Identification of Dynamic Proteome Changes Upon Ligand Activation of Trk-Receptors Using Two-dimensional Fluorescence Difference Gel Electrophoresis and Mass Spectrometry*

Barbara Sitek‡, Ognjan Apostolov‡§, Kai Stühler‡, Kathy Pfeiffer‡, Helmut E. Meyer‡, Angelika Eggert§, and Alexander Schramm§¶

The TrkA and TrkB tyrosine kinases are members of the neurotrophin receptor family and mediate survival, differentiation, growth, and apoptosis of neurons in response to stimulation by their ligands, NGF and BDNF, respectively. Expression levels of TrkA/TrkB are important prognostic factors in a variety of embryonal tumors including neuroblastoma, the most common solid tumor of childhood. Because TrkA/TrkB exhibit a high level of sequence similarity and use overlapping pathways for signal transduction, the existence of specific effector molecules crucial for receptor and cell-type-specific response is likely. To identify these effectors by analyzing biological effects of TrkA and TrkB activation in a defined model, we performed a proteome study using the human neuroblastoma SY5Y cell line stably transfected with the TrkA or TrkB cDNA. The use of the recently introduced DIGE (fluorescence two-dimensional difference gel electrophoresis) system (Amersham Biosciences, Piscataway, NJ) allowed us to monitor differences in protein expression between samples in one gel. Proteomic changes were monitored in a time course of 0, 0.5, 1, 6, and 24 h following receptor activation. Using MALDI mass spectrometry, we identified, respectively, 22 and 9 differentially expressed proteins upon the addition of neurotrophin in SY5Y-TrkB and SY5Y-TrkA cells. Functional assignment revealed that the majority of these proteins are involved in organization and maintenance of cellular structures. Molecular & Cellular Proteomics 4:291–299, 2005.

Neurotrophins mediate growth, survival, and differentiation of normal sympathetic neurons. The tyrosine kinases TrkA, TrkB, and TrkC have been identified as the biologically active receptors for the neurotrophins nerve growth factor (NGF),¹ brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT3), respectively (1). Ligand binding induces Trk receptor dimerization and autophosphorylation of cytoplasmic tyrosines leading to the activation of various signaling pathways, including the Ras/MAPK pathway, the PLCγ pathway, and the PI3K pathway (2). The physiological functions of Trk receptors in the nervous system vary markedly ranging from induction of proliferation to synaptic modulation and axonal path-finding (reviewed in Refs. 3 and 4).

There is considerable interest in aberrant Trk receptor expression or signaling as a hallmark of various diseases, including Alzheimer's disease (5) and solid tumors such as carcinomas (6) and neuroblastoma (7). Despite of the high degree of sequence similarity and broadly overlapping signaling pathways, activation of TrkA or TrkB causes divergent biological responses and phenotypes when expressed in the same cell type. For example, high expression of TrkA in neuroblastoma is associated with a favorable biology and a good patient outcome, whereas TrkB is mainly expressed on aggressive neuroblastomas with a poor patient outcome (7, 8). The biological effect of Trk receptor signaling also varies between cell types. Activation of TrkA by NGF causes differentiation in neuronal precursors and neuroblastoma cells, whereas it induces proliferation in fibroblasts and apoptosis in medullloblastoma cells (9, 10).

In medullary thyroid carcinoma, TrkB expression is associated with favorable biology and good prognosis (11), which is in contrast to its role in neuroblastoma. The high level of sequence homology and overlapping signaling pathways of Trk receptors suggest the existence of specific effector molecules critical for receptor and cell-type-specific responses. Although some target molecules for tyrosine kinase signaling elements have been reported (12, 13), there is still no comprehensive view of neurotrophin signaling at the proteome level. As the identification of specific effector proteins is of major interest for the understanding of the biological role of Trk receptors in particular in solid tumors, we here aimed
to gain deeper insights into TrkA and TrkB signaling pathways of the childhood tumor neuroblastoma by a proteomic approach.

The human neuroblastoma SY5Y cell line stably transfected with the TrkA or TrkB cDNA was used in a model system. The resulting biological phenotypes of these cell lines have been extensively analyzed (14, 15). Briefly, activation of SY5Y-TrkA cells by NGF resulted in neuronal differentiation, growth inhibition, and inhibition of angiogenesis (14, 15), whereas activation of SY5Y-TrkB cells by BDNF resulted in enhanced proliferation and resistance to chemotherapy (16). Thus, the biological phenotypes of TrkA/B-transfected SY5Y cells corresponded well with the excellent outcome of TrkA-expressing neuroblastomas and the poor outcome of TrkB-expressing neuroblastomas.

We used the difference gel electrophoresis (DGE) system together with MALDI-peptide mass fingerprinting (PMF) MS analysis to detect reproducible proteome changes caused by ligand activation of SY5Y-TrkA or -TrkB cells in a time course from 0 to 24 h in five biologically independent experiments.

**EXPERIMENTAL PROCEDURES**

*Molecular Cloning and Cell Culture—SH-SY5Y is a subclone from the human SK-N-SH neuroblastoma cell line and lacks endogenous Trk receptor expression (17). Full-length TrkA or TrkB cDNA were cloned into the retroviral expression vectors pLNCX or pLNCX2, respectively (Clontech, Palo Alto, CA). The resulting SY5Y-TrkA and SY5Y-TrkB cell lines have been characterized previously (14). In contrast to SY5Y, the SY5Y-TrkA and SY5YTrkB cell lines demonstrated high and comparable expression of the inserted receptor on mRNA and protein levels (14). Cells were grown in RPMI 1640 containing 10% FBS, L-glutamine, and 500 μg/ml geneticin (Life Technologies, Inc., Eggenstein, Germany). Neither BDNF nor NGF was detectable from 0 to 24 h in five biologically independent experiments.*

*In the dark. Proteins extracted from NGF-treated and untreated SY5Y-TrkA cells were labeled with Cy3 and Cy5, respectively, mixed with Cy2-labeled internal standards (see Fig. 1) and run in one gel. The internal standard contained equivalent amounts of pooled proteins including samples from all time points of one experimental round, which proved to be useful for internal calibration purposes (18). Proteins of BDNF-treated SY5Y-TrkB cells were labeled with Cy2 (untreated = Cy3, internal standard = Cy5).*

**IEF and 2-DE—** Labeled proteins of neurotrophin-activated SY5Y-TrkA or SY5Y-TrkB cells and untreated controls were mixed, and per 100 μl cell lysate, 10 μl DTT (1.08 g/ml; BioRad) and 10 μl Ammonium 2-4 (Amersham Biosciences) were added. IEF was performed using tube gels (20 cm × 0.9 mm) containing carrier ampholytes (CA-IEF) and applying a voltage gradient in an IEF-chamber produced in house (19). After IEF, the ejected tube gels were incubated in equilibration buffer (125 mM Tris, 40% (w/v) glycerol, 3% (w/v) SDS, 65 mM DTT, pH 6.8) for 10 min and stored on a tube gel carrier device at −80 °C. The second dimension (SDS-PAGE) was performed on (15.2% total acrylamide, 1.3% bisacrylamide) polyacrylamide gels using a Desaphe VA 300 system. IEF tube gels were placed onto the polyacrylamide gels (20 cm × 30 cm × 0.7 mm) and fixed using 1.0% (w/v) agarse containing 0.01% (w/v) bromphenol blue dye (Riedel-de-Haen, Seelze, Germany). For identification of proteins by MS, 400 μg total protein was applied to IEF tube gels (20 cm × 1.5 mm) and subsequently to preparative SDS-PAGE gels (20 cm × 30 cm × 1.5 mm). Silver staining was performed using a protocol compatible with MS (20).

**Scanning and Image Analysis—** SDS-PAGE gels were scanned using a Typhoon 9400 scanner (Amersham Biosciences). Excitation and emission wavelengths were chosen specifically for each of the dyes according to recommendations of the manufacturer. Images were preprocessed using the ImageQuant™ software (Amersham Biosciences). Intra-gel spot detection and inter-gel matching were performed using the Differential In-gel Analysis (DIA) mode and Biological Variation Analysis (BVA) mode of “DeCyder” software (Amersham Biosciences), respectively. Spot intensities were normalized to the internal standard (see above), and significantly regulated proteins were identified by Student’s t test. Protein spots differentially expressed (p < 0.05) between either SY5Y-TrkA or SY5Y-TrkB cells and untreated controls in the time course following neurotrophin treatment were identified using MS.

**In-gel Tryptic Digestion and Protein Identification by MALDI-MS—** Spots of interest were manually cut out from the preparative gels, digested with trypsin (Promega, Madison, WI) in-gel, and extracted from the gel as described previously (21). C18- ZipTips™ (Millipore, Bedford, MA) were used for MALDI target preparation according to the manufacturer’s instructions. The peptides were eluted on the MALDI target with 1.2 μl of matrix solution (α-cyano-4-hydroxy cinnamic acid in ACN and 0.1% TFA (1:1, v/v)). For analysis of tryptic peptides, MALDI-TOF MS was applied using the UltraFlex™ (Bruker Daltonik, Bremen, Germany) equipped with a Scout™ MTP MALDI target used for MALDI target preparation according to the manufacturer’s instructions. The spectra were acquired in the positive mode with a target voltage of 20 kV and a pulsed ion extraction of 17.25 kV. The reflector voltage was set to 21 kV and detector voltage to 1.7 kV. The internal calibration of PMF spectra was performed using the autolysis products of trypsin with the monoisotopic masses (m/z = 842.51 Da, m/z = 1045.5642 Da, and m/z = 2211.1046 Da). The PMF spectra were processed using the XMass™ (Bruker) or FlexAnalysis™ software (Bruker), and the generated data were compared with the ProteinScape™ (Bruker) proteome database in order to identify the proteins. Searches were started from ProteinScape™ database, using the “Profound” search algorithm (22). A score >1.65 was used as threshold for protein identification. The following search parameters were selected: fixed
cysteine modification with propionamide, variable modification due to methionine oxidation, two missed cleavage sites maximally in the case of incomplete trypsin hydrolysis, and no details about 2D-PAGE-derived protein mass and pI. The searches were run in the human protein subdatabase of NCBI (April–September 2003). The gene ontology classification for the identified proteins was obtained using OntoExpress (23).

RESULTS

To identify specific protein expression changes following TrkA or TrkB activation in neuroblastoma cells by their respective ligands, NGF or BDNF, we analyzed the proteome of SY5Y-TrkA, SY5Y-TrkB, or untreated control cells using the 2DE-based DIGE system. To detect immediate proteome changes as well as late effectors, we chose five time points between 0 and 24 h following ligand addition. Cy3-labeled neurotrophin-treated proteins were run together with Cy5-labeled untreated proteins and the Cy2-labeled protein standard (composed of equivalent amounts of all samples at a given time point) in one gel (Fig. 1).

Identification of Differentially Expressed Proteins Following TrkA/B Receptor Activation—We combined the carrier ampholyte-IEF/SDS-PAGE technique and the DIGE technology by adapting the lysis buffer system for proteome profiling of whole-cell lysates of SY5Y-TrkA/B cultures. This enabled the separation of complex protein mixtures in the range of 5–150 kDa. In whole-cell lysates of SY5Y-TrkA/TrkB, 1,700–1,900 distinct protein spots were detected by the Decyder software and subsequent manual correction (Fig. 2).

The analysis of the expression profiles of SY5Y-TrkB cells using DeCyder software resulted in 24 significantly and reproducibly regulated spots induced by BDNF (p < 0.05, ratio > 1.5; Table I) and 13 regulated spots induced by NGF in SY5Y-TrkA cells (p < 0.05, ratio > 1.3; Table I). A total of 20 spots specific for SY5Y-TrkB cells and nine regulated spots were specific for SY5Y-TrkA cells (Fig. 3). Four spots were regulated in both cell lines. While three spots demonstrated the same regulation pattern, one spot was inversely regulated between SY5Y-TrkA and SY5Y-TrkB (Fig. 2, spot no. 20, i.e. tropomyosin 3).

For identification of proteins, spots were digested in-gel and analyzed by MALDI-PMF MS. Preprocessed MALDI-PMF spectra were sent to the ProteinScape™ (Bruker) proteome database using “Profound” search algorithms (22). We successfully identified 22 of 24 BDNF/TrkB-regulated proteins and 9 of 13 NGF/TrkA-regulated proteins. An overview of these proteins in presented in Table I and includes information about the theoretical and experimental molecular masses (Mr), isoelectric points (pI), accession numbers, and assignments to the kinetic groups. Most proteins (n = 24) found to be differentially regulated were detected at late time points (regulation group 3; Table I). Proteins identified during this study were categorized using "OntoExpress" (23). Most of the proteins were assigned to "cytoskeleton organization and biogenesis" (GO: 7010). These include β-actin, dynein, galec- tin-1, vimentin, and tropomyosin-3. Two other GO groups were identified, “cell surface receptor-linked signal transduc- tion” (GO: 7166, 7167) and “pyruvate metabolism” (GO: 6090). Heterogeneous nuclear ribonucleoprotein K (hnRNP K) and RhoGDIα are members of the former GO group and dihydro- lipoamide dehydrogenase and lactate dehydrogenase are in- cluded in the latter GO group. The majority of the proteins identified in this study have not yet been implicated in TrkA/ TrkB signaling or the biology of neuroblastoma.

Expression Kinetics—The identified proteins could be divided into three groups (1–3) according to their expression kinetics during cell stimulation with their specific ligands: 1) proteins regulated throughout all time points analyzed, 2) proteins showing early regulation peaking between 0.5 and 1 h, and 3) proteins that were differentially expressed in the late phase of stimulation (after 6 h). The expression kinetics of one protein from each of these groups is diagrammed in Fig. 4. The majority of the proteins differentially expressed upon neu-
rotrophin receptor activation were regulated in the late stimulation phase (10/13 in SY5Y-TrkA and 16/24 in SY5Y-TrkB). The positions of differentially expressed spots in the 2DE-gels are shown in Fig. 2, A (SY5Y-TrkA) and B (SY5Y-TrkB).

Comparability of Silver-stained Gels and DIGE—Direct recovery of proteins using the DIGE system is only feasible in a fully automated system where spots are recovered by robotic arms. An inexpensive alternative is to isolate differentially expressed proteins from MS-compatible silver-stained gels. To ensure comparability between the DIGE system and MS-compatible silver staining, we performed silver post-staining of a Cy3-labeled probe composed of a mixture of different time points of neurotrophin-treated SY5Y-TrkA and SY5Y-TrkB cell lysates separated on 2D-PAGE gels. Within the gel section compared, MS-compatible silver-stained gels allowed the identification of ~1,550 spots, while Cy3 staining resulted in ~1,700 detected spots using the DIA software. The overall protein patterns visualized in the image overlay were highly comparable (Fig. 5), although it must be mentioned that some proteins are detected by silver staining but not by DIGE (marked as “1” in Fig. 5) and/or vice versa (marked as “2” in Fig. 5). Reliable spot alignment between silver- and Cy3-stained gels can be assumed for proteins >30 kDa. Spots in the lower molecular mass range did not exactly align, but were shifted to slightly higher masses in the Cy3-labeled proteins (marked as “3” in Fig. 5). This shift is most probably induced by the additional mass of the dye molecule (570 Da) covalently attached to the protein, which only is detectable for smaller proteins. This indicates high resolution of proteins on our large gel format (300 mm). Silver staining allowed the localization of proteins found to be differentially expressed in DIGE-labeled gels and their subsequent identification by MALDI-PMF.

DISCUSSION

We here describe the application of the DIGE technology for identification of proteins differentially expressed following Trk receptor activation in SY5Y neuroblastoma cells stably transfected with TrkA or TrkB cDNA. These cell lines are excellent tools for studying the response to external stimuli against an otherwise homogeneous genetic background. Although early signal transduction processes of Trk receptors have been analyzed (24, 25), late effector proteins have not yet been described. This report presents, for the first time, evidence for proteins associated with and potentially contributing to the biological changes induced by Trk receptor activation in neuroblastoma.

The DIGE technology allows rapid identification of protein changes between samples on the same 2-DE gel, and thus reduces interexperimental variation. Additionally, DIGE covers a dynamic detection range of three orders of magnitude, while silver staining only detects 30-fold changes (26–30). We show that DIGE and silver staining are compatible techniques allowing the identification of differentially expressed proteins by
MALDI-PMF MS (Fig. 5). The reproducibility of the system was assessed by repeating the time course in five independent cell culture experiments, allowing the comparison of different cellular extracts produced under identical conditions and application of significance analysis. A potential pitfall for identification of proteins detected by Cy-dye labeling is irregular shifting of the respective silver-stained protein band in the molecular mass range when using a high-resolution large-gel format (Fig. 5). Thus, the molecular mass of the Cy-molecule has to be taken into account when picking the spots for identification by MS without post-staining. Summarizing these technical aspects, we here present a highly reproducible 2D-based method for identification of differentially expressed proteins. The potential contribution of selected regulated proteins, identified in this study, to the observed phenotypes of SY5Y-TrkA and SY5Y-TrkB cells will be briefly discussed:

The coding gene for tropomyosin-3 was assigned to chromosome 1q23 in close proximity to TrkA (31). With this protein we identified the first protein oppositely regulated following activation of TrkA or TrkB receptors. Thus, the contribution of tropomyosin-3 to the phenotypes of SY5Y-TrkA and SY5Y-TrkB cells deserves further functional analysis. Up-regulation of tropomyosin-3 relative to other tropomyosin isoforms in transformed cells has been described (32). Introduction of tropomyosin-3 into a neuronal cell line was concordant with increased cell mobility and reduction of stress fibers (33). Thus, up-regulation of tropomyosin-3 by BDNF/TrkB might also contribute to the more aggressive phenotype of TrkB-expressing SY5Y cells. In contrast, down-regulation of tropomyosin-3 by NGF/TrkA might contribute to the differentiated and favorable phenotype of TrkA-expressing neuroblastoma cells.

**Table 1**
Summary of significantly regulated proteins following neurotrophin receptor activation in SY5Y-TrkA or SY5Y-TrkB identified by MALDI-MS

All proteins identified in this study are listed together with their theoretical and experimental molecular weights (MW), isoelectric points (pI), accession numbers, and kinetic groups assignments (Fig. 4). The direction of protein regulation in SY5Y-TrkA and SY5Y-TrkB cells is indicated by arrows (→, no regulation; ↑, up-regulation; ↓, down-regulation). Numbers next to the arrows identify the kinetic group (1, regulated at all time points analyzed; 2, early response, 0.5 or 1 h after neurotrophin addition; 3, late response between 6 and 24 h after neurotrophin stimulation).

| Spot no. | Protein                                                                 | MW[kDa] theoretical | MW[kDa] experimental | Accession no. | Regulation/group | Profound Score | Sequence Coverage (%) |
|----------|------------------------------------------------------------------------|---------------------|----------------------|---------------|-----------------|---------------|----------------------|
| 1        | Dynin intermediate chain protein                                       | 71.5 5.4            | 69.0                 | gi24307879    | → → → → → → → | → → → → → | → → → → → → →     |
| 2        | Dynin intermediate chain protein                                       | 71.5 5.5            | 95.3                 | gi24307879    | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 3        | Heat shock 70kDa protein 5                                             | 72.3 5.3            | 86.8                 | gi16507237    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 4        | Heat shock 70kDa protein 5                                             | 72.3 5.3            | 86.8                 | gi16507237    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 5        | Lamin A/C isoform 1 precursor                                          | 6.4 71.1            | 62.9                 | gi24307894    | → → → → → → → | → → → → → | → → → → → → →     |
| 6        | HnRNP/Ku                                                               | 51.5 5.4            | 72                  | gi14415437    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 7        | not identified                                                          | 5.6 67.5            | 62.9                 | gi24307894    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 8        | Lamin A/C isoform 2                                                    | 6.4 65.1            | 62.9                 | gi24307894    | → → → → → → → | → → → → → | → → → → → → →     |
| 9        | Lamin A/C isoform 2                                                    | 6.4 65.1            | 62.9                 | gi24307894    | → → → → → → → | → → → → → | → → → → → → →     |
| 10       | Protein disulfide-isomerase/ER60 precursor                             | 5.9 56.8            | 60.9                 | gi7437386     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 11       | Protein disulfide-isomerase/ER60 precursor                             | 5.9 56.8            | 60.9                 | gi7437386     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 12       | Dihydroxyacridine dehydrogenase                                       | 7.4 54.3            | 60.4                 | gi5975255     | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 13       | Adenyl cyclase-associated protein                                       | 6.1 51.3            | 57.5                 | gi5353569     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 14       | Nuclear matrix protein NMP200                                           | 6.1 55.2            | 60.5                 | gi7457381     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 15       | TATA binding protein interacting protein                               | 6.2 59.8            | 60.4                 | gi5975255     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 16       | ATP synthase, H+ transporting                                          | 6.2 59.8            | 60.4                 | gi5975255     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 17       | ATP synthase, H+ transporting                                          | 6.2 59.8            | 60.4                 | gi5975255     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 18       | Calumenin                                                               | 8.5 36.7            | 35.8                 | gi5031867     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 19       | Lactate dehydrogenase                                                  | 5.1 14.6            | 12.8                 | gi45242207    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 20       | Tropomyosin 3                                                           | 4.7 32.9            | 34.4                 | gi69828       | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 21       | Rho GDP dissociation inhibitor/GD(al)alpha                             | 5.2 19.7            | 22.1                 | gi2946816     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 22       | NM23-H1                                                                | 5.1 14.6            | 12.8                 | gi45242207    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 23       | Gagecin 1                                                               | 5.1 14.6            | 12.8                 | gi45242207    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 24       | not identified                                                          | 5.4 24              | 24                   | gi5975255     | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 25       | Lamin A/C isoform 1 precursor                                          | 6.8 71.1            | 66.6                 | gi24307894    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 26       | Lamin A/C isoform 1 precursor                                          | 6.8 71.1            | 66.6                 | gi24307894    | ↑ ↑ ↑ ↑ ↑ ↑ ↑ | → → → → → | → → → → → → →     |
| 27       | HnRNP/Ku                                                               | 5.1 51.5            | 70.5                 | gi14165437    | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 28       | Lamin B2                                                                | 5.3 67.7            | 71                   | gi24307894    | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 29       | Vimentin                                                                | 7.0 54.2            | 52.4                 | gi5457855     | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 30       | not identified                                                          | 5.5 49.4            | 49.4                 | gi5457855     | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 31       | not identified                                                          | 5.5 49.4            | 49.4                 | gi5457855     | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 32       | ACTB protein                                                            | 5.6 40.2            | 55                   | gi15277505    | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |
| 33       | not identified                                                          | 5.6 26.4            | 26.4                 | gi15277505    | ↓ ↓ ↓ ↓ ↓ ↓ ↓ | → → → → → | → → → → → → →     |

**Fig. 3.** Venn diagram of protein spots regulated following neurotrophin treatment of SY5Y-TrkA or SY5Y-TrkB. Neurotrophin treatment resulted in nine spots exclusively regulated in SY5Y-TrkA cells, 20 spots exclusively regulated in SY5Y-TrkB cells, and four spots showing differential regulation in both cell lines. The proteins identified in this study are listed in Table 1.
Isoforms of a dynein intermediate chain protein (Table I, Fig. 4A) were also differentially regulated between SY5Y-TrkA and SY5Y-TrkB cells. Dynein intermediate chain expression is involved in differentiation of rat pheochromocytoma cells and altered in response to NGF (34). Thus, it might also be important for differentiation signaling in SY5Y-TrkA cells.

Galectin-1, a multifunctional protein regulating inflammatory responses (35) as well as the induction of T cell apoptosis...
and cell adhesion (36), is up-regulated exclusively in SY5Y-TrkB cells (Fig. 4C) and might contribute to the immune escape of aggressive neuroblastoma cells. In primary neuroblastomas, galectin-1 expression was shown to be restricted to proliferating tumor cells (37). As proliferation of SY5Y-TrkB cells is enhanced following BDNF stimulation in vitro, galectin-1 up-regulation may also be a prerequisite to proliferation in these cells.

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) was transiently up-regulated in SY5Y-TrkB cells (Fig. 4B). This protein can act as a translational repressor, which functions as a “RNA silencer” in immature epithelial cells to suppress translation of proteins only needed in differentiated cells. In neuroblastoma, a role for hnRNP K remains to be elucidated.

Taken together, tropomyosin-3, galectin-1, and hnRNPK are up-regulated following TrkB activation and are associated with aggressive tumor phenotype, increased cell mobility, and suppression of differentiation in line with the phenotype of TrkB-expressing neuroblastomas. Recent data indicate that the nature of Trk responses can differ depending on whether receptors are localized on presynaptic cell axons, on postsynaptic cell dendrites, or the plasma membrane of the cell soma. Neurotrophins bind Trk receptors at nerve terminals, activate the receptor, and induce receptor internalization and retrograde transport of Trk-containing vesicles. The latter is a microtubule-dependent process involving the dynein motor (reviewed in Ref. 38). This concept may underlie the differential expression of dynein isoforms observed in our model system. Our data suggest that the protein expression of dynein isoforms is differentially regulated between SY5Y-TrkA and SY5Y-TrkB cells in response to their specific ligands. However, functional assays are needed to elucidate whether there are Trk-receptor dependent differences in retrograde transport and the exact role of all observed protein changes.

It is difficult to assess exactly how many proteins are involved in the complex process of NGF-induced differentiation of SY5Y-TrkA cells and BDNF-induced proliferation of SY5Y-TrkB cells. However, the proteins identified here are not sufficient to completely explain the observed phenotypes. Several reasons may account for the missing information. 1) Utilization of overexpressed Trk receptors in neuroblastoma cells may give rise to artifacts due to the high levels of stimulation, leading to both false positives and false negatives. Nevertheless, the biological phenotype of the transfectants corresponds well with the favorable clinical properties such as differentiation and spontaneous regression of primary neuroblastomas expressing high levels of TrkA, and the unfavorable properties including proliferation, therapy resistance, and metastasis of neuroblastomas expressing TrkB, although dosage effects cannot be ruled out. In fact, resistance to chemotherapy was correlated to the amount of TrkB expressed in this model system (16). 2) Changes in proteome composition associated with the observed phenotype might be observable only at later time points following neurotrophin receptor activation. 3) Even the employment of state-of-the-art 2D techniques monitor only a fraction of the total proteome. Therefore, some of the changes in protein composition causing the different phenotypes of SY5Y-TrkA and SY5Y-TrkB are be-
yond the technical detection limits. 4) Changes in the activity status (e.g. phosphorylation) or compartmental localization of certain proteins may differ in unstimulated and neurotrophin-stimulated SY5Y-TrkA/TrkB cells. 5) It cannot be excluded from our study design that markers of differentiation already present on RNA level 24 h following NGF treatment of SY5Y-TrkA will be detectable on protein level only at later time points. This further contributes to the notion that transcriptome and 2D gel studies are often complementary (39) and direct comparison of RNA and protein data is hampered by currently existing technical limitations. Furthermore, differences in mRNA and protein stability introduce an additional level of variance, which cannot be overcome at present. It could be argued that the opposing phenotypes of neurotrophin-treated SY5Y-TrkA and SY5Y-TrkB cells may be governed by only a small number of factors rather than global proteomic changes. At least in the case of medium- to high-abundance proteins, only a few regulated protein spots (n = 37) were detected. The contribution of low-abundance proteins as well as differences in protein activity or compartmentalization remains to be analyzed. In light of the importance of short-term activation changes, analysis of the “phospho-proteome” may be necessary to fully understand neurotrophin receptor signaling. Integration of the signaling pathways and functional analysis of effector proteins identified in this study may reveal additional insights into the underlying courses for the opposing phenotypes of SY5Y-TrkA and SY5Y-TrkB cells.

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To whom correspondence should be addressed: University Hospital Essen, Children’s Hospital, Division of Pediatric Oncology, Hufelandstrasse 55, 45122 Essen, Germany. Tel.: 49-201-723-2506; Fax: 49-201-723-5750; E-mail: alexander.schramm@uni-essen.de.

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