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Regular Article

Phosphoprotein profiling of erythropoietin receptor-dependent pathways using different proteomic strategies

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Proteomic techniques provide new tools for the global analysis of protein profiles but also for the investigation of specific protein functions. The analysis of signaling cascades has traditionally been performed by the determination of enzymatic or transcription factor activities representing a certain pathway. Functional proteomics now allows more comprehensive approaches to study cellular responses induced during ligand/receptor interactions. In this study we evaluated proteomic strategies for the investigation of structure-function relationships in the erythropoietin receptor signalling complex. After expression of epidermal growth factor/erythropoietin receptor mutant molecules in an identical cellular background we characterized their potential to induce cellular activities. Using this system we focused our efforts on post-translational modifications of signalling proteins reflecting a substantial part of receptor-dependent signaling events. Although tyrosine phosphorylated proteins were enriched by immunoprecipitation the analysis using the classical approach combining two-dimensional gel electrophoresis and identification by matrix assisted laser desorption/ionization-time of flight-mass spectrometry revealed that low expressed signaling proteins cannot be detected by this technique. An alternative strategy using one-dimensional gel separation of phosphoproteins and liquid chromatography-tandem mass spectrometry, however, allowed us to identify multiple proteins involved in intracellular signalling representing already established pathways but also proteins which have not been linked to EPO-induced signaling so far. This approach offers the potential to extend functional proteomic studies to complex signaling processes.

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Abbreviations: EGF, epidermal growth factor; **EGFR**, epidermal growth factor receptor; **EMSA**, electrophoretic mobility shift assays; **EPO**, erythro-poietin; **EPOR**, erythropoietin receptor; **FCS**, fetal calf serum; **JAK**, janus family kinase; **MAPK**, mitogen-activated protein kinase

1 Introduction

The development of erythroblasts beyond the CFU-E stage is tightly regulated by the cytokine erythropoietin (EPO), which supports cell survival and proliferation by the activation of its receptor (EPOR), a member of the superfamily of cytokine receptors [1]. The idea that cytokine receptors induce instructive signals that play an essential role during the differentiation of hematopoietic progenitor cells [2] has been brought into question by recent studies. Several homo-

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dimeric receptors including the G-CSF (granulocyte-colony stimulating factor) receptor [3], the prolactin receptor [4], the mpl (thrombopoietin) receptor [5] and the EPOR [6] were shown to substitute for their functions during cell differentiation pointing to a permissive role of hematopoietic cytokines. The exact role of these receptors in the differentiation of cells, however, is not yet defined.

The interaction between EPO and its receptor, which is expressed on late erythroid progenitor cells initiates multiple signalling pathways by the recruitment of cytosolic *src* homology 2 (SH2) domain-containing proteins to the phosphorylated receptor [7]. The phosphorylation of receptor tyrosine residues is mainly due to the activity of the Janus family kinase 2 (JAK2), a receptor-associated enzyme which is undergoing transphosphorylation after receptor dimerisation [8]. A number of proteins interacting with the receptor has been identified. They include additional kinases, adaptor proteins, transcription factors, negative effectors and other molecules involved in intracellular signal transduction (for review see [9]). The resulting multiprotein receptor complex induces a specific response at the level of transcription and translation. Studies using mutated forms of EPOR have identified single tyrosine residues that are critical for the recruitment of distinct signaling proteins. It is, however, still difficult to relate the function of single tyrosine residues/ receptor subdomains or distinct signaling molecules/pathways to the induction of proliferative, antiapoptotic or differentiative cellular responses initiated after binding of EPO to its receptor.

The signaling events involved are based to a large extent on post-translational modifications of pre-existing proteins without the need for *de novo* protein synthesis [10]. The most prevalent modification occurring in approximately one-third of eukaryotic gene products is protein phosphorylation [11]. In view of the complexity in the phosphoprotein network the recent progress made in the development of techniques to identify proteins by mass spectrometry is of increasing importance. Proteomic strategies seem to have an enormous potential in the analysis of changes in the abundance of proteins, but also in their post-translational modification. A major challenge in creating phosphoproteome maps, however, remains the fact that phosphorylated key regulatory molecules such as signaling proteins are expressed at rather low levels.

In this study we attempted to apply proteomic strategies to the investigation of structure-function relationships in the EPOR signaling complex. Using a set of EPOR mutant molecules expressed in an identical cellular background we focused on rapid changes in the tyrosine phosphorylation state of proteins. We show that the phosphoproteomes established after enrichment by immunoprecipitation, separation by 2-DE and identification by MALDI-TOF and/or LC-MS/MS analysis comprise only proteins with high abundance. An alternative strategy which combines 1-D separation of phosphoproteins and capillary LC-MS/MS, however, allowed us to identify multiple proteins involved in intracellular signaling representing already established pathways but also proteins which have not been linked to EPO-induced signaling so far.

2 Materials and methods

2.1 Cell culture and expression of EGF/EPOR

Ba/F3 cells were maintained in RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 10% v/v fetal calf serum (FCS) and conditioned medium from WEHI-3 cells as a source of murine interleukin-3 (IL-3). The chimeric receptor constructs have been generated as described previously [12]. Briefly, constructs were established by fusing wild type and mutant forms of the murine EPOR intracellular and transmembrane domains to the extracellular domain of the human epidermal growth-factor receptor (EGFR) and their stable transfection in murine Ba/F3 cells. Clones expressing high and comparable levels of the constructs designated as EGF/EPOR were selected by RT-PCR and immunoblotting using an antibody directed to the intracellular domain of the murine EPOR. All constructs used in this study were verified by didesoxy-sequencing of the complete coding region.

2.2 Viability assay

Cell viability was measured using metabolic conversion of WST-1 reagent [21] following manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Briefly, cells were deprived of IL-3 and restimulated by different concentrations of epidermal growth factor (EGF) or IL-3 for 45 h in microtiter 96-well plates (3000 cells/100 μL). For background determination a control well without cells was treated in parallel for every microtiter plate. Subsequently, 10 μL of WST-1 reagent was added to each well and metabolic cell activity was assessed by measuring absorbance at 450 nm (against 620 nm as a reference).

2.3 Immunoblotting

Cell lysates were prepared by boiling 5×10^5 cells of each sample in 50 μL SDS sample buffer (31.5 mM Tris-HCl, pH 6.8, 2.5 mM EDTA, 5% v/v glycerol, 1% w/v SDS, 5% v/v β-mercaptoethanol, 0.01% w/v bromophenol blue). Total protein was separated through SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia Biotech, Freiburg, Germany) as described previously [22]. Membranes were blocked with 1% w/v BSA and incubated with anti-JAK2, anti-phospho-STAT5 and anti-phosphotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. After a final incubation with a horseradish peroxidase-labeled anti-rabbit or anti-mouse Ig antibody, blots were developed using the ECL chemilumi-

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nescent detection system (Amersham Pharmacia Biotech). Immunoprecipitations were performed essentially as described below for anti-phosphotyrosine antibodies.

2.4 Preparation of nuclear extracts and electrophoretic mobility shift assays

Nuclear extracts were prepared essentially as described [21]. For electrophoretic mobility shift assay (EMSA) analysis nuclear proteins corresponding to 1×10^5 cells were incubated with 16 fmol double stranded oligonucleotide containing a STAT5 binding site derived from the β-casein promoter (5'-AGA TTT CTA GGA ATT CAA ATC-3') or a AP-1 consensus binding site (5'-CGC TTG ATG ACT CAG CCG ATC-3'). The oligonucleotides were end-labeled with [γ-^{32}P]-ATP (Amersham Pharmacia Biotech) by polynucleotide kinase. The shift assays were performed in a total volume of 20 µL in the following buffer: 10 mM Tris-HCl, pH 7.5, 50 mM potassium chloride, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mg/mL BSA, 5% v/v glycerol, 0.1% v/v NP40, 1 mM Pefabloc (Roche Diagnostics, Mannheim, Germany). The reactions, also containing poly(dI-dC) (1 µg/mL; Roche Diagnostics), were performed at room temperature for 30 min and initiated by the addition of nuclear extract. Complexes were analyzed by electrophoretic separation on a 6% polyacrylamide gel in $0.25 \times$ TBE buffer. Dried gels were exposed to X-ray film for autoradiographic analysis.

2.5 Cell lysis and anti-phosphotyrosine immunoprecipitation

5×10^8 Cells were lysed in 4 mL lysis buffer containing 50 mM Tris-HCl, pH 7.6 (Sigma-Aldrich, Taufkirchen, Germany), 150 mM NaCl, 1% v/v NP40 (Roche Diagnostics), 1 mM vanadate (Sigma-Aldrich), 20 mg protease inhibitor cocktail (Complete) (Roche Diagnostics), 0.6 ng/mL Pepstatin (Roche Diagnostics) and 1 ng/mL Leupeptin (Roche Diagnostics). The cell extracts were vortexed, incubated for 45 min at 4°C and centrifuged for 5 min at $15\,000 \times g$.

The lysates were incubated with a mixture of anti-phosphotyrosine antibodies: 20 µg of 4G10 monoclonal antibody coupled to agarose beads (Upstate, Lake Placid, NY, USA), 10 µg of biotin-conjugated RC20 monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) and 50 µL of streptavidin agarose (Novagen, Frankfurt/Main, Germany). Lysates were rotated for 7 h at 4°C. Agarose beads were collected by centrifugation and washed 5 times with 1 mL lysis buffer. Immunopellets were then diluted in 100 µL 2-DE sample buffer: 9 M urea (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), 2 mM CHAPS (Bio-Rad Laboratories, Munich, Germany) and 0.8% v/v Biolytes, pH 3–10 (Bio-Rad Laboratories) for 2-DE or in 50 µL sample buffer (31.5 mM Tris-HCl, pH 6.8, 2.5 mM EDTA, 5% v/v glycerol, 1% w/v SDS, 5% v/v β-mercaptoethanol, 0.01% w/v bromophenol blue) for 1-DE and LC-MS/MS-analysis. Samples were incubated for 3 min at 95°C prior to 1-DE.

2.6 2-DE

Immunoprecipitates (100 µL) were mixed with 230 µL rehydration buffer containing 8 M urea, 10 mM DTT, 1 mM CHAPS and 0.25% v/v Biolytes. Immobiline Dry Strips (Bio-Rad Laboratories) were rehydrated with 330 µL sample at 20°C for 12 h. IEF was performed for a total of 30 000 Vh, with the voltage ramped lineary from 150 to 8000 V during the first 5 h and maintained for 15.5 h at 8000 V. After focusing, the IPG strips were incubated for 30 min in 25 mL equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.01% w/v bromophenol blue and 1% DTT) at room temperature. The IPG strips were then transferred for 45 min in 25 mL equilibration buffer 2, which is identical to buffer 1 except that 260 mM iodoacetamide (ICN Biochemicals, Eschwege, Germany) replaced DTT. The samples were then separated by SDS-PAGE in a second dimension. Therefore, the equilibrated strips were loaded onto the top of the gel and electrophoresed overnight. The proteins were visualized with Colloidal CBB (Sigma-Aldrich) or by silver staining using a protocol compatible with mass spectrometric protein identification [13].

2.7 In-gel digestion of proteins and sample preparation prior to MS

The protein spots were excised, washed with 50% v/v ACN in 25 mM ammonium bicarbonate, dehydrated in ACN, and dried in a vacuum centrifuge. The gel pieces were re-swollen in 4 µL of 5 mM ammonium bicarbonate containing 120 ng trypsin (sequencing grade, Roche Diagnostics). After 15 min, 5 µL of 5 mM ammonium bicarbonate was added to keep the gel pieces wet during enzymatic cleavage (12–16 h). To extract peptides, 10 µL of 0.3% v/v TFA in ACN was added, the samples were sonicated for 5 min, and the separated liquid was taken to dryness under vacuum.

For MALDI-MS measurements, the samples were redissolved in 10 µL of 0.1% v/v TFA, 5% v/v ACN in water. The peptides were purified using a C18 RP-minicolumn fitted into a micropipette tip (ZipTip C18, Millipore, Bedford, MA, USA), prior to mass spectrometric analysis. The purification was carried out according to the manufacturers manual. Peptides were eluted from the ZipTips with 2.5 µL of 60% v/v ACN, 0.3% v/v TFA in water.

2.8 MALDI-MS PMF

MALDI-MS measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA, USA). 1 µL of the peptide solution was mixed with 1 µL of CHCA matrix solution consisting of 10 mg of matrix dissolved in 1 mL of 0.3% v/v TFA in ACN:water (1:1, v/v). From the resulting mixture 1 µL was applied to the sample plate. Samples were air-dried at room temperature. Measurements were performed in the reflection mode at an acceleration

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voltage of 20 kV, 70% grid voltage and a delay of 200 ns. Each spectrum obtained was the mean of 256 laser shots. Mass spectra were calibrated using autolytic fragments of trypsin as internal standards. Proteins were identified by searching the NCBI database using the program MS-Fit (http://prospector.ucsf.edu). The parameters for the search were as follows: the modification of cysteine-side chains by propionamide residues and the oxidation of methionine were considered as partial modifications. The maximum number of missed tryptic cleavages was 1. The monoisotopic masses were considered and the mass tolerance was set to 0.05 Da. A protein was accepted as identified if at least 25% of the complete protein sequence matched (sequence coverage > 25%).

2.9 1-DE

Immunoprecipitates were loaded onto a 12% *T* polyacrylamide gel (20 × 20 cm) and separated overnight followed by staining with Colloidal CBB (Sigma-Aldrich). The gel lanes were cut into 45 pieces of equal size and subjected to in-gel digestion with trypsin as described.

2.10 Capillary LC-MS/MS

For on-line ESI-MS/MS, the samples were reconstituted in 5 μL of 0.1% v/v TFA, 6% v/v ACN in water after in-gel digestion and dehydration under vacuum.

All experiments were performed on a quadrupole orthogonal acceleration TOF-mass spectrometer Q-TOF Ultima (Micromass, Manchester, UK) equipped with a Z-spray nanoelectrospray source. A micromass CapLC LC system was used to deliver the peptide solution to the electrospray source. Typically, 2–5 μL of the sample were injected using 0.2% v/v formic acid (FA) in water at a flow rate of 20 μL/min (eluent C) and concentrated on a precolumn (PepMap C18, 5 μm, 100 Å, 5 × 300 μm i.d.). Peptides were eluted onto an analytical column (PepMap C18, 3 μm, 100 Å, 150 × 75 μm i.d.) and separations were performed at an eluent flow rate of 200 nL/min which was achieved by splitting the flow (5 μL/min) of pumps A and B. Mobile phase A was 0.1% v/v FA in ACN:water (5:95, v/v) and B was 0.1% v/v FA in ACN:water (8:2, v/v). Runs were performed using a gradient of 3–64% B in 60 min. The mass spectrometer was operated in the positive ion mode with a capillary voltage of 1.8–2.2 kV, a cone voltage of 35 V, and a source temperature of 80°C. To perform MS/MS experiments automatic function switching (survey scanning) was employed. Doubly and triply charged ions with intensities greater than 120 counts were selected for MS/MS. The maximum number of concurrent MS/MS acquisitions from a single MS survey scan was 2. The instrument was calibrated using selected fragment ions that resulted from the collision-induced decomposition of Glu-fibrinopeptide B. The processed MS/MS spectra (MassLynx v 4.0 software) were appended to a single peak-list (pkl-file) and the MS/MS ion search option of the Mascot program (http://www.matrixscience.com) was used to search against the NCBI non-redundant protein database. The mass tolerance of precursor and sequence ions was set to 0.1 and 0.2, respectively. Addition of acrylamide onto cysteine side chains and methionine oxidation were considered as possible modifications. A protein was accepted as identified if the Mascot total score was greater than 100.

3 Results

3.1 Analysis of the cellular response triggered by mutant EGF/EPOR hybrids

To investigate the signal transduction downstream of the EPOR we used the pro-B cell line Ba/F3 which was engineered to express hybrid receptors consisting of the extracellular domain of the human EGFR fused to the transmembrane and cytoplasmic domains of the murine EPOR to circumvent activation of endogenous receptors. Ba/F3 cells have already been shown to be a useful model in the analysis of diverse receptor functions after expression of heterologous cytokine receptors. In a previous study we were able to link the activation of the transcription factor NF-κB to distinct receptor substructures and to define essential requirements for the induction of this pathway [12] using the same system. In the present study we attempted to compare two receptors mutated in the signaling competent intracellular domain to the wild type EPOR (Fig. 1). In one of the mutated receptor

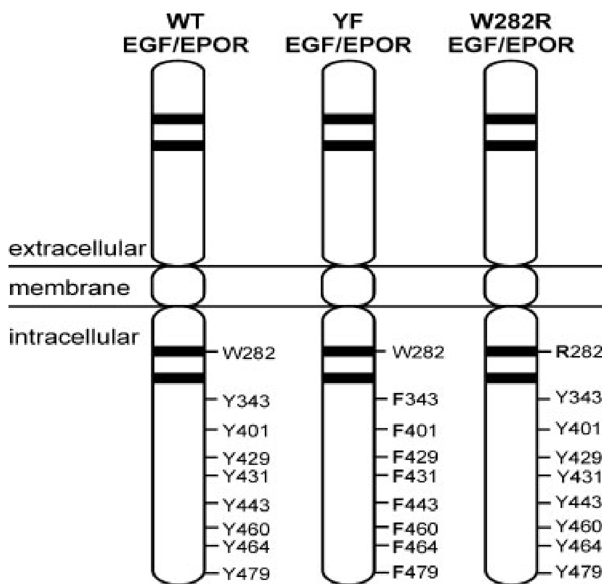

Figure 1. Schematic representation of wild type and mutated EGF/EPOR. All receptor forms consist of the human EGFR ligand binding domain and the transmembrane and cytoplasmatic domains of the murine EPOR. Amino acid numbers correspond to the mature WT EGF/EPOR protein. In contrast to the wild type all eight tyrosine residues of the intracellular domain have been converted to phenylalanine in the YF EGF/EPOR mutant protein. The W282R EGF/EPOR form contains a disrupted JAK2-binding site due to the replacement of tryptophan 282 by arginine.

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forms (YF EGF/EPOR) all eight tyrosine residues which are known to serve as docking sites for SH2-domain containing signaling proteins have been replaced by phenylalanine to explore the role of receptor phosphorylation for the induction of receptor-dependent signaling pathways. A second receptor designated as W282R EGF/EPOR contains a single amino acid substitution in a region proximal to the membrane causing an inability to associate with the cytosolic tyrosine kinase JAK2 [14]. JAK2 has been shown to be an essential part for most but not all of the known receptor functions. Consequently, the response initiated by this receptor form should reflect JAK2-independent signaling events.

After stable expression of the receptor types described and selection of clones with comparable expression levels we analyzed their ability to induce a proliferative response following stimulation by EGF (Fig. 2). In contrast to the wild type hybrid receptor both mutated receptor forms fail to induce mitogenesis. As we have described previously [12] these receptor types are not able to transmit signals which prevent apoptosis as shown by the separation of DNA preparations from cells deprived of IL-3 and exposed with or without EGF for 8 h. These data demonstrate that both cellular functions depend on the presence of receptor-associated JAK2, but also on the existence of phosphorylated tyrosine residues within the intracellular receptor domain. Additional experiments were carried out to investigate the potential of receptors to induce distinct signaling pathways. Immunoprecipitation studies revealed that JAK2 is indeed not phosphorylated on tyrosine residues in cells expressing the receptor W282R EGF/EPOR and challenged by EGF, whereas the other two forms retain the potential to activate the kinase (Fig. 3A).

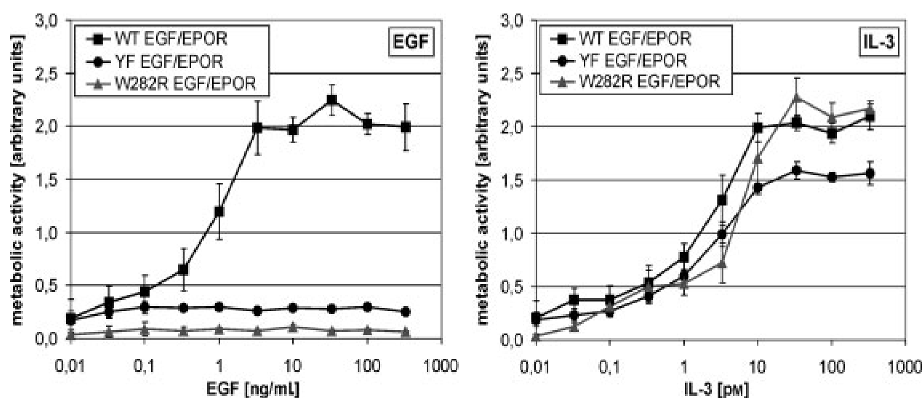

Figure 2. Tyrosine phosphorylation and JAK2-binding of hybrid EGF/EPOR are essential to transmit mitogenic activities. BaF3 cells stably transfected with the cDNAs for the WT, YF or W282R form of EGF/EPOR hybrids were cultured for 45 h in 96 well microtiter plates at 3000 cells per well in the presence of increasing concentrations of EGF or IL-3, respectively. The metabolic activity of all populations was determined by the conversion of added WST-1 reagent as described. The data shown represent the mean ±SEM of 4 independent experiments.

Furthermore, nuclear extracts of receptor-expressing cells were subjected to EMSA analysis for the presence of active transcription factors (Fig. 3B) following EGF stimulation. As expected, only the wild type receptor induces the DNA-binding activity of STAT5, a transcription factor known to be critically involved in EPO signaling. Interestingly, the JAK2-deficient receptor is able to induce the transcription factor AP-1, which is known to be a target of mitogen-activated protein kinase (MAPK)-dependent pathways, to the same extent as the wild type receptor.

Taken together the established system displays critical functions of EPO receptor specific signaling and allows a comparative analysis of different receptor types concerning their potency to modulate signaling and transcription on the basis of an almost identical cellular background.

3.2 Analysis of the tyrosine phosphorylated subproteome fraction in cells expressing EPOR variants by 2-DE and PMF

The interaction of cytokines with their specific membrane receptors results in a rapid and transient phosphorylation of cellular target proteins at tyrosine residues. In an attempt to identify these sets of protein substrates and to compare the corresponding potency of distinct receptor structures to mediate this process we prepared immunoprecipitates from both EGF-stimulated or unstimulated cells using a mixture of anti-phosphotyrosine antibodies. In an initial step we analyzed these subproteomic fractions using immunoblots for the presence of established signaling proteins known to be tyrosine phosphorylated in response to EPOR dependent signalling pathways. As shown in Fig. 4 for immunoprecipitates from cells expressing the wild type receptor, signaling molecules like STAT5 and JAK2, which are known to be tyrosine phosphorylated in response to EPOR activation are present in EGF-treated but not or to a much lower extent in control cells.

In a next step we separated immunoprecipitated phosphoproteins by 2-DE to establish phosphoprotein maps corresponding to the different receptor types in a stimulated/unstimulated condition. The corresponding CBB stained (data not shown) and silver stained gel images shown in Fig. 5 were reproduced several times. Using the image analysis software PDQuest v 7.0 (Bio-Rad Laboratories) we detected up to 44 spots (wild type receptor) in CBB stained gels and up to 168 spots in silver stained gels. As expected, immunoprecipitates from cells expressing the mutant receptor types display a lower number of detectable as well as differentially expressed

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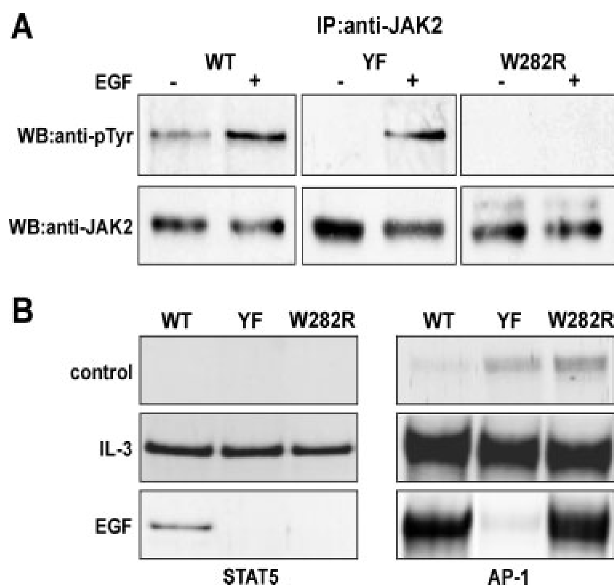

Figure 3. Activation of JAK2 and induction of STAT5 and AP-1 DNA-binding activities require specific receptor structures. (A) Cells were cultured for 5 h without IL-3, restimulated with EGF for 15 min and lysed in lysis buffer as described. JAK2 was immunoprecipitated and samples were analyzed by immunoblotting using a phosphotyrosine specific antibody. (B) Cells were maintained in medium for 5 h without any cytokine and restimulated with IL-3 or EGF for 15 min. Nuclear extracts were subjected to EMSA analysis using oligonucleotides containing STAT5 or AP-1 binding sites, respectively.

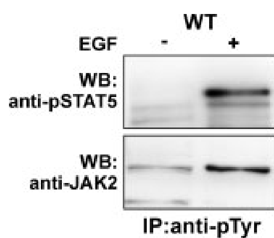

Figure 4. Anti-phosphotyrosine immunoprecipitations of EGF-stimulated cells contain STAT5 and JAK2. Tyrosine phosphorylated proteins were immunoprecipitated from unstimulated or EGF-stimulated Ba/F3 cells expressing the WT EGF/EPOR. Aliquots of the immunoprecipitates were separated by SDS-PAGE, transferred onto PVDF membrane and analyzed using antibodies directed to Phospho-STAT5 or JAK2.

phosphoproteins (see Table 1). We exploited two mass spectrometric techniques to identify the proteins from both CBB stained gels (PMF by MALDI-TOF) as well as silver stained gels (capillary LC-MS/MS). The results are summarized in Table 1 and the identity of proteins is displayed in Table 2. Almost all identified proteins are components of the cytoskeleton or known to be highly expressed metabolic enzymes. Although we have shown the presence of typical signaling proteins in the immunoprecipitates (see above), they are obviously not detectable by CBB and silver staining of 2-DE gels.

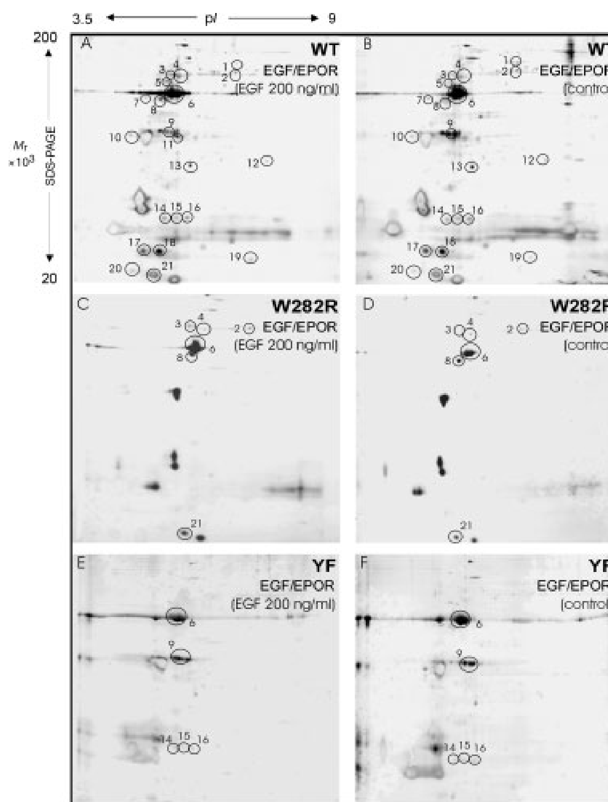

Figure 5. 2-DE phosphoprotein profiles of cells expressing different EGF/EPOR constructs. Tyrosine phosphorylated proteins were prepared by immunoprecipitation from EGF-treated or untreated Ba/F3 cells expressing the WT EGF/EPOR (A-B), W282R EGF/EPOR (C-D) and YF EGF/EPOR (E-F), separated by 2-DE and silver stained.

Table 1. Detection of phosphoproteins by 2-DE analysis

	WT EGF/EPOR		W282R EGF/EPOR		YF EGF/EPOR	
Staining	I	II	I	II	I	II
No. of proteins	44	168	19	33	30	55
Differential expressed proteins	10	14	8	6	3	6

After immunoprecipitation of tyrosine-phosphorylated proteins, their 2-DE separation, CBB (I) and silver staining (II) the total number and differential expression of proteins were compared for three different receptor types. The images were analyzed by the PDQuest v 7.0 software (Bio-Rad Laboratories).

3.3 Examination of EPOR dependent signal transduction by 1-DE and capillary LC-MS/MS

In an attempt to develop a strategy which allows us to identify low expressed proteins in the immunoprecipitates described above we combined 1-DE and capillary LC-MS/MS. Immunoprecipitated proteins were separated by SDS-PAGE,

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Table 2. Tyrosine-phosphorylated proteins identified from 2-DE gels by nanoLC-MS/MS shown in Fig. 5

No.	Identified proteins	NCBI Acc. no.	$M_r \times 10^3$	p*I*	WT	W282R	YF
					peptides matched		
1	lamin C	P11516	65.5	6.4	3	–	–
2	propionyl-CoA-carboxylase	P05166	58.2	7.6	24	17	–
3	vimentin	P20152	53.7	5.1	3	1	–
4	thymopoietin	AAC52574	50.2	9.4	10	3	–
5	inhibitor of urokinase	P35456	35.4	6.4	1	–	–
6	actin	CAA45026	41.8	5.2	3	3	2
7	inhibitor of urokinase	P35456	35.4	6.4	2	–	–
8	tropomodulin	Q9JHJ0	39.5	5.0	5	7	–
9	actin	CAA45026	41.8	5.2	14	–	10
10	tropomyosin	AAB37701	28.9	4.7	13	–	–
11	inhibitor of urokinase	P35456	35.4	6.4	1	–	–
12	hydrolase (serine proteinase)	NP_011915	27.4	5.9	2	–	–
13	myosin light chain alkali	Q60605	15.8	4.8	11	–	–
14	myosin light chain alkali	Q60605	15.8	4.8	1	–	1
15	hydrolase (serine proteinase)	NP_011915	27.4	5.9	4	–	4
16	hydrolase (serine proteinase)	NP_011915	27.4	5.9	3	–	2
17	cytochrome-c-oxidase	AAH05564	13.8	8.3	3	–	–
18	lectin	AAH57165	57.8	5.9	1	–	–
19	hydrolase (serine proteinase)	NP_011915	27.4	5.9	2	–	–
20	hydrolase (serine proteinase)	NP_011915	27.4	5.9	2	–	–
21	cytochrome-c-oxidase	AAH05564	13.8	8.3	1	–	–

each lane was cut into 45 pieces of equal size and subjected to in-gel digestion by trypsin. Using LC-MS/MS we were able to identify up to 25 protein species in a single gel slice.

In contrast to the common approach we found a substantial higher number of proteins involved in cytokine dependent cellular functions. In total, we identified 388 different proteins in cells expressing the WT EGF/EPOR, 348 proteins in W282R EGF/EPOR cells and 293 proteins in YF EGF/EPOR cells. As shown in Table 3 these proteins fall into several functional groups. In general, anti-phosphotyrosine immunoprecipitations of EGF-stimulated cells contain a higher number of proteins. As expected, the WT EGF/EPOR displays the highest potential to mediate the phosphorylation of proteins, whereas cells which are not able to recruit the tyrosine kinase JAK2 to the receptor (W282R EGF/EPOR) are clearly diminished in their response to EGF. Importantly, we were able to identify a high number (49) of signaling proteins using this approach. Some of these proteins belong to signaling pathways (i.e. JAK2, SHIP, Lyn) already established for the EPOR, but we also identified several proteins which have not been linked to this system so far (Table 4).

4 Discussion

The development of red blood cells is tightly regulated and critically dependent upon erythropoietin and its cognate receptor, which transmits specific signals to the nucleus after ligand binding. The induction of differentiation, proliferation as well as pro- or antiapoptotic decisions are triggered by receptor-dependent signaling pathways which culminate in changes at the level of gene expression. A common strategy to investigate the role of specific signaling pathways is to monitor the activity of signaling components or changes in the quantity of one or more mRNA species after challenge of cells by cytokines. However, the significance of such data has been hampered by the complexity and cross-talk of the signaling cascades involved. Therefore it will be necessary to explore new approaches to facilitate the analysis of cytokine function.

In this work we studied the complex EPOR signal transduction network by the application of two proteomic approaches. To evaluate the potential of these techniques in the analysis of signaling pathways we expressed a wild type EGF/EPOR construct and two variants containing mutations affecting the association of signaling molecules in the pro-B cell line Ba/F3. On the basis of an almost identical cellular background we compared their phosphoprotein pattern before and after receptor activation. It is well accepted that the phosphorylation status of proteins contains an extraordinary amount of information about the cellular situation. Our experimental strategy was designed for the analysis of short time stimulated (15 min) cells to focus on post-translational modifications of signaling proteins and to minimize the potential modulation of protein levels. In addition, cDNA hybridization experiments performed simultaneously (data not shown) clearly show that soon after cytokine challenge many cytokine dependent genes start to undergo transcriptional changes. In fact, the highest number of differential

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Table 3. Classification and numbers of proteins identified by nanoLC-MS/MS

Functional classification	**WT** EGF/EPOR		**W282R** EGF/EPOR		**YF** EGF/EPOR	
Signaling	**37**		**24**		**17**	
+ −	30	18	16	16	13	11
Cytoskeleton	**36**		**16**		**26**	
+ −	32	25	11	9	21	15
Metabolic proteins	**68**		**95**		**84**	
+ −	58	33	57	70	63	51
Transcription and translation	**193**		**164**		**117**	
+ −	155	134	120	112	100	67
Other proteins	**54**		**49**		**49**	
+ −	42	34	29	35	34	34
Total no.	**388**		**348**		**293**	

Numbers of identified proteins from wild type and receptor mutants W282R and YF (EGF stimulation [+], control [−]). Immunoprecipitated phosphoproteins were separated by SDS-PAGE. The lanes were cut in pieces and each gel slice was digested by trypsin as described. Samples were analyzed by nanoLC-MS/MS technology.

expressed genes was detected within 30 min after receptor activation in a time course up to 8 h. This is in agreement with the concept that the cytokine dependent modulation of signaling pathways produces rapid changes in the transcriptome and proteome specifying a certain cell phenotype. Our first approach to compare the proteins enriched by anti-phosphotyrosine immunoprecipitation in cells before and after stimulation of three different erythropoietin receptor types was based on a common strategy, the 2-DE separation of proteins and their identification by mass spectrometric techniques. Although we used an effective preselection and were able to establish reproducible phosphoprotein images the mass spectrometric identification revealed that the majority of proteins are highly expressed proteins of the cytoskeleton and the ribosomal machinery, which are, at least in part, known to undergo phosphorylation in response to receptor activation. The expected proteins already established for EPOR-dependent pathways, however, were not detectable within the 2-DE separated protein pattern in silver stained gels. This is in agreement with several recently published studies focusing on similar subjects [15, 16, 17] and underlines the limitations of 2-DE based approaches in particular the restricted detection of low abundance proteins [18]. As shown for the EPOR associated signaling proteins JAK2 and STAT5, however, such low expressed proteins can easily be detected by conventional immunoblotting techniques in the immunoprecipitates used to create 2-DE phosphoprotein patterns as described above. To make these obviously low expressed proteins accessible to proteomic analysis we evaluated a second approach based on 1-DE separation of phosphoprotein immunoprecipitates and the analysis of peptide mixtures produced by tryptic digestion of proteins in gel fragments by LC-MS/MS. This strategy enabled us to identify a wide variety of signaling proteins. Among them are well established signaling intermediates known to be part of EPOR-triggered pathways, but also a number of new candidates which have not been linked to this system so far. For example, our data clearly show the ability of the WT EGF/EPOR to activate the JAK-dependent pathway as well as the Ras/Raf/MAPK cascade, whereas the corresponding proteins were not present in the phosphoprotein pool triggered by the mutant receptor forms. Both pathways have been shown to be involved in mitogenic and antiapoptotic activities of the EPOR and depend critically on the existence of receptor associated tyrosine kinases and phosphorylated tyrosine residues in the cytosolic receptor domain [12, 19, 20]. Interestingly, the W282R EGF/EPOR cells were shown to contain active AP-1 after EGF stimulation which suggests the existence of JAK2 independent induction. A second tyrosine kinase, Lyn, which is known to be activated in response to EPO [23, 24] was also detected among the WT EGF/EPOR-triggered phosphoproteins. We have recently shown a functional implication during erythroid maturation and a physical interaction between Lyn and the EPOR using immobilized peptides representing the intracellular receptor domain [25]. Apart from these and other well established molecules we identified a panel of proteins which may represent new signaling intermediates in this system (Table 4). The phosphorylation of small ubiquitin-like modifier-1 (SUMO-1), which is restricted to wild type receptor expressing cells, has not been observed so far. SUMO-1 is involved in the post-translational modification of proteins and has been suggested to be part of strategies repressing cytokine induced signals in analogy to the covalent ubiquitin attachment [26]. Furthermore, we identified several GTP-binding proteins as well as proteins involved in the modification/regulation of G proteins which have not been reported to be involved in EPOR induced regulatory events. Most of these proteins belong to the Ras related superfamily including Ras, Rab, Rho and ARF subfamily members. It has recently been shown, that an inhibitory heterotrimeric G protein is constitutively coupled to a region in the *C*-terminal end of the EPOR [27, 28]. The corresponding data suggest a role of a Gi protein in the activation of Erk1/2. Interestingly, the receptors used in our study display distinct differences in their ability to modulate the activity of

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Table 4. Examples of signaling proteins identified by nanoLC-MS/MS

No.	Signaling protein	Acc. No.	$M_r \times 10^3$	pI	WT	W282R	YF
					peptides matched		
1	protein-tyrosine kinase JAK2	B39577	130.5	7.1	2	–	–
2	MAP kinase p38	IP38	43.3	6.0	3	–	–
3	protein kinase ERK-1	S28184	42.8	6.2	2	–	–
4	MAP/ERK kinase kinase 3	NP_002392	67.1	9.1	1	–	–
5	SH2-containing inositol phosphatase	JC6118	133.3	7.8	4	–	–
6	RAS related protein 5C	P51147	23.4	8.6	1	1	1
7	GTP binding protein SAR 1a	P36536	22.4	6.4	–	1	1
8	myb binding protein 1a	NP_058056	151.9	9.1	7	2	1
9	Sipa-1	P46062	112.0	6.0	3	–	–
10	GTPase-activating protein	S27869	35.6	9.1	2	–	–
11	GPI-anchored membrane protein 1	NP_058019	73.5	4.8	2	2	–
12	inositol triphosphate receptor type 2	CAA43852	306.8	6.1	3	–	–
13	GAP SH3 binding protein	NP_038744	51.8	5.4	2	9	–
14	p62 ras-GAP associated phosphoprotein	AAA64997	48.3	8.8	3	–	–
15	protein-tyrosine kinase lyn	A39719	58.8	7.1	2	–	–
16	RuvB-like 2	NP_006657	51.1	5.5	2	3	2
17	vaccinia related kinase 3	AAH10473	50.8	8.9	5	5	4
18	Rag D protein	NP_067067	45.6	4.8	2	–	–
19	GTP-binding protein G39	CAA31391	40.1	5.3	1	–	–
20	guanine nucleotide-binding protein	Q14344	40.4	5.3	3	1	–
21	casein kinase alpha subunit	CAA38710	45.1	7.3	3	–	–
22	casein kinase II	B30319	44.4	7.4	3	–	–
23	SUMO-1 activating enzyme subunit 1	NP_005491	38.4	5.2	1	–	–
24	protein phosphatase subunit 1-alpha	CAA30645	37.5	5.9	2	–	–
25	GTP-binding regulatory protein beta-2	RGHUB2	37.3	5.6	2	–	–
26	14–3-3 protein, epsilon	AAB22277	17.6	5.1	2	–	–
27	14–3-3 protein, zeta/delta	P29361	27.8	4.8	2	2	2
28	dolichol-phosphate mannosyltransferase 1	NP_034202	29.6	9.6	1	–	–
29	RAB27A protein	NP_034202	25.0	5.2	1	–	–
30	GTP-binding protein Ran/TC4	JC1455	14.8	5.9	1	–	4
31	Phosphatidylinositol synthase	P70500	23.5	8.2	2	–	–
32	calmodulin	MCRB	16.7	4.1	1	–	–
33	GTP-binding protein DRG	JC1349	40.5	9.0	–	1	–
34	G protein beta subunit	BAA06185	35.0	8.1	–	1	2
35	Rab5c protein	AAH23027	23.4	8.6	–	1	2
36	calreticulin	NP_031617	48.0	4.3	–	2	–
37	annexin A2	NP_004030	38.6	7.6	–	5	–
38	GTP-binding protein, 23K	S01934	22.8	5.2	–	1	–
39	ADP-ribosylation factor 4	NP_031505	20.4	6.6	–	1	–
40	PTPN6	AAC36008	63.1	6.8	–	2	–
41	carboxyl terminal LIM domain protein 1	NP_058557	35.7	6.4	–	1	–
42	phosphoprotein phosphatase	B27430	33.5	5.5	–	1	–
43	MAPK activating protein PM24	NP_037425	32.7	11.3	–	2	–
44	GDPdissociation inhibitor 3	NP_032138	50.5	5.9	–	–	1
45	rho GDP dissociation inhibitor alpha	AAH04732	23.4	5.1	–	–	1
46	GTP-binding protein rab1B	S06147	22.2	5.6	–	–	2
47	Ras-related protein Rab-6A (Rab-6)	P20340	23.6	5.4	–	–	2
48	SET protein	Q01105	32.1	4.1	–	–	1
49	S-AKAP84	AAB53740	57.9	4.8	–	–	1

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GTP-binding proteins. Work is continuing in order to examine the specific involvement of these molecules in the EPOR signaling machinery and the role of certain receptor structures. The strategy described above is, however, not suitable for the quantification of phosphoproteins. A combination with recently developed methods allowing the quantitative comparison of two phosphoprotein pools like ^{18}O labeling or the isotope coded affinity tag approach should overcome this limitation.

We can conclude from our experiments that a proteomic strategy based on an effective enrichment of phosphoproteins and their identification by a combination of 1-DE and LC-MS/MS provides the sensitivity required for the detection of low expressed signaling molecules and offers the potential for a more comprehensive analysis of complex cellular responses.

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