Temporal Perturbation of Tyrosine Phosphoproteome Dynamics Reveals the System-wide Regulatory Networks*§

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Signal transduction systems are known to widely regulate complex biological events such as cell proliferation and differentiation. Because phosphotyrosine-dependent networks play a key role in transmitting signals, a comprehensive and fine description of their dynamic behavior can lead us to systematically analyze the regulatory mechanisms that result in each biological effect. Here we established a mass spectrometry-based framework for analyzing tyrosine phosphoproteome dynamics through temporal network perturbation. A highly time-resolved description of the epidermal growth factor-dependent signaling pathways in human A431 cells revealed a global view of their multiphase network activation, comprising a spike signal transmission within 1 min of ligand stimulation followed by the prolonged activation of multiple Src-related molecules. Temporal perturbation of Src family kinases with the corresponding inhibitor PP2 in the prolonged activation phase led to the down-regulation of the molecules related to cell adhesion and receptor degradation, whereas the canonical cascades as well as the epidermal growth factor receptor relatively maintained their activities. Our methodology provides a system-wide view of the regulatory network clusters involved in signal transduction that is essential to refine the literature-based network structures for a systems biology analysis. Molecular & Cellular Proteomics 8:226–231, 2009.

It is well known that the signal transduction system within a cell leads to the determination of diverse cell fates, such as proliferation, differentiation, or apoptosis (1, 2). To date, the extensive analyses that have been performed on cell signaling under a variety of experimental conditions have revealed many of the key molecules and related events that result in various biological effects. Regarding the epidermal growth factor (EGF) receptor pathway, which is one of the most intensively studied signaling systems, the related literature has especially been accumulated and even integrated into a large scale interaction map in a recent study (3). In addition to a series of biochemical analyses of each signaling molecule, recent approaches using protein microarrays and mass spectrometry have also added a global view of ErbB signaling initiation in vitro (4, 5). Such an integrative view of this biological system, however, cannot provide a fundamental theory on how the entire signaling network is regulated without analyzing their dynamic behaviors at the system level.

Recent technological advances in mass spectrometry-based proteomics have enabled us to make a large scale identification of signaling molecules through the enrichment of phosphorylated proteins or peptides (6–11). In combination with protein/peptide labeling strategies based on stable isotope labeling by amino acids in cell culture (SILAC) (12) or by using iTRAQ (isobaric tags for relative and absolute quantitation) reagents (13), their time course activation profiles have also been generated through the relative quantitation of labeled peptides in mass spectra (14–17).

In this study, we established an integrated framework for analyzing the dynamic behavior of the SILAC-encoded tyrosine phosphoproteome through temporal network perturbation. To acquire highly time-resolved dynamic data in a high throughput manner, we developed a method for the efficient enrichment of tyrosine-phosphorylated signaling molecules to greatly shorten the time for sample preparation and subsequent LC-MS/MS analysis (see Fig. 1a). After relative quantitation based on the SILAC-encoded data is automatically performed using the AYUMS algorithm (18), the multiple quantitative data for different time points are integrated into fine activation profiles through data normalization (see Fig. 1b). A precise and highly time-resolved description of the network dynamics provides a theoretical basis for estimating the temporal perturbation effect in relation to specific signaling molecules, allowing us to obtain a system-level view of the regulatory relationships in transducing signals.

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1 The abbreviations used are: EGF, epidermal growth factor; SILAC, stable isotope labeling by amino acids in cell culture.
Temporal Perturbation of Tyrosine Phosphoproteome Dynamics

Here we applied our methodology to the signaling networks that work upon EGF stimulation in human A431 cells as a model system. A time-resolved description of their tyrosine phosphoproteome dynamics revealed the property of multiphase network activation, comprising a spike signal transmission within 1 min of ligand stimulation followed by the prolonged activation of multiple Src-related molecules. Temporal perturbation of Src family kinases with PP2 in the prolonged activation phase enabled us to clearly distinguish between sensitive and robust pathways in response to this treatment, providing a system-level view of the Src function in the EGF signaling of A431 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human epithelial A431 cells were labeled with either L-arginine (Arg 0), L-[U-13C6, 14N4]arginine (Arg 6), or L-[U-13C4,N15]arginine (Arg 10) as described previously (14). After overnight serum starvation, the three types of SILAC-encoded cells (2 × 10 cm dishes per condition) were treated with 100 ng/ml EGF for 0, 1, and 10 min, respectively, where the value t corresponds to either 0.5, 2, 5, 10, 15, 20, 25, or 30 min. The cells stimulated with each indicated time interval were washed three times with PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM sodium orthovanadate, and protease inhibitors (Complete Mini, Roche Diagnostics) one by one in the same manner. Each lysate, quantified as −3 μg of proteins through BCA assay, was mixed equally for subsequent immunoprecipitation.

**Sample Preparation for Mass Spectrometric Analysis**—Immunoprecipitation of tyrosine-phosphorylated protein complexes was performed as described previously (14). After washing the immunoprecipitates quickly with a lysis buffer containing no detergents, elution of the target molecules was conducted with 25 mM phenyl phosphate (19). The eluted protein complex (−1 μg) was digested with trypsin directly in the solution, desalted with ZipTip (C18, Millipore), and finally concentrated to a volume of as little as 20 μl to inject into the nano-LC system.

**Mass Spectrometry and Protein Identification**—After applying the peptide mixture to a C18 column (800-μm inner diameter × 3 mm long), reversed-phase separation of the captured peptides was done on a column (150-μm inner diameter × 75 mm long) filled with H-Q-Sil C18 (3-μm particles, 120-Å pores; KYA Technologies) using a direct nanoflow LC system (Dina, KYA Technologies). The peptides were eluted with a linear 5–65% gradient of acetonitrile containing 0.1% formic acid over 120 min at a flow rate of 200 nl/min and sprayed into a quadrupole time-of-flight tandem mass spectrometer (Q-Tof-2, Micromass) (20, 21). Acquisition of MS/MS spectra was performed using the following parameters: dynamic exclusion time, 120 s; duty cycle, 2 s; mass tolerance, 0.1 Da. The MS/MS signals were then converted to text files by MassLynx (version 3.5, Micromass) and processed against the RefSeq (National Center for Biotechnology Information (NCBI)) human protein database (39,397 sequences as of July 3, 2006) (22) using the Mascot algorithm (version 2.1.02, Matrix Science) with the following parameters: variable modifications, oxidation (Met), N-acetylation, pyroglutamination (Gln), phosphorylation (Ser, Thr, and Tyr), and stable isotopes of Arg 6 and Arg 10 (Arg); maximum missed cleavages, 3; peptide mass tolerance, 500 ppm; MS/MS tolerance, 0.5 Da. Protein identification was based on the criterion of having at least one MS/MS data with Mascot scores that exceeded the thresholds (p < 0.05). A randomized decoy database created by a Mascot Perl program estimated a false discovery rate at 0.93% for all the identified peptides.

**Protein Quantification and Data Normalization**—For each LC-MS/MS experiment, activation changes regarding all the peptides with Mascot scores (≥20) at 1 and t min after EGF stimulation were automatically calculated as relative ratios to the state before stimulation using the AYUMS algorithm (version 1.00) (18) where the value t corresponded to either 0.5, 2, 5, 10, 15, 20, 25, or 30 min. Relative quantification by AYUMS was performed based on their peak intensities of SILAC-encoded peptides. For each protein identified, the relative quantitation value for each peptide data was all normalized to the average value of -fold activation for 1 min of ligand stimulation. The average ratio and its standard deviation were then calculated for each stimulation time based on the normalized quantitation values, yielding time series activation profiles. We assessed the quantification variability based on all the -fold changes at 1 min regarding the proteins with the quantitative data on at least three peptides per sample. The average relative standard deviation was 24.8%, leading to more than a 95% statistical confidence in the selected threshold of 1.5-fold.

**Network Perturbation Analysis**—Serum-starved A431 cells labeled with either L-arginine or L-[U-13C6,15N4]arginine were treated with 100 ng/ml EGF as described above. After stimulation for 1 min, Src family kinase inhibitor PP2 (Calbiochem) was added to the Arg 10-labeled cells at a final concentration of 5 μM and incubated for another 1, 4, 9, and 14 min, respectively. The carrier solution for PP2 was added to the Arg 0-labeled cells and incubated for the same interval as a control. The cells were then lysed, mixed equally, and subjected to nano-LC-MS/MS analysis in the same manner as described above. For this analysis, we used a new mass spectrometer (Q-STAR Elite, Applied Biosystems) and set the parameters to 60 s for dynamic exclusion, Smart IDA (information-dependent acquisition) mode for duty cycle, and 100 ppm for mass tolerance. The perturbation effect with PP2 was represented as a relative ratio of Arg 10 to Arg 0 for each protein using the MSQuant program (version 1.4.3) (23).

**RESULTS**

**Identification of Tyrosine-phosphorylated Molecules in EGF-stimulated Human A431 Cells**—Because tyrosine phosphorylation is known to play a key role in early EGF signaling, we tried describing the time-resolved dynamics of tyrosine-phosphorylated proteins for 30 min after EGF stimulation using the SILAC technology (Fig. 1a). To make a comprehensive identification of the target signaling molecules, tyrosine-phosphorylated protein complexes were enriched by affinity selection with anti-Tyr(P) antibodies. The peptide mixture generated from the protein complexes through tryptic digestion was then subjected to nano-LC-MS/MS analysis. Our tyrosine phosphoproteome analysis of EGF-stimulated A431 cells generated 28,248 MS/MS spectra in total. A database search against the RefSeq human protein database (22) led to the identification of 3,730 peptides, which were ultimately assigned to 790 unique peptides (supplemental Table 1). Among a total of 136 proteins identified, 77 proteins were identified by more than two peptides, whereas 59 proteins were supported by single peptide identification (supplemental Table 1).

**Generation of Activation Profiles through Comprehensive Quantification and Data Normalization of Time Series -Fold Changes**—Regarding the proteins identified based on the arginine-containing peptide(s), we performed automatic relative quantitation of the SILAC-encoded MS data using the
The time course profiles for the 19 signaling molecules measured at all the time points are shown in Table I and Fig. 2. As shown in Fig. 2a, spike activation of the EGF receptor was observed within 1 min of ligand stimulation followed by sustained signaling with the -fold change lower than the first transmission. The other signaling molecules were also subjected to the fast activation upon EGF stimulation, but their dynamic behaviors in the prolonged activation phase depended on mol-

ecules (Fig. 2, a–d). Notably the effectors related to cell adhesion, such as the integrin and catenin families, mostly displayed sustained activation profiles (Fig. 2, b and c). As some of these adhesion molecules as well as the EGF receptor are classified as Src substrates in the public database (25), our time-resolved data strongly suggested the widespread cooperation of Src family kinases with the maintenance of the prolonged activation phase of EGF signaling in human A431 cells.

**Network Perturbation of the EGF-dependent Prolonged Activation Phase with the Src Family Inhibitor PP2**—Next to analyze the network-wide role of Src family kinases in the prolonged activation phase of EGF-stimulated A431 cells, we tried to make a quantitative description of the regulatory effect on the tyrosine phosphoproteome by network perturbation with the Src family inhibitor PP2. We performed time course PP2 treatment from 1 min after the EGF input was added when the phase of the EGF signaling shifted from the spike activation to the prolonged activation. The perturbation effect was described as a relative ratio to the activation state with no PP2 treatment (supplemental Table 3). The result indicated widespread down-regulation of the molecules related to cell adhesion and receptor degradation as well as some other EGF receptor effectors (Vav2, Shp2, and PLCγ1), whereas the canonical cascades (Shc, Grb2, and ERK2) as well as the EGF receptor relatively maintained their activities (Table II). Our perturbation analysis revealed many signaling effectors that were not described as c-Src interac-
tors in a series of previous studies (Table II), providing a system-level view of the regulatory clusters of Src family kinases in the early EGF signaling of A431 cells.

**DISCUSSION**

Our quantitative description of the tyrosine phosphoproteome dynamics in human A431 cells provided a global view of their network activation status with high time resolution. Fine activation profiles of the EGF-induced signaling molecules showed an overall view of the multiphase property of their network activation. The first spike activation of the EGF receptor upon EGF stimuli was quickly transmitted throughout the entire pathways within 1 min. The subsequent phase of
prolonged activation was prominently observed regarding cell adhesion and structural molecules as well as the EGF receptor. As some of the cell-cell contact molecules, such as caveolin 1, catenin δ1, and junction plakoglobin, as well as the EGF receptor are considered to be Src substrates in the context of signal transduction (25), our time-resolved description of signaling dynamics indicated a probability that Src family kinases play an influential role in regulating the prolonged activation phase of EGF signaling in A431 cells. The temporal perturbation of Src family kinases with the corresponding inhibitor PP2 demonstrated the strong correlation of Src family kinases with cell migration-associated pathways,

TABLE I

| RefSeq accession no. | Definition | 0.5 min | 1 min | 2 min | 5 min | 10 min | 15 min | 20 min | 25 min | 30 min |
|----------------------|------------|---------|-------|-------|-------|--------|--------|--------|--------|--------|
| NP_004915            | Actinin, α4| 11.25   | 10.22 | 8.54  | 7.09  | 5.86   | 4.94   | 4.50   | 3.12   | 3.56   |
| NP_001611            | AHNAK nucleoprotein isoform 1| 17.60   | 12.79 | 8.55  | 10.24 | 8.26   | 6.96   | 2.23   | 3.47   | 3.08   |
| NP_000485            | α1 type XVII collagen| 15.99   | 10.14 | 6.19  | 8.99  | 8.23   | 6.60   | 4.35   | 4.09   | 3.67   |
| NP_004351            | Cadherin 1, type 1 preproprotein| 5.66    | 5.39  | 3.90  | 2.13  | 3.20   | 3.93   | 2.03   | 2.46   | 2.53   |
| NP_001322            | Catenin (cadherin-associated protein), δ1| 12.91   | 9.63  | 7.18  | 6.62  | 5.56   | 5.46   | 3.01   | 3.73   | 3.03   |
| NP_001894            | Catenin, α1| 5.15    | 3.74  | 3.08  | 2.70  | 2.24   | 2.10   | 2.14   | 1.77   | 1.75   |
| NP_001744            | Caveolin 1| 4.20    | 4.76  | 4.29  | 4.55  | 3.91   | 3.65   | 3.56   | 3.67   | 6.04   |
| NP_055423            | Cytoplasmic FMR1-interacting protein 1 isoform a| 11.58   | 7.40  | 4.03  | 4.69  | 3.62   | 2.89   | 2.94   | 1.91   | 2.18   |
| NP_002221            | Junction plakoglobin| 12.96   | 10.54 | 8.98  | 8.24  | 7.04   | 5.82   | 5.04   | 4.45   | 5.31   |
| NP_038465            | NCK-associated protein 1 isoform 1| 5.86    | 4.11  | 3.11  | 2.60  | 2.76   | 1.83   | 1.39   | 1.26   | 1.12   |
| NP_002651            | Phospholipase C γ1 isoform a| 14.97   | 6.82  | 5.24  | 4.35  | 3.88   | 3.66   | 3.60   | 2.64   | 2.47   |
| NP_009114            | Plakophilin 3| 9.61    | 7.32  | 6.85  | 6.42  | 5.69   | 4.83   | 3.34   | 2.61   | 2.70   |
| NP_002825            | Protein-tyrosine phosphatase, non-receptor type 11| 6.03    | 5.11  | 4.49  | 3.40  | 3.12   | 2.05   | 1.84   | 1.50   | 1.96   |
| NP_005219            | Epidermal growth factor receptor isoform a| 18.74   | 13.13 | 9.42  | 9.63  | 8.88   | 8.58   | 7.36   | 7.83   | 7.36   |
| NP_001005731         | Integrin β4 isoform 3 precursor| 16.09   | 10.33 | 8.27  | 5.99  | 6.46   | 6.19   | 5.16   | 4.56   | 5.15   |
| NP_002221            | Junction plakoglobin| 12.96   | 10.54 | 8.98  | 8.24  | 7.04   | 5.82   | 5.04   | 4.45   | 5.31   |
| NP_038465            | NCK-associated protein 1 isoform 1| 5.86    | 4.11  | 3.11  | 2.60  | 2.76   | 1.83   | 1.39   | 1.26   | 1.12   |
| NP_002651            | Phospholipase C γ1 isoform a| 14.97   | 6.82  | 5.24  | 4.35  | 3.88   | 3.66   | 3.60   | 2.64   | 2.47   |
| NP_009114            | Plakophilin 3| 9.61    | 7.32  | 6.85  | 6.42  | 5.69   | 4.83   | 3.34   | 2.61   | 2.70   |
| NP_002825            | Protein-tyrosine phosphatase, non-receptor type 11| 6.03    | 5.11  | 4.49  | 3.40  | 3.12   | 2.05   | 1.84   | 1.50   | 1.96   |
| NP_005426            | Rho guanine nucleotide exchange factor 5| 3.34    | 2.80  | 1.83  | 2.51  | 1.98   | 1.86   | 1.67   | 1.62   | 1.30   |
| NP_006073            | Tubulin, α, ubiquitous| 3.32    | 2.34  | 1.64  | 2.15  | 1.95   | 1.81   | 1.28   | 1.58   | 1.38   |
| NP_003362            | Vav 2 oncogene| 10.65   | 5.97  | 3.63  | 2.40  | 2.69   | 2.42   | 1.59   | 1.84   | 1.74   |
| NP_003370            | Villin 2 | 15.14   | 13.73 | 8.78  | 8.24  | 5.43   | 4.46   | 2.92   | 2.74   | 2.31   |

Fig. 2. Time-resolved activation profiles of EGF signaling molecules in A431 cells. The data are classified into four functional categories: the EGF receptor (EGFR) and its well known effectors (a), proteins involved in cell adhesion (b), proteins related to cell structure (c), and undefined effectors in EGF signaling (d).
whereas it had only a slight impact on the EGF receptor activity. We also observed a strong down-regulation of the receptor degradation pathways through the inhibition of Src family kinases, reflecting the previous evidence of the effect of c-Src on EGF receptor endocytosis (26). Thus, our network perturbation strategy based on a highly time-resolved description of tyrosine phosphoproteome dynamics provided a network-wide view of the pathway clusters controlled by Src family kinases in A431 cells. Based on some previously described network models (27–33), our methodology enables us to refine such general network structures into a “cell type-specific” architecture. We expect that mathematical analysis based on these cell type-specific network models will lead us to the efficient identification of potential drug targets for specific disease conditions and also enable us to theoretically estimate the effect of the corresponding drugs on a network-wide scale prior to clinical application.

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| RefSeq accession no. | Definition | 2 min<sup>b</sup> | 5 min<sup>b</sup> | 10 min<sup>b</sup> | 15 min<sup>b</sup> |
|----------------------|------------|-------------------|-------------------|-------------------|-------------------|
| Classical cascades   |            |                   |                   |                   |                   |
| NP_002077            | Growth factor receptor-bound protein 2 isoform 1   | 0.48              | 0.41              | 0.44              | 0.43              |
| NP_620407            | Mitogen-activated protein kinase 1                 | 0.69              | 0.63              | 0.79              | 0.95              |
| Proteins involved in receptor internalization |            |                   |                   |                   |                   |
| NP_005179            | Cas-Br-M (murine) ecotropic retroviral transforming sequence | 0.29              | 0.14              | 0.23              | 0.35              |
| NP_733762            | Cas-Br-M (murine) ecotropic retroviral transforming sequence | 0.45              | 0.22              | 0.14              | 0.14              |
| Other EGFR effectors |            |                   |                   |                   |                   |
| NP_003362            | Vav 2 oncogene                                     | 0.10              | 0.11              | 0.13              | 0.12              |
| NP_002825            | Protein-tyrosine phosphatase, non-receptor type 11 | 0.11              | 0.31              | 0.19              | 0.24              |
| Proteins involved in cell adhesion |            |                   |                   |                   |                   |
| NP_001005731         | Integrin β4 isoform 3 precursor                    | 0.12              | 0.14              | 0.14              | 0.24              |
| NP_004351            | Cadherin 1, type 1 preproprotein                   | 0.13              | 0.12              | 0.08              | 0.22              |
| NP_001784            | Cadherin 3, type 1 preproprotein                   | 0.14              | 0.15              | 0.09              | 0.25              |
| Proteins related to cell structure |            |                   |                   |                   |                   |
| NP_000485            | α1 type XVII collagen                              | 0.16              | 0.08              | 0.05              | 0.10              |
| NP_001744            | Caveolin 1                                          | 0.40              | 0.35              | 0.66              | 0.65              |
| Other proteins       |            |                   |                   |                   |                   |
| **ND, not determined.** |            |                   |                   |                   |                   |

<sup>a</sup> The molecules annotated as c-Src interactors in the Human Protein Reference Database Release 7 (25) are indicated in underlined italics.

<sup>b</sup> In every case, PP2 treatment was performed after 1 min of EGF stimulation. For each time period of EGF stimulation, the perturbation effect on each signaling molecule is described as a ratio relative to the activation state with no PP2 treatment.

<sup>c</sup> ND, not determined.
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