1. BLUE NATIVE PAGE AND MASS SPECTROMETRY ANALYSIS OF EPHRIN STIMULATION-DEPENDENT PROTEIN-PROTEIN INTERACTIONS IN NG108-EPHB2 CELLS

COSTEL C. DARIE*[1], VIVEKANANDA SHETTY, DANIEL S. SPELLMAN, GUOAN ZHANG, CHONGFENG XU, HELENE L. CARDASIS, STEVEN BLAIS, DAVID FENYO, AND THOMAS A. NEUBERT

Department of Structural Biology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 540 First Avenue, Lab 5-18, New York, NY 10016, USA

Abstract. Receptor tyrosine kinases (RTK) are proteins that undergo dimerization and/or multimerization and autophosphorylation in response to ligand stimulation. Members of the RTK family are receptors for a series of growth factors that, upon stimulation, are able to start signaling events that promote cell growth and differentiation. A class of RTKs, the Eph receptors (EphRs), are found in a variety of cell types and play important roles in patterning the central and peripheral nervous systems, as well as in synapse and neural crest formation. Interaction of Eph receptors with their ephrin ligands activates signal transduction pathways that lead to cytoskeletal remodeling through formation of many stable or transient protein-protein interactions. However, these intracellular signal transduction pathways that lead to cytoskeletal remodeling are not well understood. Here, we combined Blue Native PAGE (BN-PAGE) and mass spectrometry (MS) to analyze protein-protein interactions as a result of ephrin stimulation. We analyzed both lysates and phosphotyrosine immunoprecipitate (pY99-IP) of unstimulated and ephrin-stimulated cells. Our experiments allowed us to characterize many constitutive homo- and hetero-protein complexes from the cell lysate. Furthermore, BN-PAGE and MS of the pY99-IPs from both unstimulated and stimulated cells allowed us to analyze protein-protein interactions that resulted upon ephrin stimulation. Combination of BN-PAGE and MS also has the potential for the analysis of stable and transient protein-protein interactions in other ligand-stimulated RTK-dependent signal transduction pathways.

1. Introduction

Receptor tyrosine kinases are proteins that undergo dimerization and/or multimerization and autophosphorylation in response to ligand stimulation. Members of the RTK family are receptors for a series of growth factors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) that, upon stimulation, are able to start signaling events that promote

*Corresponding author. E-mail: darie@saturn.med.nyu.edu

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cell growth and differentiation. A common feature for these RTKs is that their growth factor ligands are secreted proteins.

A different class of RTKs is represented by Eph receptors (EphRs) and is the largest sub-family of RTKs. These EphRs are found in a variety of cell types from developing and mature tissues and play important roles in patterning the central and peripheral nervous systems (Holland et al., 1996, 1998; Wilkinson, 2001), synapse formation (Dalva et al., 2000), neural crest formation (Robinson et al., 1997), as well as angiogenesis and vascular system remodeling (Yancopoulos et al., 1998; Cheng et al., 2002; Adams, 2003; Wang et al., 2006). EphRs differ from other RTKs in three regards: (1) EphRs do not promote cell growth and differentiation, but rather play important roles in attractive and repellent reactions between the cells. (2) Activation of EphRs is performed by ephrins that, unlike the other RTK soluble ligands, are anchored to cell membranes. (3) During stimulation, EphRs may be either receptors that are activated by ephrin ligands and trigger the downstream signaling or they may be ligands that activate upstream signaling in ephrin-bearing cells.

To date, at least 14 different EphRs have been identified, and based on the type of ligand with which they interact, they have been divided into two classes: EphAs and EphBs (Gale et al., 1996). EphA receptors comprise nine members: EphA1-A8 and EphA10. They interact with ephrins A which consist of five members: ephrin A1-A5. EphB receptors comprise five members: EphB1-B4 and EphB6. They interact with ephrins B which consist of three members: ephrinB1-B3 (Gale et al., 1996). Although all EphRs and ephrinBs are transmembrane proteins that contain extracellular and cytosolic domains, ephrinsA are connected to the cell surface by glycosylphosphatidylinositol anchors, and therefore do not contain a cytosolic domain.

It is commonly agreed that interaction of ephrins with Eph receptors leads to a gradual formation of ephrin-EphRs multimers that trigger cytoskeletal remodeling. This underlies cell adhesion and motility in the ligand and receptor bearing cells (Miao et al., 2000; Carter et al., 2002; Vearing and Lackmann, 2005). However, the intracellular signal transduction pathways that lead to cytoskeletal remodeling in the ephrin- or EphR-bearing cells are not well understood. Understanding of these pathways is further complicated by the bidirectional signaling that occurs between EphB receptors and their ephrinB ligands (Holland et al., 1996; Bruckner et al., 1997; Holland et al., 1998; Cowan and Henkemeyer, 2001).

Upon activation of signal transduction pathways, many transient protein-protein interactions occur, leading to the formation of different protein complexes. These complexes form during the transition of the cells from the unstimulated to stimulated state. Knowledge about the structural and functional interaction of the proteins into protein complexes as a result of ephrin stimulation will lead to a more comprehensive understanding of ephrin signaling.

BN-PAGE has long been used to analyze protein-protein interactions and protein complexes (Schagger and von Jagow, 1991; Schagger et al., 1994; Darie et al., 2005; Darie et al., 2007). This method separates protein complexes based on external charge induced by Coomassie dye and according to their molecular mass

(mass). Separation of protein complexes from the BN-PAGE lane by second dimension SDS-PAGE may reveal the subunit composition of a particular protein complex, as well as the interacting partners of a particular protein. MS (Aebersold and Mann, 2003) is another tool that allows scientists to identify not only one but most potential interacting partners of a protein involved in signal transduction pathways.

In this paper, we combined BN-PAGE and MS to analyze protein complexes from the lysates and pY99-IPs of unstimulated and ephrin-stimulated cells. Our experiments allowed us to determine the molecular mass and subunit composition of many constitutive homo- and hetero-protein complexes from cell lysates. Furthermore, BN-PAGE and MS of the pY99-IPs from both unstimulated and stimulated cells allowed us to analyze protein-protein interactions that resulted from ephrin stimulation. Combination of BN-PAGE and MS also has the potential to be applied to the analysis of stable and transient protein-protein interactions in other ligand-stimulated RTK-dependent signal transduction pathways.

2. Experimental Design

These studies have been designed based on the hypothesis that specific protein complexes are formed to accomplish cell signaling and that deciphering the composition of these complexes will reveal significant insights about mechanisms of ephrin signaling.

2.1. CELL SYSTEM AND ACTIVATION OF THE SIGNAL TRANSDUCTION PATHWAYS

The strategy for analyzing protein complexes from EphB2 signaling in unstimulated and ephrinB1-Fc-stimulated NG 108 cells is shown in Figure 1. Unstimulated NG 108 cells that stably express EphB2 receptors (NG108-EphB2) were stimulated with soluble, pre-clustered ephrinB1-Fc for 45 min at 37°C, according to a published procedure (Zhang et al., 2006). The control (unstimulated) cells were treated with soluble, pre-clustered IgG-Fc. Once the cells had been stimulated and the signal transduction pathways had been activated, the cells were lysed. The lysates of unstimulated and stimulated NG 108 cells were separated by BN-PAGE or were used as starting material for antibody-based purification (phosphotyrosine immunopurification: pY99-IP). The pY99-IPs were also separated by BN-PAGE. The protein complexes that resulted were then subjected to biochemical and/or MS analysis (BN-PAGE and Western blotting [WB] or BN-PAGE and MS), followed by comparison of the data that resulted from analysis of unstimulated and stimulated cells and ultimately with the current literature.

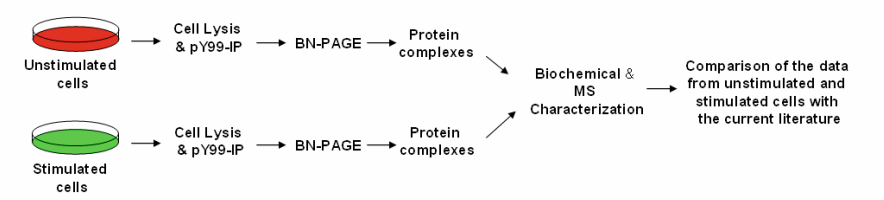

Figure 1. A workflow for the biochemical and MS characterization of ephrin signaling. The unstimulated or stimulated cells were lysed and the lysates (and/or the pY99-IPs from the lysate) were separated by BN-PAGE. The protein complexes/protein interactions were characterized biochemically and/or by MS, followed by comparison with the current literature

2.2. BIOCHEMICAL CHARACTERIZATION OF PROTEIN COMPLEXES

BN-PAGE was used for separation of stable or transient protein complexes from cell lysates or pY99-IPs by using one-dimensional (BN-PAGE; 1D) or two-dimensional (BN-PAGE; 2D) electrophoresis. A schematic of biochemical characterization of the protein complexes is presented in Figure 2. BN-PAGE (1D) gel lanes were silver stained or electroblotted and analyzed with antibodies against proteins of interest. In addition, the BN-PAGE gel lanes were used for MS analysis. The protein complexes that were separated in the BN-PAGE (1D) were also separated under reducing and denaturing conditions in (2D) and analyzed by either silver staining (2D-SS) or WB (2D-WB) (Figure 2A).

The data that resulted from these experiments were interpreted based on differences between the lysates/pY99-IPs of unstimulated and stimulated cells that were separated by BN-PAGE and either 2D-SS, 2D-WB or MS. For example, the simplest scenario for the biochemical analysis of EphB2-associated proteins upon ephrin-Fc stimulation is schematically represented in Figures 2B and C. It is known that EphRs assemble into multimers upon ephrinB1-Fc stimulation. Therefore, the mass of the EphB2-containing protein complex will shift towards a higher mass, and will be visible as a shift in BN-PAGE (1D-WB) and/or 2D-WB (Figure 2B). By comparing the WB results from the unstimulated and stimulated cells, the mass of the EphB2-containing protein complex may be determined. Furthermore, MS analysis of the BN-PAGE gel bands that contain EphB2 from both unstimulated and stimulated cells may provide the identity of the EphB2 interactors (Figure 2B). Another scenario for the biochemical analysis of EphB2-associated proteins upon ephrin-Fc stimulation is shown in (Figure 2C). In this case, it may be determined whether two different proteins (e.g., EphB2 and FAK) are or are not part of the-same protein complex before stimulation and what are their protein interactors. Furthermore, it may also be determined whether these two proteins associate into a protein complex or form two different complexes as a result of ephrin-Fc stimulation as well as their subunit composition (Figure 2C).

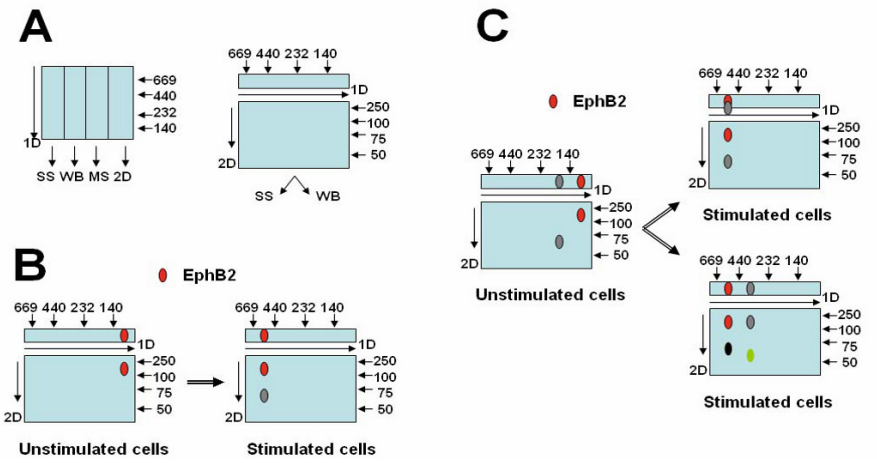

Figure 2. Biochemical characterization of ephrin signaling. (A) The starting material (lysate or pY99-IP) was separated by BN-PAGE (1D) and further used for SS, WB, MS or 2D analysis. The (2D) gel that resulted from separation of the (1D) gel lane was further used for SS or WB. (B) Comparison of the data resulting from either BN-PAGE (1D) and MS or BN-PAGE (2D) and WB of unstimulated and stimulated cells may lead to identification of the ligand stimulation-dependent protein interactors of a particular protein. (C) As in (B), except that the two different proteins investigated may interact with each other as a result of ligand stimulation, or form separate complexes with different interactors

Therefore, combination of the BN-PAGE and MS may provide information about the size of the protein complex and about the number and identities of the proteins that associate with EphB2-receptors and other signal transducing molecules. Furthermore, these experiments may allow analysis of both stable and transient protein-protein interactions as a result of ephrinB1-Fc stimulation.

2.3. MASS SPECTROMETRIC CHARACTERIZATION OF PROTEIN COMPLEXES

The general strategy for the MS characterization of the protein complexes involved in ephrin signaling is shown in Figure 3. The BN-PAGE gel lanes that contained the protein complexes were cut into 20–30 gel pieces and subjected to enzymatic digestion (tryptic digest). The resulting peptide mixture was then analyzed by reverse phase liquid chromatography and MS (LC-MS-MS/MS: NanoAquity LC and Waters Q-Tof Premier MS), and the raw data were processed and submitted to a database search using Mascot for protein identification. These experiments provided a list of proteins for each gel band that may be part of the same protein complex. The outcomes from these experiments were compared between the unstimulated and stimulated cells and then compared with the results obtained using biochemical analysis of the protein complexes. All these results were ultimately compared with the current literature.

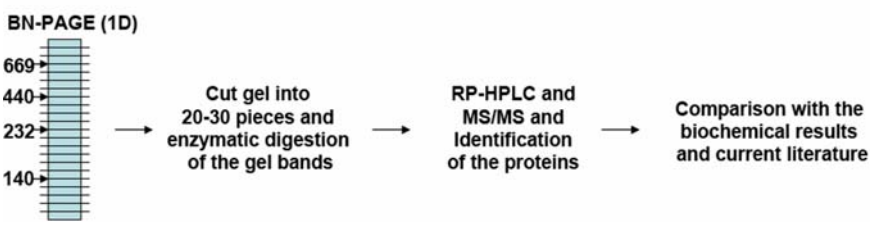

Figure 3. MS characterization of ephrin signaling. BN-PAGE (1D) gel bands were cut into pieces, digested with trypsin, and the peptides separated by LC (Nano-Aquity) and analyzed by MS (Waters Q-Tof Premier). The identified proteins were compared with the biochemical results and current literature

3. Results

3.1. ANALYSIS BY GEL ELECTROPHORESIS OF LYSATES FROM UNSTIMULATED AND EPHRINB1-FC STIMULATED CELLS

Initially, the lysates from both unstimulated and stimulated cells were separated by SDS-PAGE and then stained with either silver (Figure 4A) or Coomassie (data not shown). No significant or visible differences in the protein pattern between the two gel lanes were observed. However, when the SDS-PAGE gel was electroblotted and incubated with pY99 antibodies, significant differences between the two lysates were observed. The lysate from the stimulated cells contained more phosphorylated proteins than the lysate from the unstimulated ones, especially in the 100–200 kDa range and 60–75 kDa range. This experiment suggested that, although not visible in either silver- or Coomassie-stained gels, differences between the unstimulated and stimulated cells may be detected. Based on these data, we also concluded that the differences between the unstimulated and stimulated cells observed by WB were as a result of ephrinB1-Fc stimulation, and that the signal transduction pathways were activated.

The lysates from both unstimulated and stimulated cells were also analyzed by BN-PAGE. BN-PAGE separates protein complexes based on the external charge induced by Coomassie Blue dye under native conditions and according to their mass. Therefore, under these experimental conditions, both stable and many transient protein interactions are preserved. In order to determine the differences between the lysates from unstimulated and stimulated cells that appeared as a result of eprinB1-Fc stimulation, we separated these lysates by BN-PAGE (1D) or (2D) (Figure 5). In the silver-stained BN-PAGE (1D) gel lanes, after visual inspection and based on the mass markers, we could observe intense bands that corresponded to protein complexes with a mass of about 750, 670, 600, 440 and 400 kDa (Figure 5A). In the silver stained (2D) gels (Figure 5B), some proteins with a mass of

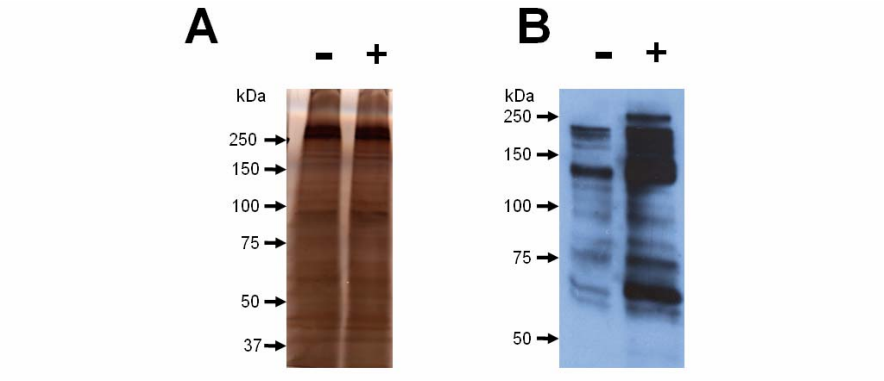

Figure 4. Analysis of lysates of unstimulated (–) and ephrin-stimulated (+) cells by SDS-PAGE. (A) The gel was silver stained (A, 1 µl loaded/lane) or electroblotted and incubated with pY99 antibodies (B, 5 µl loaded/lane). The mass markers are shown to the left of each gel

around 100–150 kDa, corresponded in (1D) gels (Figure 5A) to a mass between 150–700 kDa. This suggests that BN-PAGE separates in (1D) protein complexes that, upon reducing and denaturing are resolved into their subunits in (2D), with a mass smaller than the protein complex itself (assuming that the mass standards in the 1D [BN] gel are equivalent to the mass standards in the second [SDS-PAGE] dimension).

In order to identify differences between the unstimulated and stimulated cells as a result of ligand stimulation, we initially inspected the gel lanes that contained lysates from unstimulated or stimulated cells in either BN-PAGE (1D; Figure 5A) or 2D (Figure 5B) gels. No significant differences were observed. However, when the BN-PAGE (1D) gel lanes (data not shown) or (2D) gels (Figure 5C) were electroblotted and incubated with pY99 antibodies, significant differences between the lysates from unstimulated and stimulated cells were observed (Figure 5C). The lysate from the stimulated cells contained more phosphorylated proteins than the lysate from the unstimulated ones, in agreement with our previous data (Figure 4B) and current literature (Zhang et al., 2006), and further confirmed that the differences between the unstimulated and stimulated cells are as a result of ephrinB1-Fc stimulation. The most significant changes in the lysate from the stimulated cells in the 2D gels were observed between 100–200 kDa, 60–70 kDa, and 35–50 kDa (Figure 5C). The phosphorylated proteins from the 2D gels corresponded to a mass of approximately 100–250 kDa in the BN-PAGE (1D) under native conditions. This suggests that, under these experimental conditions (native, non-denaturing conditions), most of the tyrosine phosphorylated proteins (especially the ones with low molecular mass) observed in (2D) are part of different protein complexes in BN-PAGE (1D), with a mass ranging from 100–250 kDa (Figure 5C), again assuming the mass determinations based on standards in BN-PAGE are equivalent to

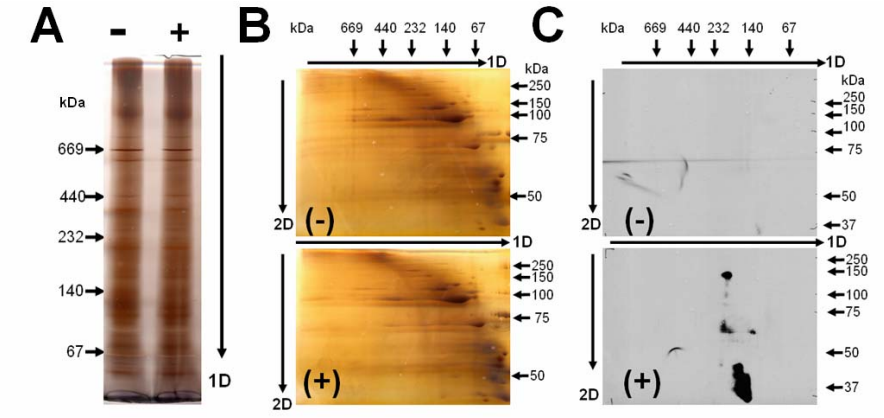

Figure 5. Analysis of the lysate of unstimulated (–) and ephrin-stimulated (+) cells by BN-PAGE. The lysates were separated in BN-PAGE (1D) (A) or BN-PAGE (1D) and SDS-PAGE (2D) (B, C) and either silver stained (A, B) or electroblotted and analyzed with pY99 antibodies. For (A), 10 µl were loaded/lane. For (B and C), 40 µl were loaded/lane in (1D). The mass markers are indicated for each gel

those in SDS-PAGE. Taken together, these data suggest that, upon stimulation, the signal transduction pathways are stimulated and the proteins involved in signaling may become phosphorylated and transiently associate in protein complexes.

3.2. ANALYSIS OF LYSATES FROM UNSTIMULATED AND BDNF-STIMULATED NEURONS BY BN-PAGE

To further confirm that BN-PAGE is suitable for analysis of protein-protein interactions as a result of ligand stimulation, we analyzed cell lysates of mouse cortical neurons that were stimulated by a different ligand, brain-derived neurotrophic factor (BDNF) that binds to Trk receptors. In this system, we focused on one particular protein, Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs, mass 115 kDa). The Hrs protein is involved in endocytic processes and delivers receptors from endosomes to lysosomes where they are degraded as a result of ligand stimulation (Lu et al., 1998; Kobayashi et al., 2005). Therefore, we separated the cell lysates of neurons that were treated or not treated with BDNF by BN-PAGE (2D-WB) and incubated the blot membranes with HRS antibodies, followed by enhanced chemiluminescence detection. As may be seen in Figure 6, the Hrs antibodies recognized a band in the 2D blot at about 120 kDa, in agreement with the theoretical mass of Hrs. The Hrs antibody-reactive band corresponded in the native (1D) blot to a mass close to 200 kDa (unstimulated lysate) or 200 and 500 kDa (stimulated lysate). It is unclear if the Hrs proteins detected at about 200 kDa in (1D) are monomers or not.

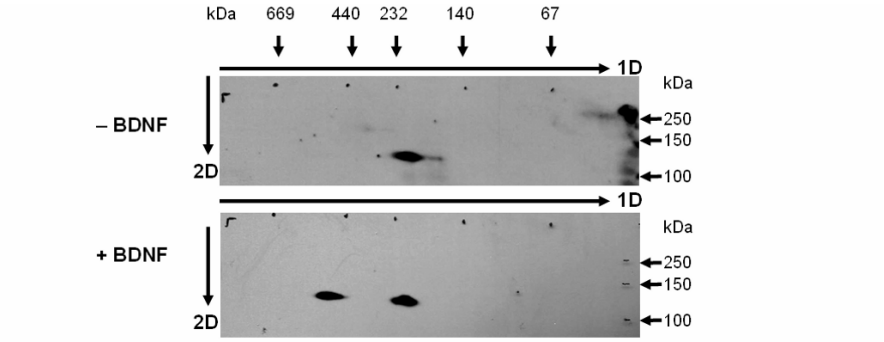

Figure 6. Analysis of lysates of unstimulated (–) and BDNF-stimulated (+) primary neuronal cell cultures by BN-PAGE. The lysates were separated in BN-PAGE (1D) and SDS-PAGE (2D), electroblotted, and visualized with Hrs antibodies. Forty µl of lysate were loaded/lane

Taken together, these experiments suggest that, upon stimulation, the Hrs protein assembles into higher mass complexes as a result of BDNF treatment. These data also suggest that, regardless of the cell system, as long as there are two different experimental conditions, they may be compared by BN-PAGE and the differences between the two states may be determined. Furthermore, the protein interactions that appear as a result of ligand stimulation (e.g., the protein(s) that interact(s) with Hrs as a result of BDNF stimulation) may be identified by MS (see below).

3.3. ANALYSIS OF LYSATES FROM UNSTIMULATED AND EPHRINB1-FC STIMULATED CELLS BY BN-PAGE AND MS

By visual inspection, in the silver-stained (Figure 5A) and Coomassie-stained (Figure 7) BN-PAGE (1D) gel lanes we could observe putative protein complexes with masses ranging from about 750 to about 400 kDa. In order to reveal the protein composition of some of these complexes, we cut out individual gel bands that corresponded to masses between 400 and 750 kDa (Figure 7), digested them with trypsin and analyzed them by LC-MS/MS. Once a subunit of a particular protein complex with a specified mass was identified, we looked for additional subunits in the same gel band and further compared these data with the current literature in terms of subunit composition, mass, protein interactions, growth factor-dependent inducibility, etc. In these experiments, we identified both homo- and hetero-complexes, most of them constitutively expressed in many mammalian cells. Among putative homocomplexes, we identified valosin-containing protein,, also named transitional endoplasmic reticulum ATPase, an 89 kDa endoplasmic reticulum membrane protein. This protein was found in bands B3 and B4, which corresponded

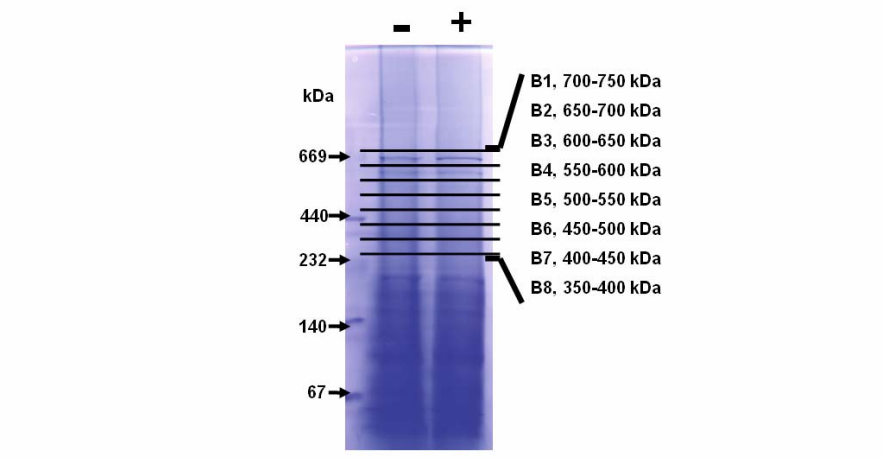

Figure 7. BN-PAGE of lysates of unstimulated (–) and ephrin-stimulated (+) NG108-EphB2 cells. The gel was Coomassie stained and the gel bands of interest (bands B1 to B8) were excised and subjected to MS analysis. The approximate masses of the bands were between 700–750 (band B1) and 350–400 kDa (band B8), as shown on the right side of the gel. The molecular mass markers are shown on the left side of the gel. Eighty microliters of lysate were loaded/lane

to masses of 550–650 kDa. A report that this 89 kDa protein is usually a homo-hexamer (DeLaBarre and Brunger, 2003), with predicted molecular mass (under native conditions) of 540 kDa, is in agreement with the mass determined experimentally by BN-PAGE.

The proteasome (prosome) was among the heterocomplexes identified by BN-PAGE and MS. This complex is involved in protein degradation and is composed of 28 alpha (mass 27 kDa) and beta (mass 29 kDa) subunits (McNaught et al., 2001), of which we identified five different subtypes of subunit alpha (alpha 1, 3, 4, 6 and 7 subunits) and five different subtypes of beta subunit (beta 1, 4, 5, 6 and 8 subunits) in a single experiment. Our experimentally determined mass of the proteasome was 650–750 kDa (detected in bands B1 and B2), in agreement with its calculated theoretical mass of 700 kDa. Examples of MSMS spectra of identified peptides that were part of either valosin-containing protein (Figure 8A) or proteasome subunits alpha 6 (Figure 8B) and beta 6 (Figure 8C) are shown in Figure 8. Taken together, these data suggest that the combination of BN-PAGE and MS is a powerful tool for determining the mass of a particular protein complex and identity of its subunits.

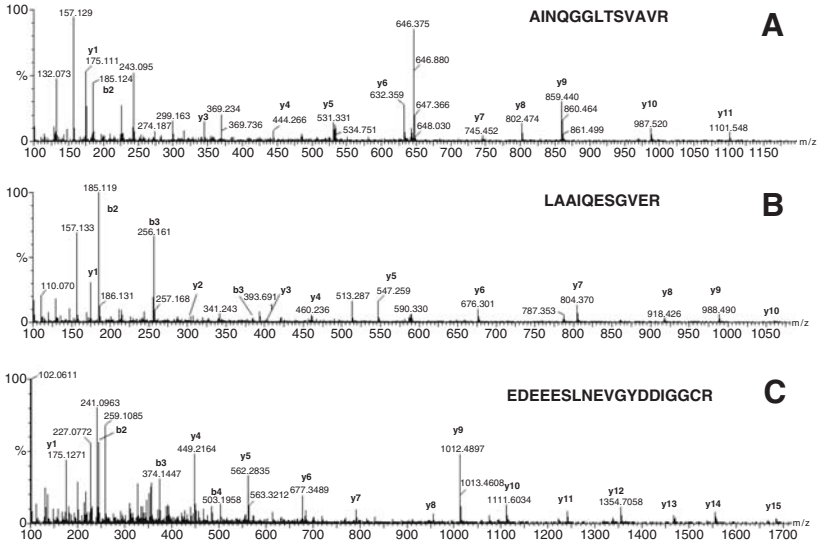

Figure 8. MS analysis of the BN-PAGE gel bands B1 (700–750 kDa) and B4 (550–600 kDa). (A) MS/MS of the *m/z* (2+) 643.33 (calculated 643.35) peak that corresponds to peptide AINQGGLTSVAVR, which is part of the proteosome alpha subunit 6 (mass 27 kDa), a 700 kDa alpha-beta heterocomplex. (B) MS/MS of the *m/z* (2+) 586.79 (calculated 586.81) peak that corresponds to peptide LAAIQESGVER, which is part of the proteosome beta subunit 6 (mass 29 kDa), a 700 kDa alpha-beta heterocomplex. (C) MS/MS of the *m/z* (2+) 1,093.49 (calculated 1,093.43) peak that corresponds to peptide EDEEESLNEVGYDDIGGCR, which is part of the valosin-containing protein (mass 89 kDa), which forms a 540 kDa homohexamer

3.4. ANALYSIS OF PY99-IPS FROM UNSTIMULATED AND EPHRINB1-FC STIMULATED CELLS BY GEL ELECTROPHORESIS AND MS

In the previous analyses of the lysates from unstimulated and stimulated cells using BN-PAGE, we could observe differences between the two states only at the WB level, with weak signals suggesting low amounts of protein. In order to identify the protein complexes that resulted upon stimulation, the cell lysates could not be used for MS analysis, due to the high complexity of the samples, large amounts of background proteins and low abundance of the proteins of interest. Therefore, we focused only on the proteins that were tyrosine phosphorylated or associated with phosphorylated proteins as a result of ephrinB1-Fc stimulation. Specifically, we performed pY99-IPs from the lysates of both unstimulated and stimulated cells. Initially we performed a small scale analysis of the pY99-IPs and separated them by either SDS-PAGE or BN-PAGE, followed by MS analysis. SDS-PAGE and MS were used to identify the differences between the proteins immunopurified by pY99 antibodies of the unstimulated and stimulated cells, while BN-PAGE and

MS were used to determine the interactions between the proteins identified by SDS-PAGE and MS that resulted upon ephrinB1-Fc stimulation.

Tables 1 and 2 show the proteins identified by SDS-PAGE and MS of pY99-IPs from unstimulated (Table 1) and stimulated (Table 2) cells. Very few proteins known to be involved in signal transduction were identified in the pY99-IPs of

TABLE 1. Proteins identified by MS analysis of the SDS-PAGE of the pY99-IP of unstimulated cells. The searching parameters were: database: NCBI_mouse_rat_FR, enzyme: trypsin, maximum missed cleavages: 1, fixed modifications: cysteine to carbamidomethyl, variable modifications: none, mass values: monoisotopic, peptide mass tolerance: 0.3 Da, fragment mass tolerance: 0.6 Da. The pkl files were recalibrated post-acquisition using in-house designed software. The NCBI accession number, protein name, Mascot score and molecular mass are shown for each identified protein

Protein Acc. #	Protein description	Score	Mw
gi|51921343	hypothetical protein LOC432987	1445	35144
gi|29244176	hypothetical protein 4732456N10	752	58230
gi|19705459	poly(A) binding protein, cytoplasmic 1	648	70656
gi|14861844	PL10 protein	507	73095
gi|13242237	heat shock protein 8	473	70827
gi|6671569	acidic ribosomal phosphoprotein P0	328	34195
gi|40068493	DEAD box polypeptide 17 isoform 1	301	72539
gi|42558248	GPI-anchored membrane protein 1	267	78121
gi|24429590	DEAH (Asp-Glu-Ala-His) box polypeptide 9	202	149489
gi|7949053	heterogeneous nuclear ribonucleoprotein A2/B1 isoform 1	198	35971
gi|31559916	heterogeneous nuclear ribonucleoprotein A3 isoform b	193	39628
gi|6754256	heat shock protein 9A	191	73483
gi|25742763	heat shock 70kD protein 5	177	72302
gi|23956214	splicing factor proline/glutamine rich	175	75394
gi|34328400	splicing factor, arginine/serine-rich 1 (ASF/SF2)	174	27728
gi|9506497	clathrin, heavy polypeptide (Hc)	168	191477
gi|6679741	PTK2 protein tyrosine kinase 2	129	119141
gi|10946928	heterogeneous nuclear ribonucleoprotein H1	110	49168
gi|6754222	heterogeneous nuclear ribonucleoprotein A/B	107	30812
gi|6754220	heterogeneous nuclear ribonucleoprotein A1 isoform a	101	34175
gi|7949051	heterogenous nuclear ribonucleoprotein U	94	87837
gi|20982845	pigpen	93	52642
gi|30794412	TAF15 RNA polymerase II, TATA box binding protein-associated	93	58566
gi|21703842	hypothetical protein LOC28088	87	55214
gi|31980689	small nuclear ribonucleoprotein polypeptide A	81	32245
gi|19527256	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	77	82448
gi|18087805	ribosomal protein S2	75	31212
gi|9789893	BRG1/brm-associated factor 53A	75	47399
gi|13385872	interleukin enhancer binding factor 2	74	43035
gi|7305247	P lysozyme structural	66	16783
gi|6671509	actin, beta, cytoplasmic	62	41710
gi|9790109	heterogeneous nuclear ribonucleoproteins methyltransferase-like 2	57	42408
gi|11024680	GERp95	57	97597
gi|13384620	heterogeneous nuclear ribonucleoprotein K	54	50944
gi|13507601	regulator of nonsense transcripts 1	51	122606
gi|31982724	MYB binding protein (P160) 1a	50	151942
gi|13242328	NS1-associated protein 1 isoform 1	50	69727
gi|34867525	PREDICTED: similar to T-complex protein 1 subunit theta	48	59550
gi|6754254	heat shock protein 1, alpha	47	84735
gi|33859724	heterogeneous nuclear ribonucleoprotein R	45	70844
gi|13592057	ribosomal protein L18	45	21645
gi|7305443	ribosomal protein L7a	42	29958
gi|51593084	SWI/SNF related, matrix associated, actin dependent regulator	38	180609
gi|6677805	ribosomal protein S4, X-linked	36	29579
gi|8567402	splicing factor, arginine/serine-rich 3 (SRp20)	36	19318

TABLE 2. Proteins identified by MS analysis of the SDS-PAGE gel of the pY99-IP of ephrinB1-Fc stimulated cells. The searching parameters were: database: NCBI_Mouse_rat_FR, enzyme: trypsin, maximum missed cleavages: 1, fixed modifications: cysteine to carbamidomethyl, variable modifications: none, mass values: monoisotopic, peptide mass tolerance: 0.3 Da, fragment mass tolerance: 0.6 Da. The pkl files were recalibrated post-acquisition using in-house designed software. The NCBI accession number, protein name, Mascot score and molecular mass are shown for each identified protein

Protein Acc.#	Protein description	Score	Mw
gi|47777351	Eph receptor B2	2306	111311
gi|51921343	hypothetical protein LOC432987	1120	35315
gi|13242237	heat shock protein 8	1111	71055
gi|6671569	acidic ribosomal phosphoprotein P0	667	34366
gi|6754256	heat shock protein 9A	521	73768
gi|25742763	heat shock 70kD protein 5	492	72473
gi|19705459	poly(A) binding protein, cytoplasmic 1	479	70884
gi|47059093	Eph receptor B4	354	110370
gi|9506497	clathrin, heavy polypeptide (Hc)	277	193187
gi|29244176	hypothetical protein 4732456N10	233	58629
gi|40068491	DEAD box polypeptide 17 isoform 2	223	46905
gi|6671509	actin, beta, cytoplasmic	206	42052
gi|42558248	GPI-anchored membrane protein 1	169	78292
gi|6679741	PTK2 protein tyrosine kinase 2	167	119939
gi|9790069	HLA-B-associated transcript 1A	166	49460
gi|9789893	BRG1/brm-associated factor 53A	160	47913
gi|6753620	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	155	73455
gi|9790109	heterogeneous nuclear ribonucleoproteins methyltransferase-like 2	155	43035
gi|9506371	actin, alpha 1, skeletal muscle	136	42366
gi|14192922	actin, alpha, cardiac	136	42334
gi|6754222	heterogeneous nuclear ribonucleoprotein A/B	131	30926
gi|7949051	heterogenous nuclear ribonucleoprotein U	131	88635
gi|34867525	PREDICTED: similar to T-complex protein 1 subunit theta	129	60121
gi|31560613	chaperonin subunit 8 (theta)	118	60087
gi|31980689	small nuclear ribonucleoprotein polypeptide A	114	32245
gi|9506571	eukaryotic translation initiation factor 2, subunit 1 alpha	110	36371
gi|8393418	glyceraldehyde-3-phosphate dehydrogenase	95	36090
gi|21450625	eukaryotic translation initiation factor 4A1	92	46353
gi|6677813	ribosomal protein S8	92	24475
gi|7305443	ribosomal protein L7a	91	30129
gi|30794450	ribosomal protein L4	90	47409
gi|6755682	serine/threonine kinase receptor associated protein	90	38774
gi|7949053	heterogeneous nuclear ribonucleoprotein A2/B1 isoform 1	89	36028
gi|22122585	splicing factor, arginine/serine-rich 7	82	27589
gi|12018306	nucleolin-related protein	82	77450
gi|27687455	PREDICTED: similar to cytoplasmic beta-actin	75	42278
gi|6981248	nucleolin	74	77158
gi|6679939	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	72	48096
gi|6678469	tubulin, alpha 6	70	50562
gi|6754254	heat shock protein 1, alpha	68	85134
gi|13385998	TNF receptor-associated protein 1	68	80501
gi|46275814	neurofilament, heavy polypeptide	66	115498
gi|6679108	nucleophosmin 1	63	32711
gi|6755110	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	62	85439
gi|8393693	laminin receptor 1	60	32917
gi|9507245	tyrosine 3-monooxgenase/tryptophan 5-monooxgenase activation protein, gamma	60	28456
gi|24429590	DEAH (Asp-Glu-Ala-His) box polypeptide 9	56	150914
gi|37674216	RIKEN cDNA G430041M01	54	32354
gi|6753324	chaperonin subunit 6a (zeta)	53	58424
gi|6756041	tyrosine 3-monooxgenase/tryptophan 5-monooxygenase activation protein, zeta	51	27925
gi|31543689	arginine/serine-rich splicing factor 6	50	39120
gi|31543313	non-catalytic region of tyrosine kinase adaptor protein 2	50	43008
gi|8394018	protein phosphatase 2a, catalytic subunit, alpha isoform	49	36156
gi|6678053	small nuclear ribonucleoprotein B	45	23811
gi|6680047	guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1	39	35511
gi|9845257	histone 1, H1c	38	21254
gi|40254593	breast cancer anti-estrogen resistance 1	37	94455

unstimulated (Table 1) compared with the stimulated (Table 2) cells. Since we analyzed pY99-IP samples, these data suggest that many of the proteins from the unstimulated cells involved in ephrinB1 signaling are not phosphorylated at tyrosine residues and not associated with a phosphotyrosine protein (Table 1). On the other hand, most of the pY99-immunopurified proteins from the stimulated cells involved in ephrinB1 signaling are either tyrosine phosphorylated, or associated with a phosphotyrosine protein, or both (Table 2). These data are in agreement with our previous experiments, in which WB with pY99 antibodies of the lysate separated by both SDS-PAGE (Figure 4B) and BN-PAGE (Figure 5C) showed a higher degree of tyrosine phosphorylation of the proteins from the stimulated cells. These data are also in agreement with a study from our laboratory, in which SILAC technology was employed (Zhang et al., 2006). Perhaps one of the most important proteins identified in the SDS-PAGE of pY99-IP of unstimulated cells is PTK2 (protein tyrosine kinase 2), a 130 kDa protein, also named focal adhesion kinase (FAK) (Table 1; see below). On the other hand, among the proteins identified in the SDS-PAGE of pY99-IP of stimulated cells and known to be involved in ephrin signaling (Zhang et al., 2006), two important proteins were identified: PTK2 (protein tyrosine kinase 2 or FAK, 130 kDa) and breast cancer anti-estrogen resistance 1, a 95 kDa protein, also named p130Cas. These two proteins are known to interact with each other via a SH3 domain (Polte and Hanks, 1995) and their identification in the pY99-IP from the stimulated cells (but only of FAK in the unstimulated ones) may suggest that FAK and p130Cas interact with each other as a result of ephrin stimulation. Since FAK was also identified in the pY99-IP of unstimulated cells, it is possible that the phosphorylated FAK interacts with p130Cas (and phosphorylates it) as a result of ephrin stimulation. Alternatively, FAK may not be phosphorylated in the pY99-IP of stimulated cells, but rather associated with phosphorylated p130Cas or with other unknown phosphorylated proteins.

In order to evaluate these hypotheses, we separated the pY99-IPs from both unstimulated and stimulated cells by BN-PAGE (1D), cut out the gel bands as shown in Figure 9 (left) and analyzed them by MS (Darie and Neubert, 2008). Not surprisingly, we identified FAK in the gel bands from unstimulated (estimated masses in the 130–170 kDa and 190–210 kDa range) and stimulated (estimated masses in the 150–170 kDa and 230–250 kDa range) cells (Figure 9, right). Since the mass of FAK is 130 kDa, this suggests that the protein identified at about 130–170 kDa is in a monomeric state, while the 190–210 kDa (from unstimulated cells) and 230–250 kDa (from stimulated cells) bands contain FAK in protein complexes. We also identified p130Cas (95 kDa) in the gel band from stimulated (estimated mass 230–300 kDa) but not unstimulated cells (Figure 9, right), in agreement with the previous SDS-PAGE and MS experiments (Tables 1 and 2). Since (1) FAK shifted from the monomeric state in unstimulated cells towards a higher mass in the stimulated cells upon stimulation; (2) p130Cas was identified in both SDS-PAGE & MS and BN-PAGE & MS as a result of ephrin stimulation; (3) it is well established that FAK and p130Cas interact with each other; and (4) FAK

(130 kDa) and p130Cas (95 kDa) were identified in the same BN-PAGE gel band with an approximate mass of 230–300 kDa, it is reasonable to conclude that FAK and p130Cas form a heterocomplex as a result of ephrin stimulation. It remains to be determined which one of these proteins (FAK, p130Cas, neither or both) are phosphorylated in the FAK-p130Cas complex. Taken together, these data suggest that SDS-PAGE, MS and BN-PAGE and MS of the pY-IPs from both unstimulated and stimulated cells are a useful system for analysis of both stable and transient protein-protein interactions.

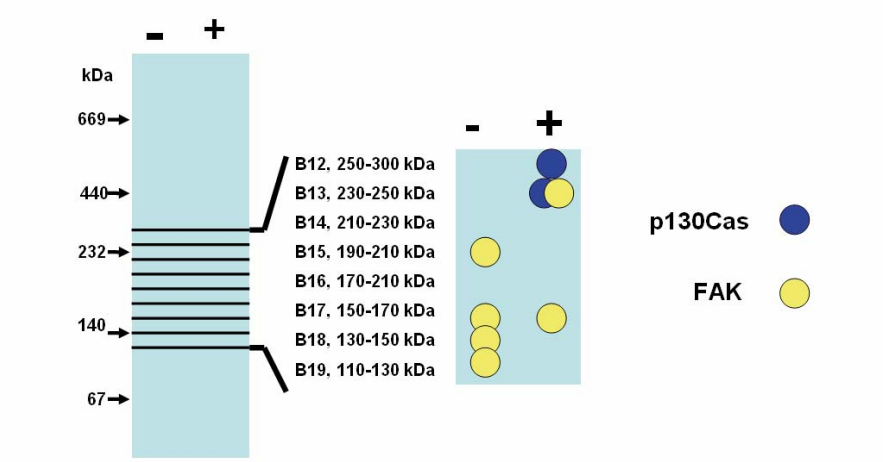

Figure 9. Representation of BN-PAGE of the pY99-IPs of unstimulated (–) and ephrin-stimulated (+) cells. Left: The gel was Coomassie stained and the gel bands of interest (bands B12 to B19) were excised and analyzed by MS. The approximate mass of the bands was between 250–300 (band B12) and 110–130 kDa thousand (band B19), as shown on the right side of the gel. The molecular mass markers are shown on the left side of the gel. Right: The approximate position in the BN-PAGE gel (mass) of the FAK and p130 Cas proteins, as determined by MS experiments. The legend of the FAK and p130 Cas proteins is also shown (right)

4. Discussion

Upon activation of signal transduction pathways, many transient protein-protein interactions occur, leading to the formation of different protein complexes. These complexes form during the transition of the cells from the unstimulated to stimulated state. Analysis of signal transduction pathways generally starts with the identification of proteins that interact with the activated receptors. Once these proteins are initially identified, further interacting partners may be identified and the function of these proteins may be studied. A very common method for studying protein-protein interactions is immunoprecipitation by one antibody followed by WB of the immunoprecipitate with a different antibody. Unfortunately, this method may be employed only if preliminary data already exists and candidate proteins

are identified. For example, if the interaction of two proteins is already established by a different method such as two-hybrid screen, immunoprecipitation and WB experiments may be confirmative. Therefore, analysis of protein-protein interactions has been limited to immunopurifications or two hybrid screens (Ito et al., 2000; Uetz et al., 2000; Gavin et al., 2002; Ho et al., 2002) that led to construction of interaction networks (Schwikowski et al., 2000; von Mering et al., 2002).

Recent advances in mass spectrometry have allowed scientists to identify by proteomic approaches not only one but many or most potential interacting partners of a particular protein involved in a signal transduction pathway by analyzing its immunoprecipitate by SDS-PAGE and MS (Steen et al., 2002; Wang et al., 2006). Other methods such as isotope-coded affinity tag (ICAT) (Gygi et al., 1999) and stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002; Blagoev et al., 2003; Ong et al., 2003) have allowed detection and quantification of the phosphorylation levels of proteins involved in signal transduction from unstimulated and stimulated cells (Zhang et al., 2006). These and similar approaches are known as functional proteomics.

Although the above mentioned approaches have advanced studies of signal transduction pathways, many questions still remain unanswered due to the technical limitations of the methodology. For example, combining SDS-PAGE (1D or 2D) with MS–although a very powerful method–is limited at the gel electrophoresis level. High or low mass proteins, very hydrophobic proteins, and proteins with high or low pI may not be identified via 2D PAGE. In addition, in experiments with unstimulated and stimulated cells, when a particular protein is identified as a participant in signal transduction pathways, no functional assignment of the protein is made unless its post-translational modifications such as phosphorylation are dramatically increased or decreased. Furthermore, even when the interacting partners of the proteins involved in signaling are identified and characterized, the functional significance of the association of these proteins into multi-subunit protein complexes via ligand stimulation is still missed. Therefore, any optimization of the current technology would be a great improvement. Analysis of protein complexes from signal transduction pathways in terms of size, composition, post-translation modifications, multimerization level, and abundance would also benefit from the introduction of new methods.

Blue Native PAGE (BN-PAGE) has long been used to analyze protein-protein interactions and protein complexes (Schagger and von Jagow, 1991; Schagger et al., 1994; Darie et al., 2005; Darie et al., 2007; Litscher et al., 2008). This method separates protein complexes based on external charge induced by Coomassie dye and according to their mass. BN-PAGE experiments may provide information about the size, number, subunit composition, stoichiometry and relative abundance of these protein complexes. Compared with previous methods used to study protein-protein interactions, there are several advantages of BN-PAGE: (1) separation of the protein complexes takes place under native conditions, so even the transient interactions between proteins may be identified, (2) separation of the protein complexes in the second dimension may reveal their subunit composition, (3) the

method may analyze the association of proteins into protein complexes as a result of ligand stimulation, independent of their levels of phosphorylation, (4) BN-PAGE may confirm the results obtained by immunopurification experiments. For example, if proteins A, B and C co-immunopurify, BN-PAGE will often distinguish between A-B-C, A-B, A-C, and B-C complexes. (5) By combining BN-PAGE with MS, both structural and functional information may be obtained (Camacho-Carvajal et al., 2004; Darie et al., 2005; Aivaliotis et al., 2006; Reifschneider et al., 2006).

In this paper, we analyzed proteins and protein complexes involved in EphB2 signal transduction pathways as a result of in ephrinB1-Fc stimulation by using SDS-PAGE, BN-PAGE, WB and MS. These experiments allowed us to conclude that the differences between the unstimulated and stimulated cells in terms of protein tyrosine phosphorylation are small and detectable only by WB experiments. These experiments also allowed us to conclude that, upon stimulation, the proteins involved in ephrin signaling associate into protein complexes that may be studied by BN-PAGE and MS. In addition, we concluded that BN-PAGE and MS analysis are suitable for separation of the protein complexes by BN-PAGE, which preserves their integrity, regardless of whether it is a homo- or a hetero-complex. Furthermore, combination of BN-PAGE and MS to study protein complexes from unstimulated and ephrin-stimulated cells also has the potential to be applied to other ligand-stimulated RTK-dependent signal transduction pathways.

The first consequences of EphB2 receptor activation are dimerization and multimerization of the receptor, and autophosphorylation of its tyrosine residues from the cytoplasmic side of the receptor (Vearing and Lackmann, 2005). The phosphorylated sites provide docking sites for a number of SH2-domain containing signaling and adaptor proteins that modulate cytoskeletal plasticity and a number of proteins that interact directly or indirectly with the phosphorylated EphRs or mediate downstream signaling have been identified (Vearing and Lackmann, 2005). Recently, a study of EphB2 signaling from unstimulated and ephrinB1-stimulated cells has been performed in our lab using SILAC technology (Zhang et al., 2006). Our group not only confirmed that some known proteins are components of the EphB2 signaling, but also identified previously unknown members that participate in the EphB2-dependent pathways (Zhang et al., 2006).

In our SDS-PAGE and MS experiments (Tables 1 and 2) we identified a number of proteins that became phosphorylated or associated with phosphorylated proteins as a result of ephrin stimulation, some of which are well known to be involved in ephrin signaling (Zhang et al., 2006). Eph receptors (B2 & B4), PTK2 protein tyrosine kinase 2/FAK, serine/threonine kinase receptor associated protein, tyrosine 3-monooxgenase/tryptophan 5-monooxgenase activation proteins (gamma & zeta polypeptides), non-catalytic region of tyrosine kinase adaptor protein 2, protein phosphatase 2a, G protein and breast cancer anti-estrogen resistance 1/p130Cas are some of the proteins identified by SDS-PAGE and MS analysis of pY99-IP of stimulated cells. Two of these proteins were of great interest to us: PTK2 protein tyrosine kinase 2 (FAK; mass 130 kDa) and breast cancer anti-estrogen resistance 1 (p130Cas; mass 95 kDa). By using BN-PAGE and MS, we were able to

conclude that these two proteins interact with each other as a result of ephrin stimulation, in agreement with the published literature (Polte and Hanks, 1995). Because we analyzed pY99-IP samples, it is likely that at least one of these proteins is phosphorylated on tyrosine. Therefore, the combination of BN-PAGE and MS is also suitable for analysis of transient protein-protein interactions.

In conclusion, we used BN-PAGE and MS to analyze protein-protein interactions from signal transduction pathways that resulted upon ephrinB1-Fc stimulation. These experiments are a starting basis for further analysis of transient protein-protein interactions in ligand-stimulated cells.

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References

Adams, R. H. (2003). "Molecular control of arterial-venous blood vessel identity." *J Anat* **202**(1): 105–12.

Aebersold, R. and M. Mann (2003). "Mass spectrometry-based proteomics." *Nature* **422**(6928): 198–207.

Aivaliotis, M., M. Karas, et al. (2006). "High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of cytoplasmatic protein complexes from Chlorobium tepidum." *Photosynth Res* **88**(2): 143–57.

Blagoev, B., I. Kratchmarova, et al. (2003). "A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling." *Nat Biotechnol* **21**(3): 315–8.

Bruckner, K., E. B. Pasquale, et al. (1997). "Tyrosine phosphorylation of transmembrane ligands for Eph receptors." *Science* **275**(5306): 1640–3.

Camacho-Carvajal, M. M., B. Wollscheid, et al. (2004). "Two-dimensional Blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates: a proteomics approach." *Mol Cell Proteomics* **3**(2): 176–82.

Carter, N., T. Nakamoto, et al. (2002). "EphrinA1-induced cytoskeletal re-organization requires FAK and p130(cas)." *Nat Cell Biol* **4**(8): 565–73.

Cheng, N., D. M. Brantley, et al. (2002). "The ephrins and Eph receptors in angiogenesis." *Cytokine Growth Factor Rev* **13**(1): 75–85.

Cowan, C. A. and M. Henkemeyer (2001). "The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals." *Nature* **413**(6852): 174–9.

Dalva, M. B., M. A. Takasu, et al. (2000). "EphB receptors interact with NMDA receptors and regulate excitatory synapse formation." *Cell* **103**(6): 945–56.

Darie, C. C., M. L. Biniossek, et al. (2005). "Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of Zea mays." *Febs J* **272**(11): 2705–16.

Darie, C. C., W. G. Janssen, et al. (2007). "Purified trout egg vitelline envelope proteins VEbeta and VEgamma polymerize into homomeric fibrils from dimers in vitro." *Biochim Biophys Acta* doi:10.1016/j.bbapap.2007.10.011

Darie, C. C. and T. A. Neubert (2008). "Analysis of protein-protein interactions in EphB2-NG108 cells as a result of ephrinB1 stimulation." *In preparation*.

DeLaBarre, B. and A. T. Brunger (2003). "Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains." *Nat Struct Biol* **10**(10): 856–63.

Gale, N. W., S. J. Holland, et al. (1996). "Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis." *Neuron* **17**(1): 9–19.

Gavin, A. C., M. Bosche, et al. (2002). "Functional organization of the yeast proteome by systematic analysis of protein complexes." *Nature* **415**(6868): 141–7.

Gygi, S. P., B. Rist, et al. (1999). "Quantitative analysis of complex protein mixtures using isotope-coded affinity tags." *Nat Biotechnol* **17**(10): 994–9.

Ho, Y., A. Gruhler, et al. (2002). "Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry." *Nature* **415**(6868): 180–3.

Holland, S. J., N. W. Gale, et al. (1996). "Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands." *Nature* **383**(6602): 722–5.

Holland, S. J., E. Peles, et al. (1998). "Cell-contact-dependent signalling in axon growth and guidance: Eph receptor tyrosine kinases and receptor protein tyrosine phosphatase beta." *Curr Opin Neurobiol* **8**(1): 117–27.

Ito, T., K. Tashiro, et al. (2000). "Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins." *Proc Natl Acad Sci U S A* **97**(3): 1143–7.

Kobayashi, H., N. Tanaka, et al. (2005). "Hrs, a mammalian master molecule in vesicular transport and protein sorting, suppresses the degradation of ESCRT proteins signal transducing adaptor molecule 1 and 2." *J Biol Chem* **280**(11): 10468–77.

Litscher, E. S., W. G. Janssen, et al. (2008). "Purified mouse egg zona pellucida glycoproteins polymerize into homomeric fibrils under non-denaturing conditions." *J Cell Physiol* **214**(1): 153–7.

Lu, L., M. Komada, et al. (1998). "Human Hrs, a tyrosine kinase substrate in growth factor-stimulated cells: cDNA cloning and mapping of the gene to chromosome 17." *Gene* **213**(1-2): 125–32.

McNaught, K. S., C. W. Olanow, et al. (2001). "Failure of the ubiquitin-proteasome system in Parkinson's disease." *Nat Rev Neurosci* **2**(8): 589–94.

Miao, H., E. Burnett, et al. (2000). "Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation." *Nat Cell Biol* **2**(2): 62–9.

Ong, S. E., B. Blagoev, et al. (2002). "Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics." *Mol Cell Proteomics* **1**(5): 376–86.

Ong, S. E., L. J. Foster, et al. (2003). "Mass spectrometric-based approaches in quantitative proteomics." *Methods* **29**(2): 124–30.

Polte, T. R. and S. K. Hanks (1995). "Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas." *Proc Natl Acad Sci U S A* **92**(23): 10678–82.

Reifschneider, N. H., S. Goto, et al. (2006). "Defining the mitochondrial proteomes from five rat organs in a physiologically significant context using 2D blue-native/SDS-PAGE." *J Proteome Res* **5**(5): 1117–32.

Robinson, V., A. Smith, et al. (1997). "Roles of Eph receptors and ephrins in neural crest pathfinding." *Cell Tissue Res* **290**(2): 265–74.

Schagger, H., W. A. Cramer, et al. (1994). "Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis." *Anal Biochem* **217**(2): 220–30.

Schagger, H. and G. von Jagow (1991). "Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form." *Anal Biochem* **199**(2): 223–31.

Schwikowski, B., P. Uetz, et al. (2000). “A network of protein-protein interactions in yeast.” *Nat Biotechnol* **18**(12): 1257–61.

Steen, H., B. Kuster, et al. (2002). “Tyrosine phosphorylation mapping of the epidermal growth factor receptor signaling pathway.” *J Biol Chem* **277**(2): 1031–9.

Uetz, P., L. Giot, et al. (2000). “A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae.” *Nature* **403**(6770): 623–7.

Vearing, C. J. and M. Lackmann (2005). “Eph receptor signalling; dimerisation just isn’t enough.” *Growth Factors* **23**(1): 67–76.

von Mering, C., R. Krause, et al. (2002). “Comparative assessment of large-scale data sets of protein-protein interactions.” *Nature* **417**(6887): 399–403.

Wang, Y., R. Li, et al. (2006). “Proteomic analysis reveals novel molecules involved in insulin signaling pathway.” *J Proteome Res* **5**(4): 846–55.

Wilkinson, D. G. (2001). “Multiple roles of EPH receptors and ephrins in neural development.” *Nat Rev Neurosci* **2**(3): 155–64.

Yancopoulos, G. D., M. Klagsbrun, et al. (1998). “Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border.” *Cell* **93**(5): 661–4.

Zhang, G., D. S. Spellman, et al. (2006). “Quantitative phosphotyrosine proteomics of EphB2 signaling by stable isotope labeling with amino acids in cell culture (SILAC).” *J Proteome Res* **5**(3): 581–8.