Immunoprecipitation and Western Blot Screening for Phosphorylation States Of Over 900 Signal Transduction Proteins

J. Stevenson, A. Siemers, P. Wethington, E. Heywood, K. Franklin, R. Campos-Gonzales

BD Transduction Laboratories, Lexington, KY, USA

Abstract

The importance of phosphorylation in signal transduction has been known for many years. Recently, tools have been made available that allow a more specific, efficient and comprehensive study of protein phosphorylation. These tools include phospho-specific antibodies and monoclonal antibodies recognizing total protein, whether phosphorylated or unphosphorylated. In this study, we surveyed the phosphorylation states and general expression levels of more than 900 different signal transduction proteins during stimulation of EGF-responsive cells. Our methods include 1) multiplex Western blotting with >900 monoclonal antibodies; 2) Western blotting using >30 phospho-specific monoclonal antibodies; and 3) anti-phospho-tyrosine immunoprecipitation followed by Western blot screening with >200 monoclonal antibodies. We found numerous phosphorylated proteins; many already described in the literature, such as MAPK and Stats. Also, we detected novel tyrosine phosphorylation changes. One such novel change was identified on Bad, a member of the Bcl-2 family. Furthermore, we compared early phosphorylation events in cells bearing a wild-type EGF receptor versus those containing an EGF-inactive receptor. Cells with the inactive receptor are unable to proliferate in response to EGF. Significant differences were detected in the phosphorylation of Stat1, ERK1/2, p38MAPK and Caveolin-1, implicating these proteins in a cell proliferation pathway. In summary, we were able to utilize an arsenal of cell biology reagents to examine changes in the general expression pattern and phosphorylation profile of EGF stimulated cells. Our methods provide a rapid and comprehensive view of the signaling pathways and proteins activated by EGF and will prove to be an invaluable tool for examining cell states that have been perturbed by various compounds or cellular defects.

Introduction

The study of receptor tyrosine kinases began with the Epidermal Growth Factor Receptor (EGFR). Activation of EGFR leads to a complex signaling cascade involving a multitude of protein:protein interactions, protein phosphorylations, translocations and transcriptional activation. EGFR possesses an extracellular ligand binding domain, transmembrane domain and a cytoplasmic domain with tyrosine kinase activity. Growth factor induces oligomerization, autophosphorylation and activation of EGFR. Autophosphorylation also creates docking sites for recruited SH2 and PTB domain containing proteins such as PLCg. Once bound, EGFR phosphorylates and induces PLCg to generate second messengers that activate PKC and stimulate release of calcium. Additionally, EGFR stimulation leads to rapid phosphorylation and translocation of Stats to the nucleus where they initiate transcription. Other signaling molecules are recruited when SHC, an adapter protein important for signaling via many types of receptors, is phosphorylated by EGFR. Once phosphorylated, SHC can recruit Grb2/Sos through SH2 binding. This brings Sos close to Ras and stimulates guanine nucleotide exchange. Once active, Ras interacts with Raf and PI-3 kinase to stimulate MAP kinase-kinase (MAPKK, MEK) which in turn activates MAP kinase (ERK). Activated ERK rapidly translocates to the nucleus to activate transcription factors. This signaling cascade plays important roles in metabolism, cell cycle, cell migration and cell proliferation. EGF activation of PI-3 kinase and MAPK also promotes cell survival through generation of additional second messengers, activation of PKB and phosphorylation and inactivation of BAD. To avoid overactivation and malignant transformation, the signal must be attenuated and terminated. This occurs at least in part through rapid endocytosis and degradation of EGFR. Overexpression and overactivation of EGFR and other family members has been implicated in many cancers, including mammary and squamous carcinomas and glioblastomas. This has led to extensive characterization of EGF signaling and the factors required for cell proliferation and survival.

In this study, we used the BD PowerBlot and BD Phospho-PowerBlot Western array screening services to identify EGF-induced early protein expression and protein phosphorylation changes in cells bearing normal, overexpressed and kinase inactive EGFR. We also used a novel dual color Western blot detection method to simultaneously measure phospho and non-phospho protein levels during a time course of EGF treatment of cells overexpressing EGFR.

Methods

Cell Culture

A431, WT(GEN) or EGF Receptor mutant (M721) cells that were 80-90% confluent were serum starved overnight, then exposed to 100 ng/ml EGF for 5 minutes at 37°C or for the times indicated in Panel V. Control cells were serum starved without subsequent stimulation with EGF. Cell lysates were prepared by the "Sample Preparation" procedure in our "Western Blotting with Monoclonal Antibodies" protocol (see"Support: Pharmingen/Transduction Laboratories Protocols", www.bdbiosciences.com).

BD PowerBlot and BD Phospho-PowerBlot Western Array Analysis

Protein sample (200 µg) was run on a 8x11 cm preparative, 4-15% polyacrylamide gradient SDS gel (Criterion, Bio-Rad) and transferred to Immobilon-P nylon membrane (Millipore). After transfer, the membrane was blocked with LI-COR® Odyssey® Blocking Buffer for 1 hour. The blocked membrane was placed in a western blotting manifold (Immunetics) that isolated 40 longitudinal channels across the membrane. In each channel, a complex monoclonal antibody cocktail was added and allowed to hybridize for one hour. A total of 920 different monoclonal antibody specificities and 31 phospho-specificities were used. The blot was removed from the manifold, washed and allowed to hybridize for 30 minutes with secondary antibodies (goat anti-rabbit IRDye™ 800, Rockland; goat anti-mouse Alexa Fluor™ 680, Molecular Probes). The membrane was washed, dried and captured with an Odyssey® Infrared Imaging System (LI-COR). Signals were measured using PDQuest 2-D analysis software (Bio-Rad).

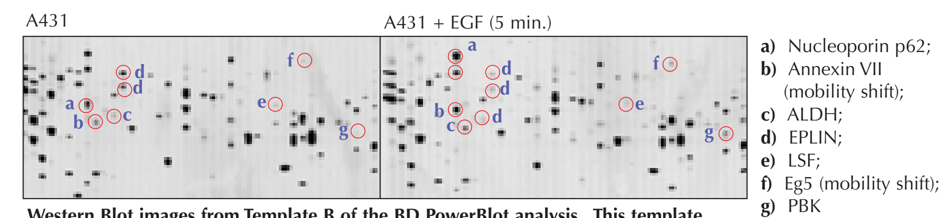
Immunofluorescence

Cells were prepared following the "Cell Staining for Immunofluorescence Microscopy" protocol (See "Support: Pharmingen/Transduction Laboratories Protocols" at www.bdbiosciences.com).

Conclusions

- BD PowerBlot analysis shows that EGF treatment of A431 cells leads to a decrease in expression of proteins involved in inhibition of cell proliferation and cell migration, and an increase in expression of proteins involved in nuclear import, chromosome regulation and MAPK signaling.
- BD Phospho-PowerBlot analysis shows that EGF treatment of A431 cells leads to phosphorylation of proteins involved in promotion of cell survival, proliferation and migration. A time course analysis of changes in phosphorylation shows a rapid increase after addition of EGF, followed by a decline in ERK and STAT phosphorylation, while caveolin phosphorylation remained high for the duration of the time course.
- Immunoprecipitation followed by western blot screening revealed a potential novel EGF-induced tyrosine phosphorylation of Bad that may be involved in promotion of cell survival
- A comparison of WT and kinase inactive EGFR showed significant differences in the EGF induced phosphorylation of Stat1, ERK1/2, p38MAPK and Caveolin-1, implicating these proteins in a cell proliferation pathway.

Panel I: BD PowerBlot Western array data: protein expression changes after exposure to EGF.



a) Nucleoporin p62;
b) Annexin VII (mobility shift);
c) ALDH;
d) EPLIN;
e) LSF;
f) Eg5 (mobility shift);
g) PBK

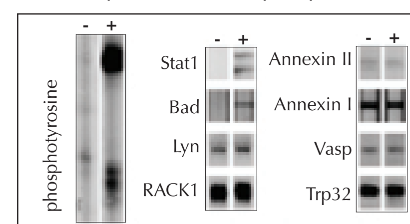
Western Blot images from Template B of the BD PowerBlot analysis. This template screens 212 of the 920 different antibody specificities used in the screen *a* through *i*, showing signals that significantly changed after exposure of A431 cells to EGF.

Panel II: Summary of PowerBlot Data, Templates A-E: EGF-Induced Changes in Expression

MAPK Pathway	Change	Proteome Summary
MEK1 (mobility shift)	+ 1.60	Activates ERK1/2
PBK	+ 4.02	MAPKK family member, may phosphorylate p38MAPK in mitotic cells
Regulators of cell proliferation		
ALDH	- 1.50	Biosynthesis of retinoic acid from retinol
Mxi-1	- 2.28	Inhibition of Myc DNA binding
Chromosome maintenance and regulation during mitosis		
Bub3	+ 2.65	Mitotic spindle checkpoint, responds to unattached kinetochores
Eg5 (mobility shift)	+ 2.61	Mitotic motor protein, regulates spindle formation
Lamin A/C	+ 1.69	Structural protein of the nuclear envelope and chromatin anchor site
LAP2	+ 2.24	Binds to lamins and chromosomes during disassembly and reassembly
RCC1	+ 1.69	Chromatin-associated, transcription, splicing, 3′-end formation, RNA export
Calcium Signaling		
Annexin VII (mobility shift)	+ 1.76	Ca2+ and phospholipid-binding protein; PDGF-induced Tyrosine phosphorylation
Cell Motility, Cell adhesion and Cytoskeletal Regulation		
ACTIN	+ 1.84	EGF induces actin stress fiber disassembly
EPLIN	- 1.86	Cytoskeletal protein: protein interactions
Gelsolin	- 1.58	Phosphoinsotide 4,5-bisphosphate binding; released during EGF induced motility
Headpin	- 5.50	Serine proteinase inhibitor that may be involved in regulation of cell migration
Cell Cycle Regulation		
LSF	- 2.78	Transcription factor that supports progression through S-phase
PKC delta	- - - -	Inhibits growth, enhances differentiation and apoptosis
Nuclear Transport, Protein Trafficking		
Nucleoporin p62	+ 3.33	Involved in the import of proteins containing nuclear localization signals
Regulators of apoptosis or differentiation		
Fas/CD95/APO-1	- - - -	Activates cell death upon ligand binding
Mcl-1	- - - -	Very labile due to PEST sequences, important early in differentiation

- - - = total loss of expression; - = decreased expression; + = increased expression

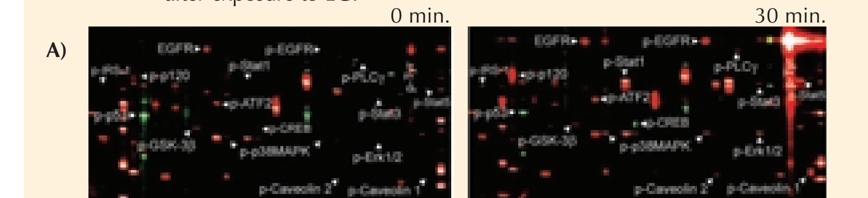
Panel III: Comparative Immunoprecipitation and Western Blot Screening of A431 Cells -/+ EGF



Anti-phosphotyrosine immunoprecipitation followed by Western Blot screening with ~200 monoclonal antibodies, summary of proteins detected.

Panel IV: BD Phospho-PowerBlot Western array data: changes in phosphorylation after exposure to EGF

A)

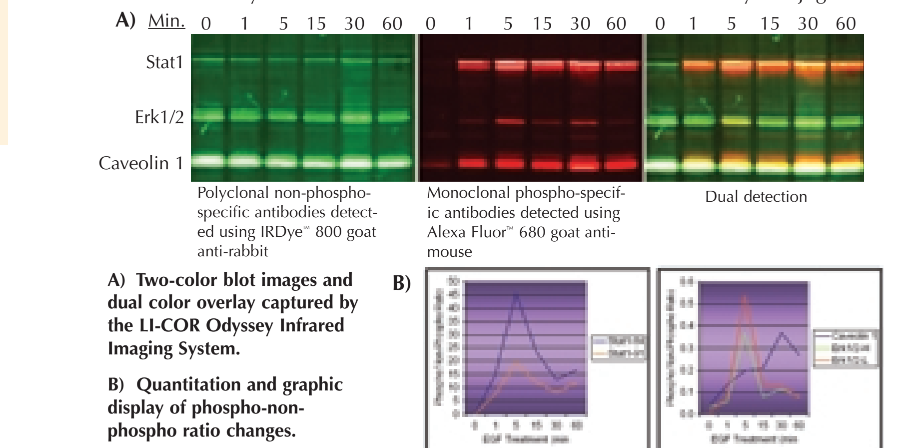


Western Blot images and summary from BD Phospho-PowerBlot analysis. This analysis screens with 31 phospho/non-phospho antibody pairs. A) 2-color blot images captured by the LI-COR® Odyssey® Infrared Imaging System. Polyclonal antibodies were detected using IRDye™ 800 goat anti-rabbit. Monoclonal antibodies were detected using Alexa Fluor™ 680 goat anti-mouse. B) Summary of changes in protein phosphorylation.

B)

EGF induced	Site	Change	Proteome Summary
Caveolin 1	Y14	++	Caveole formation
Caveolin 2	Y27	+	Forms hetero-oligomeric complex w/Caveolin 1
CREB	S133	+	Cis-acting cAMP response element mediated transcription
EGFR	activated	+++	Growth factor receptor tyrosine kinase
IRS-1	S616	++	Cell cycle progression and survival
p-Tyr		+++++	
p120 Catenin	Y228	++++	Cell adhesion/signaling
Phospholipase Cγ	Y783	+	Cell motility/mitogenesis
Stat1	Y701	++++	Activator of interferon stimulated genes
Stat3	Y705	++	Binds IL6-responsive elements/promotes acute-phase response
Stat5	Y694	++	Activated by EGF in cells overexpressing EGF receptor
p38MAPK	T180/Y182	+	Integration point for multiple biochemical signals
Erk1/2	T202/Y204	+	Integration point for multiple biochemical signals
Uninduced			
GSK-3b	Y216	no change	Ser/Thr Kinase involved in glycogen metabolism
p53	S392	no change	Growth control
FAK	Y397	no change	Adhesion, motility, cell growth and cytoskeletal organization
ATF2	T71	no change	CRE-mediated transcription, histone acetyltransferase

Panel V: EGF Treatment Time Course: Measuring the Ratio of Phospho-Protein Versus Total Protein by Simultaneous 2-Color Detection of Infrared Antibody Conjugates.



A) Two-color blot images and dual color overlay captured by the LI-COR Odyssey Infrared Imaging System.

B) Quantitation and graphic display of phospho-non-phospho ratio changes.

Panel VI: EGF Treatment of Cells Bearing A Wild-Type or EGF-Inactive Receptor.

EGF induced changes in phosphorylation.
A) IP/Westerns show the overall difference in induction of tyrosine phosphorylation, phospho-specific antibodies, and
B) Western blotting, or
C) Immunofluorescence, show more specific changes.