Receptor tyrosine kinase signaling: a view from quantitative proteomics

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Growth factor receptor signaling *via* receptor tyrosine kinases (RTKs) is one of the basic cellular communication principals found in all metazoans. Extracellular signals are transferred *via* membrane spanning receptors into the cytoplasm, reversible tyrosine phosphorylation being the hallmark of all RTKs. In recent years proteomic approaches have yielded detailed descriptions of cellular signaling events. Quantitative proteomics is able to characterize the exact position and strength of post-translational modifications (PTMs) providing essential information for understanding the molecular basis of signal transduction. Numerous new post-translational modification sites have been identified by quantitative mass spectrometry-based proteomics. In addition, plentiful new players in signal transduction have been identified underlining the complexity and the modular architecture of most signaling networks. In this review, we outline the principles of signal transduction *via* RTKs and highlight some of the new insights obtained from proteomic approaches such as protein microarrays and quantitative mass spectrometry.

Introduction

In multicellular organisms cellular survival is dependent on the proper performance of intercellular communication networks. Most cellular functions and activities are controlled by extracellular signaling molecules and one generic way to transmit the signal across the plasma membrane is by transmembrane receptors. The stimulus is transduced *via* different signaling cascades to distinct sets of terminal effectors localized throughout the entire cell, *e.g.* the cytoskeleton, the nucleus, and other cellular compartments. One large group of receptors possesses intrinsic protein tyrosine kinase activity, thus forming the family of receptor tyrosine kinases (RTKs).[1–3] These include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and insulin receptor (InsR) subfamilies. RTKs are involved in the regulation of crucial cellular processes such as cell metabolism, growth, mobility, survival, and most notably cell proliferation and differentiation. It is therefore not surprising that deregulated forms of these receptors (as a result of overexpression or activating mutations) are involved in the genesis of many malignancies.[1,4]

The term proteomics stands for the large scale study of proteins.[5] However, lately the term has been used more broadly enclosing all kinds of elaborate protein-centric studies. The analyses of organelles, protein interactions, protein modifications, are, amongst others, all part of proteomics.[6–9] Proteomics is getting more and more popular due to the higher sensitivity and throughput of current techniques. The automation of large scale protein studies allows experimental set-ups similar to genomic approaches which would not have been possible a decade ago.[10] In the first part of this review we will briefly discuss the principles of signal transduction of RTKs and in the second part we will highlight the impact of quantitative proteomics on the current view of RTK signaling. We will discuss quantitative proteomics approaches focusing on quantitative mass spectrometry (MS)-based proteomics and its influence on the perception of EGFR signaling.

Signal transduction by receptor tyrosine kinases

All RTKs share some common structural and functional features. They are single polypeptide chains (one exception is the InsR family where the receptors exist as disulfide-linked dimers), which consist of an extracellular ligand-binding domain, one transmembrane helix, and a C-terminal cytoplasmic portion.[3,11] The most distinguishing feature of RTKs is the presence of a tyrosine kinase domain in their intracellular portion. RTKs also employ similar strategies to trigger downstream signal transduction.[12] Binding of the ligand to the extracellular parts triggers dimerization and subsequent autophosphorylation of the receptor subunits, a result of major significance for kinase activation and for downstream signaling of RTKs.[3,13–15] Phosphorylation of conserved tyrosine residues within the activation loop of the catalytic domain (*e.g.*, Tyr 1162 for InsR or Tyr 857 for PDGF β receptors) results in displacement of the activation loop from the active site and increased enzymatic activity.[16–18] The importance of such an autoregulatory mechanism is supported by several examples of point mutations in the activation loops that result in cell transformation due to constitutive kinase activity.[1,4] In contrast to virtually all other RTKs, members of the EGFR family appear not to employ this autoregulatory mechanism. Substitution of the conserved tyrosine in the

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activation loop appears not to affect the kinase activity of these receptors.[19] As a result of this loose autoinhibitory function ErbB2 displays kinase activity even in the absence of ligand stimulation. Furthermore, as found in numerous malignancies, the kinase activity of this receptor is strongly enhanced by its overexpression, causing cell transformation.[1,4,20,21]

A common theme of the enhanced kinase activity of RTKs is that it leads to their subsequent autophosphorylation on tyrosine residues outside the catalytic domain, which are the key to downstream signaling (Fig. 1). Since the discovery of protein tyrosine kinases, tyrosine phosphorylation has been systematically recognized as a major mechanism of transmembrane signaling, in particular in RTK-mediated signal transduction. Tyrosine autophosphorylation of RTKs is critical for the recruitment and activation of a variety of downstream cytoplasmic targets and subsequent initiation of dynamic signaling cascades. Indeed, most of the tyrosine autophosphorylation sites are located outside the catalytic domain and function as specific docking sites for Src homology 2 (SH2) and/or phosphotyrosine binding (PTB) domains of otherwise structurally and functionally diverse signaling proteins that are able to propagate the signal in various ways.[3,22–25] The major signaling pathways downstream of RTKs being the Ras/MAPK (mitogen-activated protein kinase) signaling cascades, signaling pathways involving phospholipid second messengers *via* PLCγ (phospholipase C-γ) and PI3K (phosphatidylinositol 3′-kinase), and the STATs (signal transducers and activator of transcription) pathway (Fig. 1). These have been extensively reviewed over the years and will not be further described here (for reviews see ref. 2, 3 and 23).

The specificity of signal transduction at this initial stage is directed to a large extent by the amino acid sequence surrounding the phosphorylated tyrosine residue. This is the key factor that determines which of the phosphotyrosine-binding proteins will be recruited to the activated RTK (from the plethora of SH2/PTB domain-containing proteins expressed in a given cell). For example, phosphorylation on a tyrosine residue which is followed by an asparagine residue at the +2 position results in recruitment of Grb2 (growth factor receptor-bound protein 2) and subsequent MAPK activation, whereas the presence of a methionine residue at the +3 position is the key to PI3K binding and the consequent switch to phospholipid-driven downstream signaling. Some of the target molecules of RTKs possess intrinsic enzymatic activities, which are altered by their binding to activated receptors, resulting in subsequent phosphorylation and/or a conformational change.[26–32] Examples include Src protein tyrosine kinase, Shp2 protein tyrosine phosphatase, GTPase activating protein for Ras (Ras GAP), PI3K and PLCγ among many others. For some of these enzymes (*e.g.*, PI3K and PLCγ), the recruitment to RTKs seems to serve a dual function, since it also translocates them to the inner side of the cell membrane where their substrates are to be found (Fig. 1).[33–35] Other signaling molecules do not contain any catalytic domains and function as adaptors that bind several proteins at the same time, thus linking activated receptors to additional proteins involved in downstream signal propagation.[36–38] A prototypical example is the pivotal adaptor molecule Grb2, which connects a variety of RTKs to the Ras/MAPK signaling pathway.[39,40]

Another major aspect of RTK signaling is the proper attenuation and termination of signal transmission. In order to maintain their normal cellular functions and physiological outcomes, the activities of RTKs must be tightly regulated. Indeed, simultaneous with cell activation, activated receptors initiate a cascade of events responsible for the subsequent attenuation and termination of signals. Attenuation and termination of RTK signaling occurs at various cellular levels and involves structurally and functionally diverse signaling molecules. Some of these molecules are protein kinases downstream of the activated receptors (*e.g.*, PKC and ERK) that phosphorylate specific residues, usually serine or threonine within the juxtamembrane receptor region, and inhibit RTK activity.[41–44] Other important regulators are protein tyrosine phosphatases (PTPs), which dephosphorylate key tyrosine residues on activated receptors or downstream signaling molecules, thereby downregulating RTK-mediated signal transduction.[45,46] A major mechanism responsible for the attenuation and termination of RTK-mediated signaling, parallel to the regulatory processes described above, is the endocytic removal of active receptor–ligand complexes from the cell surface. Central to these processes appears to be the covalent conjugation of ubiquitin (monoubiquitination) or a chain of ubiquitins (polyubiquitination) to the receptor and downstream signaling molecules (Fig. 1).[47–50] Several studies demonstrated that RTK monoubiquitination on multiple sites is sufficient for both endocytosis and degradation of EGFR and PDGFR.[51,52] However, it was also recently shown that EGFR can be internalized independently of ubiquitination events as well.[53,54] These studies strongly suggest a robust and complex internalization process, which can happen dependently and independently of multiple kinase and ubiquitination mechanisms.

Due to the importance of RTKs for many aspects of human biology, RTK signaling has been extensively studied over the last few decades and a large number of proteins that are involved in the signaling pathways are already known. However, most of these proteins do not fit into the canonical dogma "one gene-one protein-one function" but implement complex biological functions and are often involved in signaling networks with distinct or even opposite terminal effects. Also, in the past few years it became evident that signaling pathways do not operate as linear cascades, but rather as networks, allowing cross-talk between different pathways involving various positive and negative feedback signals. In this regard, large-scale proteomics studies have contributed significantly to the current awareness of the enormous complexity and dynamics of RTK signaling networks.

Proteomic approaches for elucidation of signaling pathways

In proteomics, generally large numbers of proteins of given cell states are analyzed by techniques ranging from protein microarrays to MS-based approaches. Whereas a few years ago the mere presence of a protein was analyzed, nowadays proteomic approaches are quantitatively characterizing changes

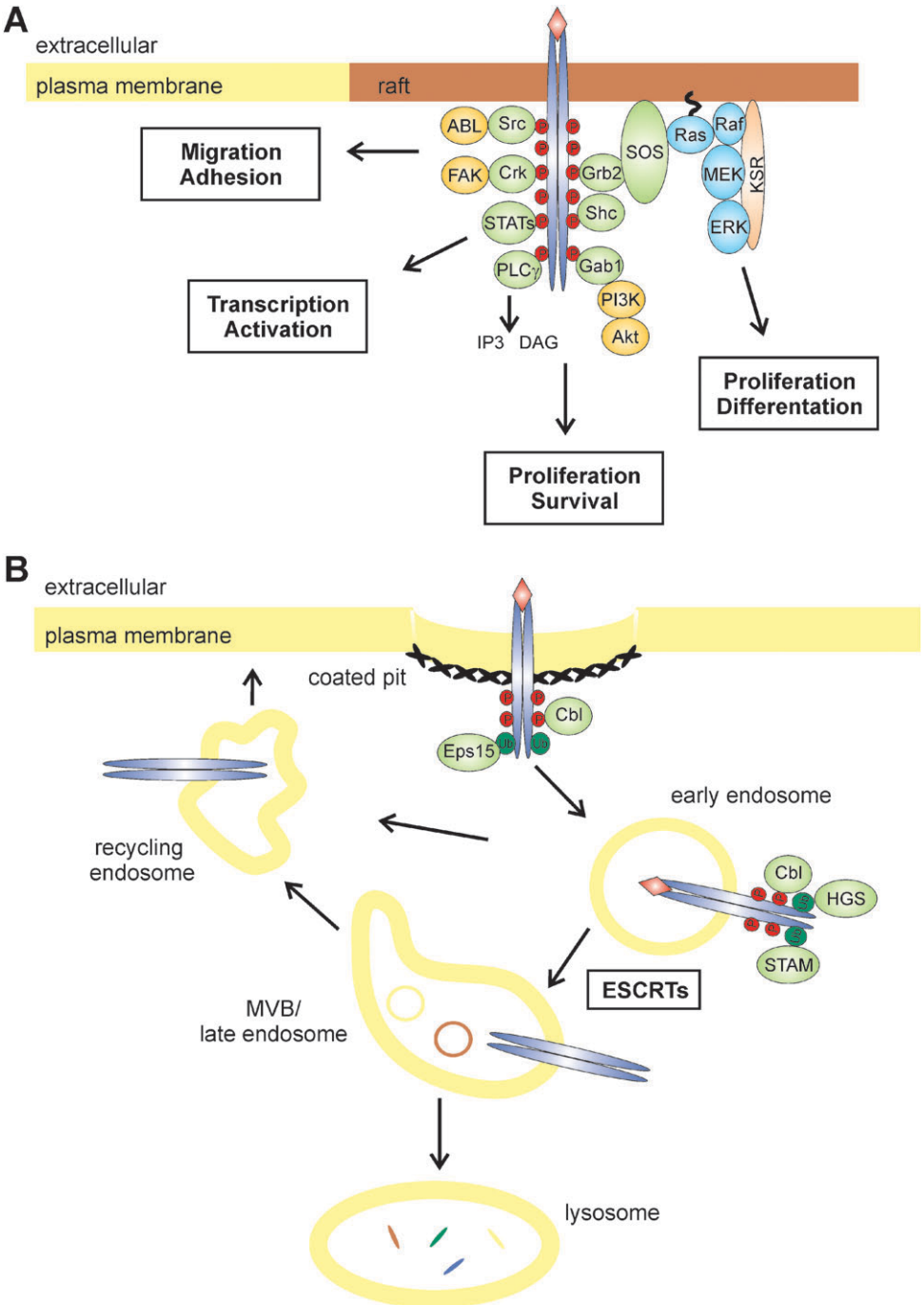

Fig. 1 Major pathways of the EGFR signaling network. **A**: **Signal initiation at the plasma membrane**. Ligand binding induces receptor dimerization followed by transphosphorylation of tyrosine residues. Signaling pathways downstream of EGFR include the Ras/MAP kinase signaling cascade, signaling pathways involving phospholipid second messengers *via* PLCγ and PI3Ks, signaling *via* non-receptor tyrosine kinases, and the STAT pathway. **B**: **Receptor internalization and signal attenuation**. EGFR is internalized *via* caveolae or clathrin-coated pits. Cbl family E3-ligases are responsible for ubiquitination of receptors. Sorting of receptors for recycling or degradation takes place in early endosomes as well as in multi-vesicular bodies (MVBs) and it is tightly regulated by different endosomal sorting complexes required for transport (ESCRTs) and ubiquitin binding proteins (for review see ref. 47).

on a proteome-wide scale.[55] Recent developments of vigorous quantitative proteomic strategies allow truly "functional proteomics" experiments. Temporal changes in protein composition can now be recorded allowing, for example, cellular signal transduction to be tracked. Data obtained from temporal analyses of phosphorylation events reveal insights into cellular decision making, enabling the construction of time-resolved signaling networks.[56] These network models may allow *in silico* predictions of the consequences of network perturbations happening, *e.g.* in specific diseases. Currently, significant efforts are being made into the mathematical modeling of RTK signaling networks.[57] For example, a computational linear mapping

technique has been used to successfully predict cell migration and proliferation after ErbB2 stimulation, identifying critical phosphorylation events.[58] Pathway analysis tools for the characterization of high-throughput ''omics'' data are complementing these undertakings.[59,60] Ultimately, models could help the greater understanding of diseases like cancer, enabling the design of better-directed potent therapies. In general, targeted and discovery-driven quantitative proteomic strategies can be distinguished. Targeted analyses such as protein microarrays, western blot analyses, and phospho-FACS can be ideally used to analyze single signal nodes in greater detail over many time points.

The discovery-driven technique par excellence is quantitative MS-based proteomics, which allows an unbiased global analysis of cellular protein dynamics and differential comparison of entire signaling networks.[61,62] MS-based quantitative proteomic approaches have thereby become the method of choice for the analysis of PTM-based cellular signaling events.[9,63] Thus, global phosphorylation changes can now be studied in an unbiased approach and a site-specific characterization and quantitation of signaling events *in vivo* is feasible. In the remaining part of this article we will focus on EGFR signaling dynamics and discuss the various proteomic strategies, their findings and implications on the current view of RTK signaling.

Non-MS-based analyses

A large fraction of the classical non-MS-based analyses rely on the availability of recombinant proteins and specific antibodies. As the sensitivities of antibody-based approaches are very high, these are optimal if only a limited sample amount is available, *e.g.* in a clinical setting. It was however shown that over 50% of antibodies tested in a whole yeast proteome screen reacted with multiple targets.[64] Nevertheless, when carefully designed targeted non-MS-based analyses are of great value in establishing detailed protein interaction networks. For example, microarrays containing virtually all human SH2 and PTB domains were used to measure the dissociation constants for each domain of 61 tyrosine-phosphorylation sites of the four human ErbB receptors.[65] 116 new interactions were identified. Additionally, when EGFR and ErbB2 were overexpressed, as often occurs in cancer, they became more promiscuous with lower affinity thresholds, whereas ErbB3 stayed unchanged. This could mean that the extent of the protein concentration-dependent promiscuity change might have an influence on the oncogenic potential of a receptor. In a similar approach new interactions of ErbB4 were described, such as the binding of STAT1.[66] It appeared that ErbB4 interacts more selectively than the other ErbB receptors. As there is no ligand known for ErbB2, and ErbB3 is enzymatically inactive, the selectivity of ErbB4 could enable ErbB2 and ErbB3 to signal independently of EGFR. In a different methodology, laser capture-microdissected material of human non-small cell lung cancer and a reverse phase protein array was used to evaluate whether EGFR mutations influence phosphorylation levels of EGFR and downstream components *in vivo*.[67] It was shown that EGFR mutant carcinomas exhibit increased signaling *via* the Akt (PKB)/mTOR pathway, possibly providing survival advantages compared to cells baring wild type receptors.

Quantitative mass spectrometry

A whole panel of quantitative MS-based proteomic strategies exist, mostly relying on isotopically labeled peptide counterparts (Fig. 2).[68] It is possible to absolutely quantify signaling events using techniques such as the AQUA method.[69] An isotopically labeled peptide is synthesized and added in known amounts to the sample to absolutely quantify its endogenous counterpart. As this is rather cost intensive, relative quantitation techniques reporting fold-changes have become widely appreciated. Two of the best known examples are isobaric tag for relative and absolute quantitation (iTRAQ) and stable isotope labeling by amino acids in cell culture (SILAC).[70,71] iTRAQ employs isotope coded covalent tags which are used to chemically label peptide or protein mixtures. Classically it was used as a 4-plex strategy. However, recently 8-plex iTRAQ has been introduced.[72] Using SILAC, the entire cellular proteome is metabolically labeled by isotopic variants of amino acids added to the growth medium. Most commonly, different populations of cells are grown in medium containing three distinct forms of both arginine (Arg) and lysine (Lys) allowing 3-plex experiments.[73] Peptides are labeled by one amino acid variant when trypsin is used for enzymatic digestion, which simplifies data analysis as compared to ^{15}N labelling.[74,75] Several strategies for label-free protein quantitation have been suggested, which generally rely on extracted ion currents, peptide, or spectral counting.[10,76–78] Whereas label-free quantitation has the advantage of reduced costs it is still less accurate when compared to isotope-labeled analyses.[10,79] Thus, to confidently detect *e.g.* two-fold changes, larger numbers of experimental replicas are required and significantly more peptides from the corresponding proteins have to be recorded compared to label-based strategies.[80,81]

Targeted MS analyses

Targeted MS analyses, *e.g. via* multiple reaction monitoring (MRM) or using the AQUA strategy, are not limited by specific reagents and yield the highest possible sensitivity for MS-based type of experiments. Nevertheless, to be included into these analyses, phosphorylation sites have to be identified by discovery-driven approaches, because peptides carrying regulated phosphorylation sites have distinct physico-chemical properties, and not all are readily detectable by common mass analyzers.[82] In an initial MRM study a discovery phase was followed by a subsequent monitoring and quantitation phase. 222 EGF-regulated tyrosine phosphorylation sites (of which 31 were not known) were monitored over seven time points achieving an experimental reproducibility of 88% compared to 34% in an unbiased approach.[83] Additionally, this set-up increased the dynamic range significantly. The high sensitivity and reproducibility of this technique makes it especially applicable for the analysis of clinical biopsies, as it will allow an in-depth monitoring of phosphorylation levels at multiple signaling nodes.

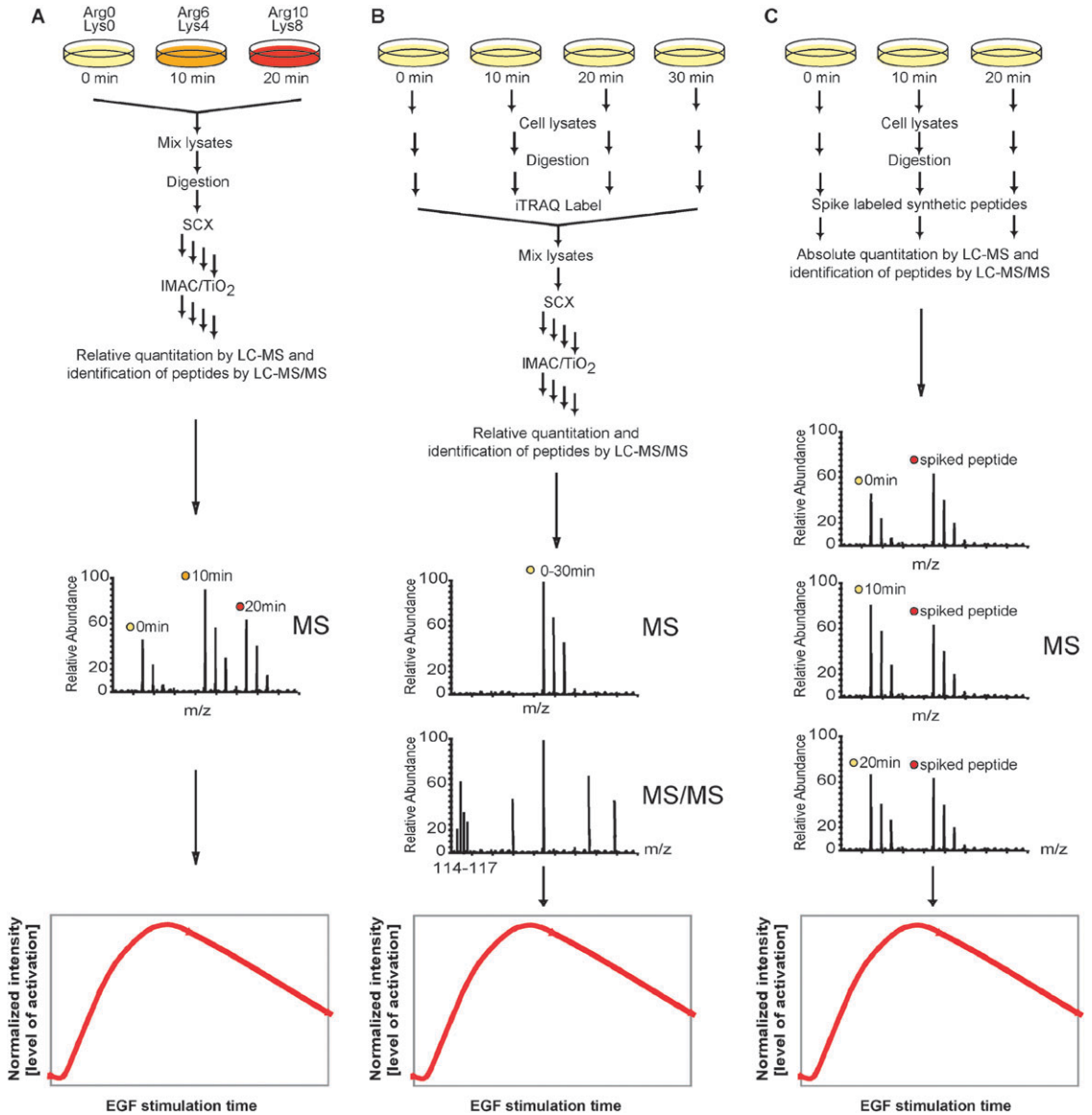

Fig. 2 Experimental design outlines for the analysis of cellular signaling events by quantitative MS. Examples are given for phosphosite-specific time course studies of ligand stimulation. **A**: **SILAC approach.** SILAC labeled cells are stimulated for various times, lysed, mixed, and ezymatically digested. Respective peptide mixtures are fractionated by strong cation exchange (SCX) chromatography, phosphopeptides purified by IMAC or TiO_2, and finally analyzed by LC-MS/MS (upper panel). Whereas peptides containing not regulated phosphosites show a ratio of 1 : 1 : 1 corresponding to the mixing ratio, peptides bearing positively regulated phosphosites show an increasing ratio. Quantitation is performed in the MS spectra (middle panel). Ratios can be combined to generate an activation profile (lower panel). **B**: **Four-plex iTRAQ approach**. Cells are stimulated for various times, lysed, ezymatically digested, labeled, and mixed. Respective peptide mixtures are treated like in A (upper panel). In the MS modes iTRAQ-labeled peptides show one signal, yielding higher intensities and reduced sample composition. Quantitation is done in the MS/MS modes *via* the reporter ions 114, 115, 116, and 117 (middle panel). Ratios can be combined to generate an activation profile (lower panel). **C**: **AQUA approach**. Cells are stimulated for various times, lysed, enzymatically digested, and synthetic peptides are spiked into the samples. Each sample has to be analyzed separately (upper panel). Using spiked peptides allows the absolute quantitation of endogenous counterparts, which is done in the MS mode (middle panel). Ratios can be combined to generate an activation profile (lower panel).

Discovery-driven MS analyses

As modified peptides are present in a substoichiometric amount, cell fractionation and analyses of subproteomes enriched in proteins or peptides involved in signal transduction are very useful. For the analysis of phosphopeptides several enrichment steps are often combined to multi-stage enrichment strategies, as for example strong cation exchange chromatography (SCX) and metal-affinity purification procedures such as immobilized metal affinity chromatography (IMAC) and TiO_2 (Fig. 2) (for a recent review see ref. 84). Fractionation can happen on different levels: on the protein level, which may also allow the identification of signal dependent protein–protein interactions, and on the peptide level. The enrichment of proteins involved in signaling can again be achieved by different means. In one of the first studies

to use quantitative MS as a read-out for signal transduction, proteins associated with activated EGFR were purified using the SH2 domain of Grb2 fused to GST as bait.[85] SILAC encoded cells were left untreated or treated with EGF and the combined lysates were affinity enriched with the recombinant bait protein. As the SH2 domain binds specifically to activated EGFR and Shc, EGF-dependent protein interactions could readily be analyzed. A total of 228 proteins were identified of which 28 were selectively enriched upon stimulation. Next to well established binding partners such as EGFR, Shc, Vav-2 and Cbl, proteins at that time not known to be involved in EGFR-dependent signal transduction could be identified, *e.g.* CD59.

Next to domains, peptides can be used to specifically enrich proteins involved in signaling events. Using this approach, interaction partners to all cytosolic tyrosine residues of the four members of the ErbB-receptor family were determined.[86] Pull-down experiments with pairs of phosphorylated and nonphosphorylated synthetic peptides in combination with SILAC were performed, indicating that each receptor has characteristic preferences for interacting proteins and most interaction partners have multiple binding sites on each receptor. STAT5 was identified as a direct binding partner to EGFR and ErbB4, and new recognition motifs were discovered for Shc and STAT5.

Protein complexes can also be purified using antibody-based strategies, especially regarding phosphotyrosine signaling. As the relatively large size of the modified tyrosine residue makes phosphotyrosines rather immunogenic, very good antibodies to be used for immunoprecipitation are available. Immunoprecipitations employing phosphotyrosine antibodies have been used to delineate EGFR,[87–90] InsR,[91,92] Her2/neu,[93] FGFR,[94] and EphB2 receptor signaling.[95] On comparison of PDGF- and EGF-based signaling in mesenchymal stem cell differentiation, it was shown that more than 90% of the signaling proteins were used by both ligands, whereas the PI3K pathway was exclusively activated by PDGF.[90] Chemical inhibition of PI3K in PDGF-stimulated cells removed the differential effect of the two growth factors, implicating it as a control point and highlighting the capability of quantitative proteomics to directly compare entire signaling networks and discover critical differences capable of changing cell fate. An antibody-based approach was used to characterize EGFR ubiquitination as well. After stimulation with EGF, six distinct ubiquitination sites were identified by tandem MS. Using an AQUA approach it could be shown that more than 50% of all EGFR-bound ubiquitin derived from polyubiquitin chains. The majority was Lys63-linked implicating a role of this linkage in receptor sorting.[96] In another study an antibody-based approach in combination with SILAC was utilized to study the influence of protein-tyrosine phosphatase (PTP) 1B on EGF and PDGF signaling.[97] PTP1B-deficient mouse embryonic fibroblasts were treated and tyrosine phsophorylation events were compared to wild-type counterparts. Eighteen proteins were identified that harbored hyperphosphorylated phosphotyrosine sites that could be functionally linked to PTP1B.

Antibody-based screening for interaction partners in combination with the analysis of signaling kinetics allows exciting insights into signal initiation and propagation. By using SILAC and 5 time points over 20 min of EGF treatment virtually all known EGFR substrates plus 31 novel effectors were identified.[88] In addition, the recorded kinetics could be used to position the proteins in the signal transduction network. A more recent study utilized a quench-flow system for the quantitative proteomic assessment of very early cellular signaling events (qPACE) in combination with SILAC, which allowed signaling kinetics to be recorded after 0, 1, 5, 10, and 60 s of EGF treatment.[89] It could be shown that receptor autophosphorylation on several tyrosine residues occurs within seconds of ligand binding. Shc tyrosine phosphorylation followed almost immediately the phosphorylation of EGFR indicating that the molecules have to be in close spatial proximity even without a stimulus being present.[98] The extended range proteomic analysis (ERPA) technique, an intermediate approach between protein-centric and peptide-centric proteomics, and label-free quantitation was used to analyze, in addition to phosphorylation changes, the dynamics of extracellular glycans on EGFR after stimulation of up to 4 h.[77] Although no changes in glycosylation were detected, this approach might become useful for the comparison of glycan structures in different disease settings.

Antibodies might as well be used for the purification of phosphorylated peptides. Information about non-phosphorylated interaction partners would be lost, however the number of identified phosphorylation sites should increase due to less non-phosphorylated peptide signals. This approach was applied in combination with iTRAQ labeling to study EGFR signaling networks, allowing clustering of activation profiles of single sites.[99] Comparing new and known sites in one cluster provided evidence for the biological role of so far unknown sites and proteins. This strategy has also been successfully used for the characterization of Her2/neu and InsR signaling. Her2/neu signaling was analyzed after EGF and heregulin (HRG) treatment and it could be shown that EGF treatment activated multiple signaling pathways stimulating migration, whereas HRG treatment resulted in stimulation of a specific subset of the migratory signaling network.[100] After up to 45 min of insulin treatment 122 tyrosine phosphorylation sites on 89 proteins could be quantified. 69 of these sites were not known to respond to insulin treatment.[101] Global surveys of tyrosine-phosphorylated peptides in cancer cell lines and non-small cell lung cancer tumors (NSCLC) demonstrated the feasibility of global screenings using antibody-based approaches, identifying in one study more than 4500 phosphotyrosine sites on more than 2700 proteins. Known oncogenic kinases such as EGFR and c-Met as well as RTKs so far not known to be involved in NSCLC, such as PDGFR, ALK, ROS, and DDR1, were identified.[102,103]

As described above, the characterization of phosphotyrosine-based signaling events has been performed successfully using immuno-affinity protocols. However, phosphoserine and phosphothreonine-based signaling events, which play an important role in signal propagation and negative feedback mechanisms of RTKs,[3] are more difficult to assess using antibody-based approaches as these residues are less immunogenic. Nevertheless, phosphoserine and phosphothreonine-antibodies,

usually targeting defined phosphorylation motifs of specific serine/threonine kinases, have been successfully applied,[104] *e.g.* to study the ATM/ATR signaling pathway.[105] To capture a larger fraction of cellular signaling events happening after growth factor treatment, global analyses have to be performed. In a first attempt to globally characterize the EGFR signaling network in an unbiased way, SILAC and EGF treatment at multiple time points were combined with SCX fractionation and TiO_2 enrichment.[106] Activation profiles of 6600 phosphorylation sites on 2244 proteins were recorded. More than 1000 sites responded more than 2-fold to EGF treatment. Among the regulated sites were many known from EGFR, Shc, and the ERKs. In addition, this study revealed that there were at least 46 transcriptional regulators involved in the signaling pathways downstream of EGFR within the 20 min stimulation period. Numerous novel phosphorylation sites were identified including sites from the transcription factors WBRS9 and DAFT-1. Clustering the kinetic data revealed functional groups of proteins allowing the signal cascade to be followed from the plasma membrane through the cytosol and into the nucleus (Fig. 3). Numerous cases were detected where sites on the same protein were regulated differently, thus highlighting the importance of analyzing signaling networks both site-specifically and quantitatively in order to unveil which of the protein's phosphorylation sites are the relevant regulatory switches for the corresponding functional context or biological system.

Conclusion and future perspectives

Growth factor signaling *via* RTKs is able to elicit diverse cellular reactions such as migration and adhesion, differentiation and proliferation, survival and apoptosis. The deregulation of RTK signaling is a hallmark of many cancer types. Extracellular signals trigger intracellular signaling cascades based on reversible PTM, phosphorylation being the most prominent one. Phosphorylation is by far the best studied cellular PTM which is largely due to the relative ease of detecting cellular phosphorylation changes. In the last decade, powerful proteomic approaches have been developed for the elucidation of cellular signaling cascades which can be based not only on phosphorylation changes, but on other diverse PTMs such as glycosylation, acetylation, methylation, and ubiquitination.[107] The new generation of mass spectrometers have the sensitivity and dynamic range to allow standardized screenings for modified peptides and proteins. Thus, MS-based proteomics in combination with new powerful software tools promises to holistically uncover the variety and density of

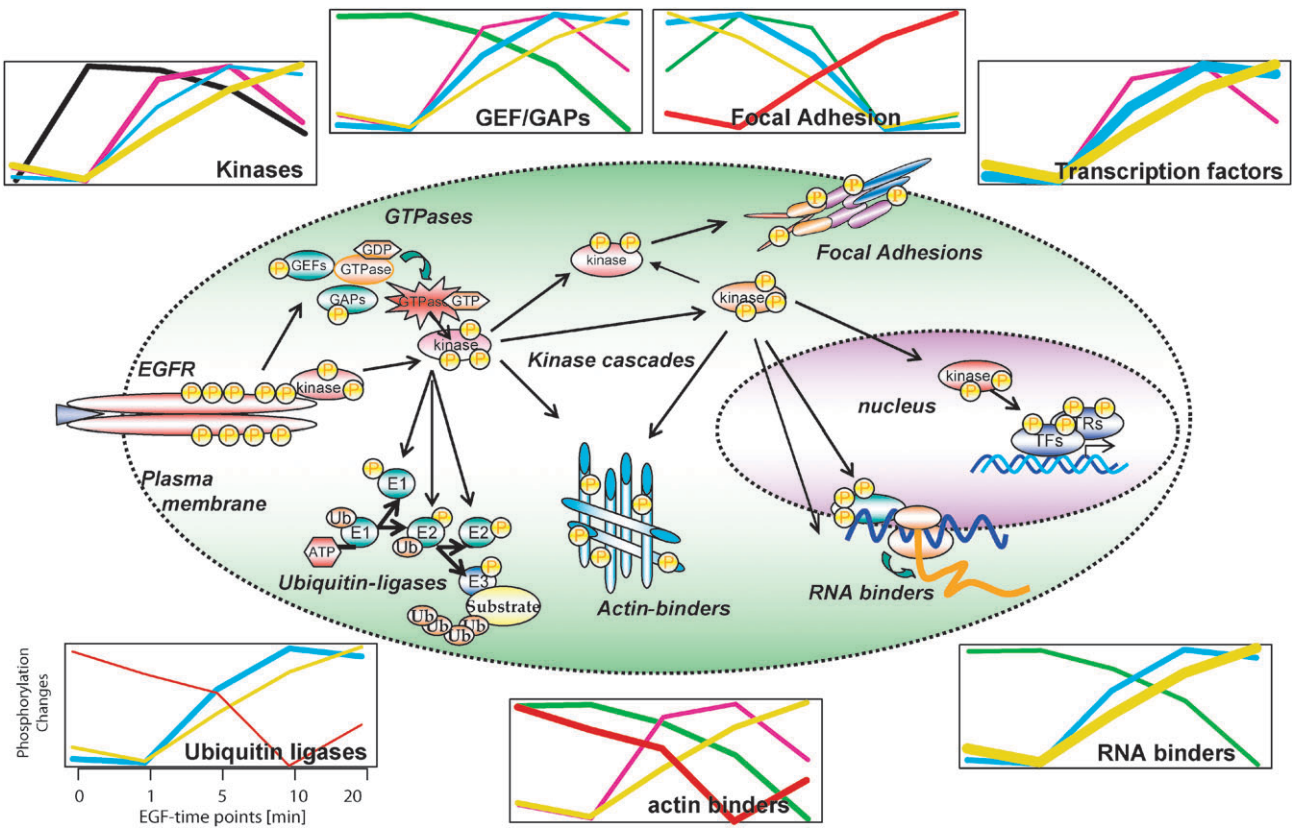

Fig. 3 EGFR signaling network: a view from quantitative phosphoproteomics. Within one minute of EGF treatment tyrosine sites on the receptor are getting phosphorylated. Direct interactors follow immediately and within five minutes multiple signaling pathway are fully activated. After ten minutes, active transcription factors reach the nucleus and set off gene transcription. Transmitting the signals from the membrane throughout the entire cell *via* reversible phosphorylation is a highly dynamic and complex process that involves numerous proteins from various functional categories and cellular localization. Some of the major categories of regulated phosphoproteins are shown in boxes with corresponding consensus kinetic curves (modified from ref. 106).

PTM combinations on a given protein. Due to MS-based proteomics the stimulus specific cross-talk of proteins can be understood allowing the design of comprehensive network models which will help to explain cellular decision making.

A major challenge for the future will be to unravel the highly dynamic nature of signal transduction, to determine the critical fine-tuning mechanisms that are responsible for specific biological outcomes, and to understand how these processes are aberrant in human diseases. In this regard, quantitative proteomics has already provided a treasure trove of data. Numerous novel signaling proteins have been revealed in the EGFR signaling network alone and thousands of EGF-dependent kinetics of phosphorylation events have been recorded as well. The information-rich data generated by quantitative proteomic approaches allows the formulation of new hypotheses which have to be tested by experimental cellular biology approaches to provide a deeper understanding of their biological significance. This is a challenge for the whole scientific community and as such is a research endeavor beyond the operability of a single group or institute.

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