesized using 9-fluorenylmethyl chloroformate (Fmoc) chemistry, high-performance liquid chromatography (HPLC) purified, conjugated to keyhole limpet hemocyanin (KLH), and used to immunize rabbits following standard polyclonal antibody production protocols. Four rabbits were immunized with each peptide immunogen. Antibodies from rabbit sera were affinity purified using the immobilized peptide target and stored at 4°C. Each rabbit antibody was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for purity, and concentrations were determined by absorbance at 280 nm using a NanoDrop N-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

**Cell culture conditions and treatments**

A431 human epidermoid carcinoma cells (American Type Culture Collection, Manassas, VA, USA) were seeded in 10-cm culture dishes and maintained in Dulbecco’s modified Eagle’s medium supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 U/ml penicillin, 10 U/ml streptomycin, and 10% fetal bovine serum (SAFC Biosciences, Waltham, MA, USA) at 37°C with 5% CO2. At 80% confluency, cells were serum starved for 24 h immediately prior to treatment with EGF. Serum-starved A431 cells were stimulated with 100 ng/ml EGF for 0, 4, 8, 16, 32, 64, 128, or 256 min in the presence or absence of 10 μM of an MEK1/2 inhibitor (SL327, Z-&-E-α-amino-4-aminophenyliithiophene-2-trifluoromethylbenzene-acetonitrile) in 0.1% dimethyl sulfoxide (EMD Chemicals, Madison, WI, USA). For inhibition studies, serum-starved A431 cells were pretreated with the MEK1/2 inhibitor for 15 min before exposure to EGF.

**Cell lysis**

Adherent cells were rinsed once with ice-cold phosphate-buffered saline (PBS) to remove residual media, and whole cell lysates were prepared in SDS-based lysis buffer (50 mM triethanolamine hydrochloride [pH 8.5] and 0.2% SDS) containing 2 U/ml Benzonase Nuclease (EMD Chemicals) and 1× Hercules Phosphatase Inhibitor Cocktail (Epitome Biosystems, Waltham, MA, USA). Total protein concentration was determined by the RC/DC protein assay (Bio-Rad, Hercules, CA, USA).

**Immunoblot analysis**

Here 20 μg of total A431 cell lysates was resolved by SDS-PAGE through 4 to 12% Bis–Tris gradient gels in 1× Mes running buffer (Invitrogen, Carlsbad, CA, USA) for 35 min at 200 V and transferred to 0.45-μm nitrocellulose membranes for 60 min at 30 V. Membranes were rinsed and blocked using reagents and protocols in the Western Breeze Chromogenic Detection Kits for rabbit and mouse primary antibodies (Invitrogen) according to the manufacturer’s protocols. Commercially available primary antibodies used were anti-EGFR [pY1068] phosphospecific antibody (cat. no. 44-788G, Invitrogen), anti-ERK1/ERK2 [pTpY185/187] phosphospecific antibody (cat. no. 44-680G, Invitrogen), pan-specific 4G10 anti-phosphotyrosine antibody (cat. no. 05-321, Millipore, Billerica, MA, USA), anti-ERK2 (cat. no. AHO1082, Invitrogen) anti-EGFR antibody for total protein (Epitome Biosystems), and pMEK1/2 [pS217/221] (cat. no. 9121, Cell Signaling Technology, Danvers, MA, USA).

**Enzymatic digestion and sample processing**

Whole cell lysates in SDS-based lysis buffer containing 5 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) were heated at 85°C for 10 min to denature proteins. Free cysteines were alkylated with 10 mM iodoacetamide (Sigma-Aldrich) for 30 min at room temperature in the dark. Endoproteinase Lys-C (Wako Chemicals, Richmond, VA, USA) was added at an enzyme/protein ratio of 1:20, and samples were incubated at 37°C with constant agitation overnight. Complete proteolytic digestion was assessed by SDS-PAGE prior to use in immunoassays. Further proteolytic digestion was prevented by the addition of 0.1 mM N-α-p-tosyl-L-lysyl chloromethyl ketone (TLCK, Sigma–Aldrich) and 1× Complete, Mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany).

**Multiplex immunoassays using the Luminex 100 system**

Whole cell lysates prepared from EGF-stimulated A431 cells were digested with Lys-C and used to measure total EGFR, MEK1, MEK2, ERK1, and ERK2, as well as phospho-EGFR [pY110/1125], phospho-MEK1/2 [pS217/221], phospho-ERK1 [pY204], and phospho-ERK2 [pY187], in the presence and absence of a specific MEK1/2 inhibitor, SL327. Epitope tag-specific antibodies (capture reagents) were coupled to bead sets (Table 2) and checked for adequate coupling efficiency according to the manufacturer’s instructions. Multiplex readout of the immunoassays used xMAP technology and the Luminex 100 system (Luminex, Austin, TX, USA).

Each assay was tested in single-plex to determine the optimal concentration of detection antibody following the manufacturer’s suggested protocol for sandwich immunoassays and using biotinylated detection antibodies, each
at 1 μg/ml, and R-phycocerythrin-linked streptavidin (SA–PE) at 2.5 μg/ml. Calibration curves for each assay were generated with threefold dilutions using synthetic peptide standards in assay buffer (1 × PBST containing 1% bovine serum albumin [BSA]) and compared with calibration curves generated when the nine assays were multiplexed. Each measurement was made in triplicate with readout on the Luminex 100 analyzer.

**Biotinylation of detection antibodies**

Here 100 μg of each Detection antibody (100 μg) was buffer exchanged into 50 mM sodium bicarbonate buffer (pH 8.5) using Microcon YM-30 spin columns (cat. no. 42410, Millipore) and biotinylated via standard amine coupling chemistry using sulfo-NHS-LC-Biotin coupling reagent (cat. no. 21335, Pierce Biotechnology, Rockford, IL, USA) following the manufacturer’s recommended protocol.

**Results**

**Development of a multiplex EGFR–mitogen-activated protein kinase signaling assay**

Based on amino acid sequence, Lys-C-specific cleavage sites, and the resulting protein fragments containing the targeted phosphosite(s) were identified for each protein (Table 1). The sites targeted included pY1110/1125 on EGFR, pS217/222 on MEK1, pS221/226 on MEK2, pY204 on ERK1, and pY187 on ERK2. Computational algorithms (Epitome Biosystems) were used to identify continuous linear sequences in EGFR, MEK1, MEK2, ERK1, and ERK2 proteins that were unique in the entire proteome and located near the phosphosite(s) of interest. The selected sequences (hereafter referred to as epitope tags) were used to design synthetic peptide immunogens for the generation of antibodies with predetermined specificity. For total protein assays, additional protein fragments that did not contain the targeted phosphosite, but did contain at least two different, non-overlapping epitope tags, were identified. Fig. 1 depicts the overall design of the multiplexed, site-specific sandwich immunoassay approach. All antibodies were evaluated for target specificity by Western blots of whole cell lysates and recombinant proteins. Antibodies that detected a dominant reactive band at the expected molecular weight on Western blots were selected for further immunoassay development. Antibodies used for phosphorylation-specific detection were obtained from commercial sources and validated internally by binding to phosphorylated and nonphosphorylated peptide standards as well as by immunoblotting with serum-starved and EGF-stimulated control A431 lysates (unpublished data).

ERK1 and ERK2 are highly homologous family members with identical amino acid sequence in the region containing the phosphorylation sites of interest (Table 1). Phosphospecific antibodies alone have been unable to differentiate between the two phosphoproteins. In sequences proximal to the targeted phosphorylation sites, the proteins differ by a conservative change in two amino acids (isoleucine [ERK1] versus valine [ERK2] and aspartic acid [ERK1] versus glutamic acid [ERK2]). To discriminate between ERK1 and ERK2, distinct epitope tags were selected in these regions and capture antibodies were generated. The ERK2 capture antibody is highly specific for ERK2 and detects a single 42-kDa band in A431 lysates and a 67.8-kDa recombinant ERK2 protein but not the 70-kDa recombinant ERK1 protein. The ERK1 capture antibody is less specific and detects both the 70-kDa recombinant ERK1 protein and the 67.8-kDa recombinant ERK2 protein but with greater than 80% preference for ERK1 (based on unpublished N1 cross-reactivity data). Homologous proteins MEK1 and MEK2 also have identical amino acid sequences in the regions containing the phosphorylation sites of interest. The regions proximal to these phosphosites contain a single, conservative amino acid difference between MEK1 and MEK2 (methionine [MEK1] and leucine [MEK2]) that was not sufficient to discriminate between the two proteins (unpublished results). Therefore, an epitope tag was selected in a region with 100% homology, and an assay was developed to measure the combined amount of phosphorylated MEK1/2 [pS217/221]p.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Epitope tag selection for EGFR-MAPK immunoassays</th>
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</thead>
<tbody>
<tr>
<td><strong>Target</strong></td>
<td><strong>PTM</strong></td>
</tr>
<tr>
<td>EGFR</td>
<td>Total</td>
</tr>
<tr>
<td>EGFR</td>
<td>pY1110/1125</td>
</tr>
<tr>
<td>MEK1</td>
<td>Total</td>
</tr>
<tr>
<td>MEK2</td>
<td>Total</td>
</tr>
<tr>
<td>MEK1&amp;2</td>
<td>pS217/221</td>
</tr>
<tr>
<td>ERK1</td>
<td>Total</td>
</tr>
<tr>
<td>ERK1</td>
<td>pY204</td>
</tr>
<tr>
<td>ERK2</td>
<td>Total</td>
</tr>
<tr>
<td>ERK2</td>
<td>pY187</td>
</tr>
</tbody>
</table>

*PTM = post-translational modification.*
Once antibodies were selected based on specificity for the target of interest, both antibodies of a pair were demonstrated to bind to the corresponding synthetic peptide standard and form a sandwich. For phosphorylation-specific assays, the absence of signal was observed using non-phosphorylated peptide standards (unpublished results). The assay range and sensitivity of each assay were characterized. Ten-point calibration curves starting at five nanomolar concentrations of peptide standards with threefold serial dilutions were generated to cover a large dynamic range (250 fM–5 nM) and the wide variation in protein abundance found in biological samples (Fig. 2). Cross-reactivity between components of the nine-plex assay was examined with N-1 dose–response curves, where each standard was omitted, one at a time, from the multiplex assay. There was no significant signal for eight of the nine assays when its cognate peptide was not included, suggesting no significant cross-reactivity with other standards (data not shown). However, some signal was detected above background with the pERK1 assay in the absence of the pERK1 peptide standard. This was expected because the ERK1 capture antibody binds recombinant ERK2 protein, albeit to a much lesser degree than to recombinant ERK1 protein. The amount of cross-reactivity was within assay variance and did not affect the quantitative accuracy of the assays.

To demonstrate recovery of each target in complex matrices, known concentrations of synthetic peptide standards and/or digested recombinant proteins were spiked into digested whole cell lysates from serum-starved A431 cells. Sandwich assays with the capture and detection antibody pairs were performed in multiplex. The interpolated values for the spiked materials were determined after correcting for the endogenous level of each target measured in serum-starved A431 cells. For eight of the nine assays, recovery from the spiked samples averaged from 83 to 120%. Recovery of total EGFR was lower than expected (~40%) but likely was due to the high endogenous levels of EGFR in A431 cells that made it difficult to measure exogenous levels within the linear detection range of the assay.

Quantitation of EGF-induced activation of EGFR

EGFR (ErbB1, NCBI acc. no. NP_005219) is a 170-kDa transmembrane receptor tyrosine kinase involved in transducing signals from a variety of extracellular stimuli,
including mitogenic and stress-related factors [14–16]. On stimulation, EGFR undergoes dimerization, conformational change, and receptor autophosphorylation, leading to increased tyrosine kinase activity and activation of intracellular substrates [14,17], including MEK1, MEK2, ERK1, and ERK2 [18]. The utility of the multiplex epitope tag technology described herein was demonstrated by analyzing temporal changes in total and phosphorylated protein during EGF-induced EGFR–mitogen-activated protein kinase (MAPK) signaling in A431 cells. Cells were serum starved for 24 h prior to stimulation with 100 ng/ml EGF in the presence or absence of the MEK1/2-specific inhibitor, SL327. Lysates were prepared as described, and measurements were made in multiplex, for total protein (EGFR, MEK1, MEK2, ERK1, and ERK2) and phospho-protein (pEGFR [pY1110/1125], pMEK1/2 [pS222], pERK1 [pY204], and pERK2 [pY187]) targets. Total EGFR protein remained relatively constant for the first 2 h following EGF stimulation at approximately 10 nM (Fig. 3A). Activation of EGFR, as measured by phosphorylation on Y1110/1125, was detected within 4 min of EGF stimulation and continued to increase, resulting in approximately 93% of total EGFR being phosphorylated 4 h after EGF stimulation (Table 2). Although a slight decrease in total EGFR protein was detected after 2 h, the amount of phosphorylated EGFR continued to increase. Pretreatment with the MEK1/2 inhibitor, SL327, did not affect the amount of total or EGF-induced phosphorylation of EGFR at early time points but did result in slightly elevated levels of phosphorylated EGFR between 32 and 128 min. Western blot analyses confirmed the general EGFR activation trends (Fig. 3B).

Quantitation of EGF-induced activation of MEK1/2

Activation of downstream targets of the EGFR–MAPK pathway were measured concomitant with EGFR activation. Site-specific activation of MAPK kinases (MAPKKs) (MEK1 and MEK2) and their targets, MAPKs (ERK1 and ERK2), was observed. Equivalent amounts of total MEK1 (2.14 ± 0.314 nM) and MEK2 (1.77 ± 0.665 nM) proteins were measured in A431 cells, and these amounts did not change appreciably during the time course of the experiment (Fig. 4A). In contrast, the amount of pMEK1/2 [S217/221] increased after EGF treatment, peaked at approximately 80 pM between 8 and 16 min, and accounted for 2% of the total MEK1 and MEK2 protein (Fig. 4A and Table 2). The level of pMEK1/2 declined back to basal levels 1 h after EGF stimulation. Pretreatment of A431 cells with SL327 delayed phosphorylation of MEK1/2 on S217/221 but resulted in delayed/prolonged activation, possibly affecting negative feedback signaling. The data correlated

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Fig. 2. Standard curves for peptide-based immunoassays. (A) Standard curves for total protein assays using peptide reference standards for EGFR, MEK1, MEK2, ERK1, and ERK2. (B) Standard curves for phosphoprotein assays using peptide reference standards for EGFR (pY1110/1125), MEK1/2 (pS222), ERK1 (pY204), and ERK2 (pY187). Average MFI is the average (N = 3) of the mean fluorescent intensity.
well with results from immunoblots using commercial phosphospecific antibodies (Fig. 4B).

**Quantitation of EGF-induced activation of ERK1 and ERK2**

Similar to EGFR and MEK1 and MEK2 total protein, the amounts of total ERK1 and ERK2 protein remained relatively constant at 1.69 ± 0.252 nM and 4.26 ± 0.768 nM, respectively, during EGF stimulation of A431 cells (Fig. 5A). These data support the involvement of negative feedback mechanisms rather than protein degradation in the deactivation of MAPKKs and MAPKs.

Maximal amounts of pERK1\[Y204\](/C24 39%) and pERK2\[Y187\](/C24 92%) were detected within 4 min following EGF stimulation of A431 cells (Fig. 5A).

**Table 2**

<table>
<thead>
<tr>
<th>Target</th>
<th>PTM</th>
<th>Bead set</th>
<th>Time&lt;sup&gt;a&lt;/sup&gt; (min)</th>
<th>Concentration&lt;sup&gt;b&lt;/sup&gt; at Time&lt;sup&gt;a&lt;/sup&gt; (nM)</th>
<th>Assay CV&lt;sup&gt;c&lt;/sup&gt;%</th>
<th>% Phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Total</td>
<td>29</td>
<td>256</td>
<td>7.85 ± 0.362 (n = 9)</td>
<td>3.9</td>
<td>4.6</td>
</tr>
<tr>
<td>EGFR</td>
<td>pY&lt;sup&gt;1110/1125&lt;/sup&gt;</td>
<td>32</td>
<td>256</td>
<td>7.30 ± 1.263 (n = 12)</td>
<td>4.8</td>
<td>17.3</td>
</tr>
<tr>
<td>MEK1</td>
<td>Total</td>
<td>4</td>
<td>16</td>
<td>2.14 ± 0.314 (n = 11)</td>
<td>8.1</td>
<td>14.7</td>
</tr>
<tr>
<td>MEK2</td>
<td>Total</td>
<td>4</td>
<td>16</td>
<td>1.77 ± 0.665 (n = 11)</td>
<td>9.5</td>
<td>37.6</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>pS&lt;sup&gt;222&lt;/sup&gt;</td>
<td>7</td>
<td>16</td>
<td>0.08 ± 0.002 (n = 3)</td>
<td>2.5</td>
<td>ND</td>
</tr>
<tr>
<td>ERK1</td>
<td>Total</td>
<td>21</td>
<td>4</td>
<td>4.26 ± 0.768 (n = 9)</td>
<td>8.4</td>
<td>18.0</td>
</tr>
<tr>
<td>ERK2</td>
<td>pY&lt;sup&gt;204&lt;/sup&gt;</td>
<td>15</td>
<td>4</td>
<td>0.66 ± 0.219 (n = 8)</td>
<td>17.4</td>
<td>33.2</td>
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<tr>
<td>ERK2</td>
<td>pY&lt;sup&gt;187&lt;/sup&gt;</td>
<td>28</td>
<td>4</td>
<td>3.93 ± 0.886 (n = 8)</td>
<td>13.0</td>
<td>22.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>PTM = post-translational modification.

<sup>b</sup>Time<sub>max</sub> = time (min) at maximum signal for phosphorylated protein.

<sup>c</sup>Concentration of each target in 1 mg/mL total protein from A431 lysates, interpolated from calibration curves generated with known amounts of synthetic peptide standards and averaged across two separate experiments.

<sup>d</sup>CV (coefficient of variance) = standard deviation/mean of replicate measurements from a single assay (Intra) or replicated measurements across two separate assays (Inter).

<sup>e</sup>n = number of replicate measurements averaged.
stimulation (Fig. 5A and Table 2) and began to decrease 16 min after EGF stimulation, reaching basal levels 2 h poststimulation. Immunoblotting with a commercial anti-phospho-ERK1/ERK2 antibody (Invitrogen) confirmed the time-dependent phosphorylation of both ERK1 and ERK2 in response to EGF treatment (Fig. 5B). Pretreatment with SL327 abrogated these effects, indicating that SL327 is a potent inhibitor of MEK activation of ERK.

Discussion

Intracellular signaling is regulated by a complex network of interconnected pathways, and dysregulation in these pathways is associated with human diseases such as cancer, diabetes, and immune dysfunction. Protein phosphorylation is the key currency of intracellular signaling, and enzymes regulating protein phosphorylation, such as kinases, are popular targets in drug development pipelines [14,16,19–24]. However, most kinases and kinase substrates are expressed, at the protein level, in low abundance relative to other cellular proteins. As a result, phosphorylation can be extremely low [25], transient, and highly dynamic. Quantitative protein measurements, particularly for post-translationally modified proteins, have been difficult due to the absence of specific capture antibodies, the complexity and distinctive properties of proteins, and the challenges in obtaining and qualifying protein standards. The epitope tag-based approach described herein addresses these issues by generating highly specific, anti-peptide antibodies against virtually any protein sequence, including membrane receptors. Isolated or purified protein antigens are not needed to make the antibodies because epitope tags are computationally defined and chemically synthesized. Fragmenting proteins into predicted peptides ensures that the predefined epitopes are available to be captured by the antibody regardless of native protein structure. In addition to being independent of native protein conformations and protein–protein interactions that could interfere with detection, the processing of samples immediately after a given treatment regime preserves the samples and prevents further alterations in content during storage and analysis. Phosphopeptides can be made synthetically and used as quantitative assay standards. Unlike current reverse-phase arrays, sandwich immunoassays have high specificity because they require that each target binds two independent antibodies.

In this work, a novel epitope tag-based, sandwich immunoassay approach was developed to enable quantitative, multiplexed total and phosphorylated protein analyses on a single sample. To demonstrate the power of this approach, site-specific temporal changes in protein phosphorylation during EGF-induced signaling were analyzed in the presence and absence of a MEK1/2-specific inhibitor, SL327, in A431 cells. Dysregulation of EGFR (ErbB1)
expression and/or signaling is widely implicated in angiogenesis, tumor growth, and progression and is the target of several anticancer drugs. Unfortunately, drug development efforts have been hindered by the lack of measurement tools to accurately access aberrant EGFR signaling [19–23]. Due to the unique peptide-based strategy, these assays are the first to quantify total and phosphorylated EGFR and downstream targets MEK1, MEK2, ERK1, and ERK2 from a single source, at the same time, with site-specific phosphorylation measurements using a robust immunoassay approach. A431 cells overexpress EGFR due to EGFR gene amplification [26] and are used extensively to study EGFR signaling. Our results demonstrate that MEK1, MEK2, ERK1, and ERK2 activation is transient and that the attenuation of these responses is due to negative feedback mechanisms and not protein degradation. Although SL327 is more effective at inhibiting ERK activation, it also inhibits and delays MEK activation by interfering with MEK activation by Raf and not just MEK phosphorylation of ERK. This may be caused by direct inhibition of Raf and MEK or could be due to increased phosphatase activity in the absence of downstream ERK activation (i.e., negative regulation of a negative feedback loop) [27]. Unlike the transient activation of MAPKKs and MAPKs, phosphorylation of EGFR at Y1110/1125 continues to increase for at least 4 h with EGF stimulation and is further enhanced by inhibiting downstream MEK. This suggests that feedback mechanisms downstream of ERK likely regulate EGFR phosphorylation. Prolonged EGFR phosphorylation may result in additional cellular events outside the direct activation of the MAPK pathway, and we currently are applying our epitope tag-based approach to generate broad phosphoprotein profiling arrays to explore these issues. The ability to accurately measure several EGFR pathway members in the context of other signaling events, simultaneously and in a temporal fashion, will further elucidate pathway dynamics and their potential role in disease pathogenesis and modulation by kinase inhibitors.

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