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Review

Signal perception and transduction: the role of protein kinases

Paul W. Schenk¹, B. Ewa Snaar-Jagalska^{*}

Section of Cell Biology, Institute of Molecular Plant Sciences, Leiden University, P.O. Box 9505, 2300 RA Leiden, Netherlands

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Abstract

Cells can react to environmental changes by transduction of extracellular signals, to produce intracellular responses. Membrane-impermeable signal molecules are recognized by receptors, which are localized on the plasma membrane of the cell. Binding of a ligand can result in the stimulation of an intrinsic enzymatic activity of its receptor or the modulation of a transducing protein. The modulation of one or more intracellular transducing proteins can finally lead to the activation or inhibition of a so-called 'effector protein'. In many instances, this also results in altered gene expression. Phosphorylation by

Abbreviations: AC, adenylyl cyclase; ANP, atrial natriuretic peptide; aPKC, atypical PKC; cAK, cAMP-dependent protein kinase; CaMK, Ca²⁺/calmodulin-dependent kinase; cAMP, cyclic AMP; CDK, cyclin-dependent kinase; cGK, cGMP-dependent protein kinase; cGMP, cyclic GMP; CK1, casein kinase I; CK2, casein kinase II; Clk, Cdc-like kinase; cPKC, classical or conventional PKC; CREB, cAMP responsive element binding protein; Csk, C-terminal Src kinase; DAG, diacylglycerol; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal regulated kinase; FAK, focal adhesion kinase; G α_i , inhibitory G α subunit; GAIP, G alpha interacting protein; GAP, GTPase activating protein; G α_s , stimulatory G α subunit; GC, guanylyl cyclase; GEF, G-nucleotide exchange factor; GNRP, G-nucleotide releasing protein; GPCR, G-protein-coupled receptor; G-protein, guanine-nucleotide binding protein; Grb2, growth factor receptor-bound protein 2; GSK-3, glycogen synthase kinase-3; His K, histidine kinase; Hog1p, high osmolarity glycerol response 1 protein; Ins, insulin; IP₃, inositol 1,4,5-trisphosphate; IRS, insulin receptor substrate; JAK, *Janus* kinase; JNK, cJun N-terminal kinase; KHD, protein kinase homology domain; Ksr-1, kinase suppressor of Ras 1; LIM domain, *lin-11/ISL1/mec-3* homology domain; LIMK, LIM kinase; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MAPKAP2, MAPK activated protein kinase 2; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MKK, MAPK kinase; MKP-1, MAPK-specific phosphatase 1; MTK1, MAP three kinase 1; Myr modification, myristoyl modification; nPKC, non-classical or novel PKC; PAK, p21-activated kinase; PDGF, platelet-derived growth factor; PDK1, 3-phosphoinositide-dependent protein kinase 1; PDZ domain, PSD-95/Dlg/ZO-1 homology domain; PI3K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTK, protein tyrosine kinase; PY, phosphotyrosine; RACK, receptor for activated C-kinase (PKC); RBD, Ras binding domain; RGS, regulator of G-protein signaling; RICK, receptor for inactive C-kinase (PKC); RPTP, receptor-like protein tyrosine phosphatase; RSK, p90 ribosomal S6 kinase; RTK, receptor tyrosine kinase; SAPK, stress-activated protein kinase; SEK1, SAPK/ERK kinase 1, SH2, Src homology domain 2; Shc, Src homology and collagen; S6K, p70 S6 protein kinase; Smad, Sma/Mad homolog; Sos, son of sevenless; STAT, signal transducer and activator of transcription; TAK1, TGF- β activated kinase 1; TESK1, testis-specific protein kinase 1; TGF- β , transforming growth factor- β ; TGFR, activin/TGF- β receptor; TM domain, transmembrane domain; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate

^{*} Corresponding author. Fax: +31 (71) 527-4999; E-mail: jagalska@rulbim.leidenuniv.nl

¹ Present address. Department of Medical Oncology, Josephine Nefkens Institute–Daniel den Hoed Cancer Center, University Hospital Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands.

protein kinases is one of the most common and important regulatory mechanisms in signal transmission. This review discusses the non-channel transmembrane receptors and their downstream signaling, with special focus on the role of protein kinases. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Protein kinase; Signal perception; Signal transduction

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1. Introduction

Cells react to environmental changes, which they perceive through extracellular signals. These signals can be either physical (e.g. light, temperature, pressure and electricity) or chemical (e.g. food, hormones and neurotransmitters). Cells can both sense and produce signals. This makes it possible that they communicate with each other. In order to achieve this, there are complex signal-sensing and -producing mechanisms in uni- and multi-cellular organisms.

Two groups of chemical signals can be distinguished: membrane-permeable and membrane-impermeable signals. The membrane-permeable signal molecules comprise the large family of steroid hormones, such as estrogens, progesterone and androgens. Steroids pass the plasma membrane and bind to specific receptors, which are localized in the cytoplasm or nucleus of the cell. After binding of the hormone, the receptor undergoes a conformational change. The receptor is then able to bind to DNA itself or to proteins which can in turn interact with

DNA. In general, steroid hormones can directly regulate gene expression by means of this process [1]. The membrane-impermeable signal molecules include acetylcholine, growth factors, extracellular matrix components, thrombin, lysophosphatidic acid, the yeast mating factors and, for the social amoeba *Dictyostelium discoideum*, folic acid and cyclic AMP. They are recognized by receptors, which are localized on the plasma membrane of the cell. The receptors are specific for one particular signal molecule or a family of closely related signal molecules. Upon binding of their ligands, these receptors transduce the signals by several mechanisms.

Binding of a ligand to an ion channel receptor can directly lead to its altered opening, which results in a changed membrane potential. There are, for instance, acetylcholine-regulated cation channels in nerve terminals [2]. Alternatively, binding of a ligand may result in the stimulation of an intrinsic enzymatic activity of its receptor or the modulation of a transducing protein. The modulation of one or more intracellular transducing proteins can finally lead to the activation or inhibition of a so-called 'effector protein'. Activity modulations can be achieved by covalent modifications at the molecular level. Among the most common and important modifications are protein phosphorylation and dephosphorylation on serine, threonine or tyrosine residues. Phosphorylation and dephosphorylation are carried out by kinases and phosphatases, respectively. As described below, changes in the phosphorylation state of cellular components can alter their properties in several ways. An interesting yet complicating phenomenon is cross-talk: intracellular responses to extracellular signals which influence each other. While stimulation of a cell with one specific ligand may lead to more than one response, different signals may lead to an analogous response via identical components.

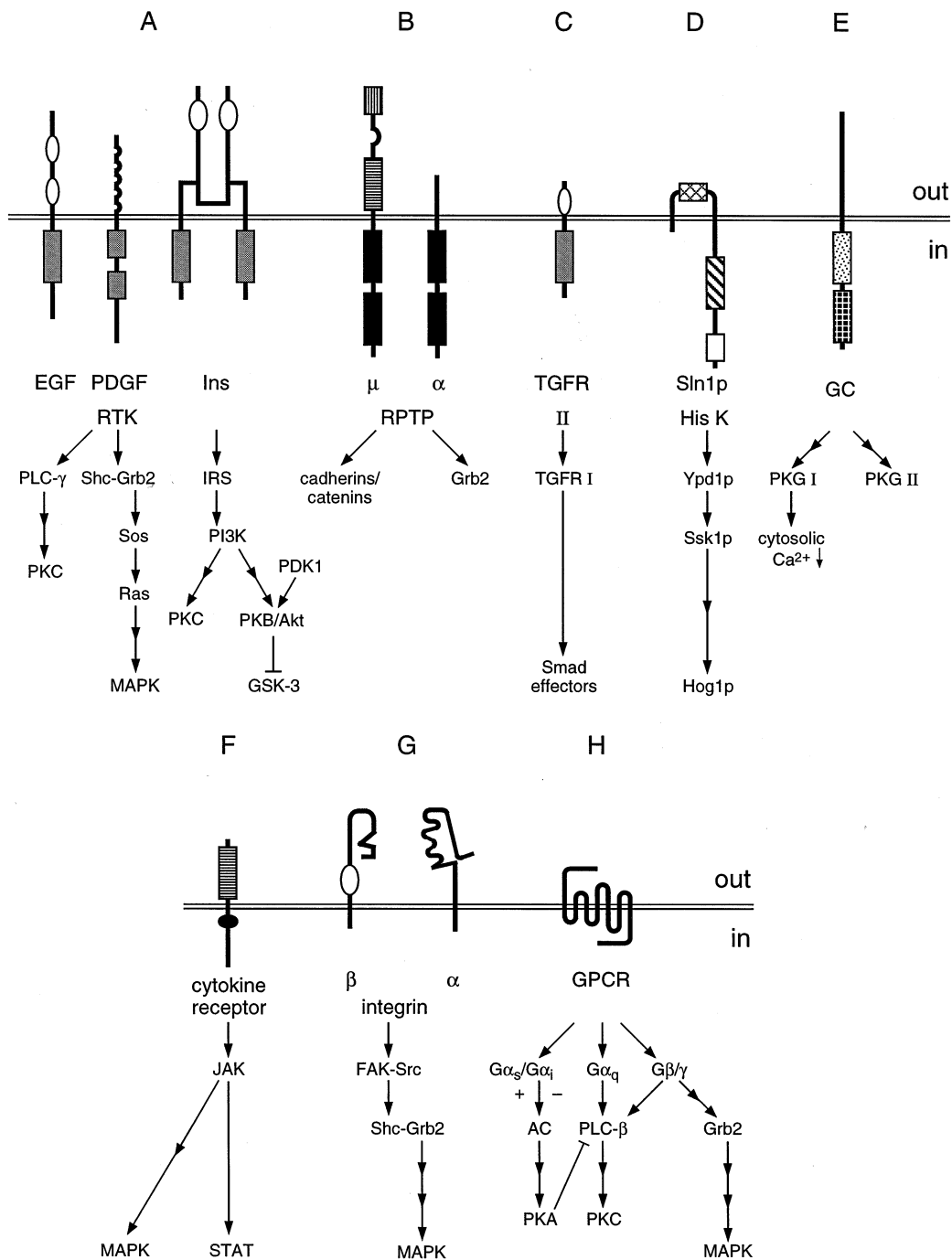
The non-channel transmembrane receptors and their downstream signaling will be discussed, with special focus on the role of protein kinases. Particular attention will be drawn to protein kinase C and mitogen-activated protein kinases. We will present current knowledge and attempt to place this into the perspective of possible future developments.

2. Receptors with enzymatic activity

2.1. Receptor tyrosine kinases

Extracellular signal molecules, like epidermal growth factor, platelet derived growth factor and insulin bind to receptor tyrosine kinases (RTKs). These receptors possess one or two intracellular tyrosine kinase regions (Figs. 1A and 2B). Upon ligand binding, RTKs auto-phosphorylate. The resulting phosphotyrosine (PY) residues act as highly selective binding sites for so-called 'SH2' (Src homology domain 2)-containing proteins, which transduce the signal by changing their enzymatic activity or recruiting other proteins. Among these SH2-containing proteins are Ras-GTPase activating protein (GAP) and phospholipase C- γ . The latter hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ releases Ca²⁺ from intracellular stores; DAG activates protein kinase C (PKC) (Fig. 1A) [3]. The SH2-containing adapter molecules, Shc and Grb2 (Src homology and collagen and growth factor receptor-bound protein 2, respectively), also bind to phosphorylated RTKs. They are importantly involved in the recruitment of the son of sevenless (Sos) protein towards the plasma membrane [4]. Sos, which is called a GEF (G-nucleotide exchange factor) or GNRP (G-nucleotide releasing protein), stimulates the exchange of GDP for GTP on the small G-protein Ras, thereby activating it. Ras-GTP in turn stimulates a protein kinase cascade resulting in mitogen-activated protein kinase (MAPK, also called ERK, for extracellular signal regulated kinase) activation (Fig. 1A) [5].

The p85 subunit of phosphatidylinositol 3-kinase (PI3K) is recruited to PY residues on insulin receptors by IRS (insulin receptor substrate) adapters. The PI3K p85 subunit is linked to the p110 subunit, which is able to phosphorylate PIP₂. This generates phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which has several intracellular targets, such as PKC and protein kinase B (PKB)/Akt. The activation of PKB/Akt requires phosphorylation by PDK1 (3-phosphoinositide-dependent protein kinase 1) and leads to serine phosphorylation and inactivation of GSK-3 (glycogen synthase kinase-3) (Figs. 1A and 2B) [6,7]. The C-terminus of the cytoplasmic PDZ



domain protein, Enigma, interacts with Tyr-containing motifs outside the tyrosine kinase cores of the Ret RTK and the insulin receptor. Enigma does so via two of its three so-called 'LIM domains' (see below). The LIM2–Ret and LIM3–insulin receptor interactions are required for mitogenic signaling

by Ret/*ptc2* and receptor endocytosis, respectively [8,9].

2.2. Receptor-like protein tyrosine phosphatases

Receptor-like protein tyrosine phosphatases

Fig. 1. Structures of cell surface receptors and schematic diagrams of their downstream signaling. In plasma membrane receptors, the N-terminus is generally extracellular, while the C-terminus is cytoplasmic. Main structural features are indicated. Open ovals, cysteine-rich regions; shaded boxes, protein kinase catalytic domains; semi-circles, immunoglobulin-like regions; horizontally striped boxes, fibronectin-like regions. Double and triple arrows represent multiple signal transduction steps (activation via second messenger formation or a cascade). (A) Receptor tyrosine kinases (RTKs). Structures of the EGF (epidermal growth factor), PDGF (platelet derived growth factor) and Ins (insulin) receptor subclasses. The protein tyrosine kinase domain of the PDGF receptor contains an insertion sequence. (B) Receptor-like protein tyrosine phosphatases (RPTPs). Structures of RPTP μ and RPTP α . Vertically striped box, specific cell–cell interaction domain; solid boxes, protein tyrosine phosphatase catalytic domains. Some RPTPs contain a single phosphatase domain. (C) Receptor serine/threonine kinases. Structure of the activin/TGF- β type II receptor (TGFR II). (D) Histidine kinases. The *Saccharomyces* Sln1p His K (histidine kinase) contains an extracellular input region (double diagonally striped box) and an intracellular transmitter module (diagonally striped box) and receiver domain (open box). (E) Transmembrane guanylyl cyclases (GCs). Dotted box, protein kinase homology domain; black and white box, cyclase catalytic domain. (F) Cytokine receptors. Solid oval, membrane-proximal JAK binding box1/box2 motifs. (G) Integrins. Structures of β and α subunits. The extracellular ligand binding region is made up from both subunits in $\alpha\beta$ heterodimers. (H) G-protein-coupled receptors (GPCRs). The C-terminal intracellular domains of GPCRs greatly vary in sequence and length. For further abbreviations, details and references, see main text.

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(RPTPs) contain a variable extracellular domain, a transmembrane domain and one or two intracellular tyrosine phosphatase domains (Fig. 1B). Depending on the RPTP subtype, the extracellular domain exhibits immunoglobulin- and fibronectin-like regions and other sequence motifs involved in cell–cell adhesion [10,11]. The subtype I RPTP CD45 is involved in the activation of B- and T-lymphocytes. It stimulates the Src-like kinases Lck and Fyn by dephosphorylating them [12]. The subtype II RPTPs μ and κ have been shown to modulate cell–cell interaction. There is some debate on the association of the cytoplasmic domain of RPTP μ with a protein complex containing cadherin transmembrane adhesion molecules and catenins (which are linked to the underlying actin cytoskeleton). It appears that Brady-Kalnay et al. recently reinforced their original observation, that cadherins are part of such complexes, which has been doubted by other authors (Fig. 1B) [11,13]. The subtype IV RPTP α tightly binds to Grb2 (Fig. 1B). Although the precise biological function of the resulting complex remains to be determined, it has already been suggested that binding of Grb2 may inhibit RPTP α activity [14]. The establishment of the exact repercussions of ligand binding to RPTPs is currently in progress.

2.3. Receptor serine/threonine kinases

The effects of transforming growth factor- β (TGF- β) superfamily signal molecules are mediated by receptors with a cysteine-rich extracellular domain and a cytoplasmic serine/threonine kinase activity (Figs.

1C and 2BC). In addition to the three TGF- β isoforms, this superfamily comprises activins, bone morphogenetic proteins and other secreted factors. Members of the TGF- β superfamily have been implicated as being crucial for many specific developmental events in vertebrates, *Drosophila melanogaster* and *Caenorhabditis elegans*; TGF- β induces growth arrest in epithelial cells. TGF- β -related factors signal through type I and type II receptor serine/threonine kinases, which form a heteromeric complex. Whereas type II receptors bind TGF- β 1 independently, this ligand does not bind to the type I receptor in the absence of the type II receptor. In the heteromeric complex, the type II receptor phosphorylates the type I receptor [15,16]. A type I–type II complex (but not type II alone) can associate with the effector Smad3 (for Sma/Mad homolog 3; formerly called hMAD-3), which is phosphorylated in vitro by type I. A complex of Smad3 and its homolog Smad4 is thought to finally function as a transcriptional regulator of downstream genes in the nucleus (Fig. 1C) [17]. The Smad2 protein has recently been reported to act as a common positive effector of both receptor serine/threonine kinases and RTKs; this provides one of the many examples of cross-talk during signal transduction from the cell surface to the nucleus [18].

2.4. The two-component regulatory system: histidine kinases

Prokaryotic organisms commonly employ the two-component regulatory system; homologous pathways have recently been identified in eukaryotes, in-

cluding *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Neurospora crassa* and *Dictyostelium discoideum*. The prototypical two-component pathway consists of two proteins: a protein histidine kinase (also called sensor kinase) and a response regulator. The N-terminal part of the histidine kinase functions as an input domain, detecting extracellular signals directly or via an upstream receptor. The C-terminal portion contains the transmitter module. This domain includes a histidine residue at which the protein auto-phosphorylates. The response regulator has a receiver domain, which catalyzes the transfer of the phosphoryl group from the histidine of the sensor kinase to a conserved aspartate residue on the receiver. The phosphorylation state of the regulator modulates the activity of its output domain, which is often involved in the regulation of transcription [19]. Hybrid histidine kinases combine an input region, a transmitter module and a response regulator in one molecule. Sensing domains can be integrated within the histidine kinase or contained within a separate protein [20]. Histidine kinases are very distinct from the superfamily of conventional protein serine/threonine and tyrosine kinases.

There are complex signal transduction pathways built from two-component circuit elements: one important motif is the His–Asp–His–Asp phosphorelay. One of the most striking examples is the Sln1p–Ypd1p–Ssk1p pathway, which governs osmoregulation in the budding yeast *S. cerevisiae*. The transmembrane protein Sln1p contains an extracellular input region and cytoplasmic histidine kinase and receiver domains (Fig. 1D); the cytoplasmic Ssk1p protein has a receiver domain. Ypd1p binds to both Sln1p and Ssk1p and mediates the phosphorelay. During this phosphorelay, a phosphate is first transferred from a His in the Sln1p kinase domain to an Asp in its receiver domain, then to a His in Ypd1p, and finally to an Asp in Ssk1p. Ssk1p in turn modulates the activity of the downstream Ssk2p/Ssk22p–Pbs2p–Hog1p (for high osmolarity glycerol response) MAPK cascade (Fig. 1D) [21–23].

2.5. Guanylyl cyclases

Guanylyl cyclases (GCs) usually serve as receptors that produce cyclic GMP (cGMP) from GTP in response to ligand binding. In general, the plasma

membrane forms of GC possess a variable extracellular domain, a single transmembrane domain, an intracellular protein kinase homology domain (KHD) and an intracellular cyclase catalytic domain (Fig. 1E). The known ligands for mammalian transmembrane GCs fall into two families: (1) the *Escherichia coli* heat-stable enterotoxins and their endogenous homologs; and (2) the natriuretic peptides, such as the vasorelaxing ANP (atrial natriuretic peptide). Binding of ATP to the KHD is believed to potentiate the activation of the GC catalytic domain upon ANP binding; protein kinase activity is, however, not required [24,25]. ANP is able to inhibit the proliferation of mesangial cells in a cGMP-dependent manner. Interestingly, it does so by inducing the expression of the downstream MAPK-specific phosphatase MKP-1, which inhibits MAPK [26]. Intracellular cGMP can activate cGMP-dependent protein kinase (cGK or PKG) I or II. The action of PKG I can then, for instance, reduce cytosolic Ca²⁺ levels in several cell types (Fig. 1E) [27].

3. Receptors without enzymatic activity

3.1. Cytokine receptors

Many cytokine receptors lack intrinsic catalytic domains. These receptors consist of a conserved extracellular domain, a transmembrane region and an intracellular domain containing the membrane-proximal so-called ‘box1’ and ‘box2’ motifs (Fig. 1F). Cytokine receptors couple ligand binding to tyrosine phosphorylations by using non-covalently associated protein tyrosine kinases: the *Janus* kinases (JAKs). These JAKs bind to the box1 and box2 motifs. The known members of the JAK family (JAK1, JAK2, JAK3 and TYK2) have a C-terminal kinase domain, which is immediately preceded by a pseudo-kinase domain; several other homologous regions in the N-terminal sequences have been implicated in interactions with various cytokine receptors. Among the signaling proteins that are recruited to the receptor complex and tyrosine phosphorylated are the cytoplasmic signal transducers and activators of transcription (STATs) (Fig. 1F). Phosphorylated STATs dimerize through reciprocal SH2–PY interactions and translocate to the nucleus, where they bind to

specific DNA elements and stimulate transcription. STATs are not only activated by cytokine receptors, but also by RTKs and the so-called ‘serpentine’ angiotensin II receptor (see below). There is evidence that STATs are at least partly modulated by the Ras–MAPK pathway; conversely, JAK2 has been shown to activate MAPK through the upstream kinase Raf-1 [28–30].

Recently, the STAT genes *dstA* and *stat92E* were cloned from *Dictyostelium* and the *Drosophila* fruit fly, respectively. Like its metazoan counterparts, the *Dictyostelium* STAT protein functions via a reciprocal SH2–PY interaction; it is activated by a serpentine cyclic AMP receptor. In the fly, the STAT92E protein is believed to be activated by a JAK homolog encoded by *hopscotch*. In both model organisms, pathways employing STATs have been implicated in developmental decisions [31,32].

3.2. Integrins

Integrins are the major type of cell surface receptors that bind to ligands on adjacent cells or in the extracellular matrix. Integrins are heterodimers of α and β subunits, which consist of a large extracellular, a single transmembrane and a short cytoplasmic domain (Fig. 1G). Most integrins bind extracellular matrix components like fibronectin, collagen or vitronectin. Upon ligand binding, the integrins cluster. This leads to the formation of focal adhesions, where integrins link to intracellular cytoskeletal complexes. The cytoplasmic domains of integrins do not have intrinsic enzymatic activities and appear to activate intracellular tyrosine kinases by clustering [33,34]. Integrin engagement elevates focal adhesion kinase (FAK) PY levels and FAK-associated tyrosine kinase activity. The non-receptor tyrosine kinase Src is thought to associate with an ‘auto-phosphorylated’ form of FAK and to phosphorylate FAK in turn. Both FAK and Src phosphorylate the adapter molecule Shc at multiple sites to create SH2 binding sites for the Grb2 adapter protein; phosphorylation of a FAK tyrosine residue near the C-terminus (by a Src family kinase) also promotes Grb2 binding. This provides a link to the proliferative Ras–MAPK cascade (Fig. 1G). Certain levels of integrin-dependent signaling to MAPK can also be achieved in the absence of either Src family or FAK activity [35–37].

3.3. G-protein-coupled receptors

A third, large group of receptors without intrinsic catalytic activity is that of the G-protein-coupled receptors (GPCRs). The GPCRs include adrenergic, muscarinic, serotonin, dopamine, adenosine, angiotensin II, thrombin, extracellular Ca^{2+} , lysophosphatidic acid (LPA), yeast mating factor and *Dictyostelium* cyclic AMP receptors [38–41]. These receptors all contain seven transmembrane regions; they are, therefore, also referred to as serpentine receptors. The N-termini of GPCRs are extracellular, while the C-termini are found in the cytoplasm (Fig. 1H). Upon binding of its ligand, a GPCR generally interacts with a heterotrimeric guanine-nucleotide binding protein (G-protein). GPCRs like the β_2 -adrenergic receptor can be desensitized by uncoupling from their G-proteins and internalization. This process is initiated by phosphorylation of the agonist-occupied receptor. Strikingly, GPCR endocytosis is required for the β_2 -adrenergic receptor-dependent activation of MAPKs [42].

Heterotrimeric G-proteins consist of three subunits: α , β and γ . The α subunit binds the G-nucleotide, contains a GTPase activity and modulates effector enzymes. The β/γ complex enhances receptor interaction with α subunits and also regulates a plethora of effectors directly. Upon ligand binding to a GPCR, the interacting G-protein exchanges bound GDP for GTP and undergoes a conformational change. This results in the dissociation of the GTP-bound α subunit from the β/γ subunit complex. The α and β/γ subunits are then able to activate or inhibit enzymes which produce an intracellular signal. The intrinsic GTPase activity of the α subunit terminates its signaling. The α subunit dissociates from the target molecule to reunite with the β/γ complex, so that the resting state is reached again [43]. RGS (regulators of G-protein signaling) family members such as GAIP (G alpha interacting protein) and RGS4 have been identified as GAPs for $G\alpha$ subunits [44,45]. Several lines of evidence suggest that RGS can switch off signaling through β/γ complexes by modulating the activity of the α subunits [46].

The activation of G-proteins leads to the regulation of channels and second messenger-producing enzymes such as adenylyl cyclase and phospholipase C. Active adenylyl cyclase (AC) produces the second

messenger cyclic AMP (cAMP) from ATP. All nine cloned mammalian ACs can be activated by stimulatory α subunits ($G\alpha_s$); several are modulated by inhibitory α subunits ($G\alpha_i$) and/or $G\beta/\gamma$ complexes. cAMP can activate the cAMP-dependent protein kinase (cAK or PKA) (Fig. 1H), which in turn phosphorylates a wide range of substrates, such as the cAMP responsive element binding protein (CREB). When PKA translocates to the nucleus and phosphorylates CREB, the latter is stimulated to regulate gene transcription. CREB does so by binding to the *cis*-acting cAMP responsive elements of several genes [47,48].

There are three mammalian phospholipase C (PLC) isoform families: PLC- β , PLC- γ and PLC- δ . Members of the PLC- β family are activated by serpentine receptors and PLC- γ isoforms are stimulated through RTKs. The mechanisms involved in PLC- δ activation have been poorly understood for a long time; it was only recently reported that PLC- δ 1 is an effector for $G\alpha_{12}$ -dependent GPCR signaling [49]. All known forms of PLC- β are stimulated to various extents by the $G\alpha_q$ family of α subunits. PLC- β 2 and - β 3 are also activated by G-protein β/γ subunits. There is cross-talk between AC and PLC pathways: PKA can specifically inhibit $G\beta/\gamma$ -stimulated PLC- β 2 (Fig. 1H) [50]. Active PLCs catalyze the hydrolysis of PIP_2 to generate the second messengers IP_3 and DAG. On the endoplasmic reticulum, there are IP_3 -specific receptors, which regulate Ca^{2+} release into the cytoplasm [51]. Cytosolic Ca^{2+} can modulate the activity of serine/threonine Ca^{2+} /calmodulin-dependent kinases (CaMKs) via calmodulin. Neuronal CaMK is, for instance, stimulated to phosphorylate and activate tyrosine hydroxylase, which is the rate-limiting enzyme in the synthesis of catecholamine neurotransmitters [52]. One of the other effects mediated by Ca^{2+} is the regulation of the conventional PKC isoforms (see below). Increased intracellular Ca^{2+} promotes binding of Ca^{2+} to inactive PKC in the cytosol. This leads to membrane-association of PKC, which then binds DAG. Binding of DAG and Ca^{2+} increases the affinity of PKC for phosphatidyl-serine, resulting in a tighter association of PKC with the membrane and full physiological activation of PKC (Fig. 1H) [53].

Mitogenic GPCRs, such as those for the lipid messenger LPA, can also activate the Ras–MAPK cascade. At least four G-protein mediated signaling pathways, involved in the action of LPA, have been identified: (1) stimulation of PLC; (2) inhibition of AC; (3) activation of Ras and the downstream Raf–MAPK pathway; and (4) remodeling of the actomyosin cytoskeleton in a $G\alpha_{12}/G\alpha_{13}$ - and Rho-dependent manner [54]. G_q links the receptor to PLC, while the inhibition of AC is mediated by G_i [55,56]. The activation of the Ras–MAPK cascade involves $G_i\beta/\gamma$ subunits, tyrosine kinase activity and recruitment of the adapter protein Grb2 (and therefore Sos, etc.) to the plasma membrane (Fig. 1H) [57–59]. The p110 PI3K γ subunit is thought to be involved in connecting G_i to the Ras–MAPK cascade in COS-7 cells [56,60]. A recent paper by Luttrell et al. [58] suggests that Src family tyrosine kinases, Shc and Grb2 link LPA receptors and $G_i\beta/\gamma$ subunits to this cascade in COS-7 cells; the authors propose that, during this process, the epidermal growth factor receptor is used as a scaffold. Data by Kranenburg et al. [59] and Roche et al. [61], on the other hand, indicate that Src and Shc are not involved in linking LPA receptors and G_i to the Ras–MAPK pathway in fibroblasts and COS-7 cells. The results of Kranenburg et al. suggest that a 100-kDa tyrosine phosphorylated protein–Grb2 complex, together with an upstream non-Src tyrosine kinase and PI3K, couples G_i to Ras–MAPK activation. Actomyosin cytoskeleton remodeling results from serine/threonine phosphorylation and inhibition of myosin light chain phosphatase [62].

After years of unsuccessful efforts, several groups have recently reported the isolation of cDNA clones encoding putative functional LPA receptors, with predicted molecular masses of approximately 40 kDa. The most recent evidence now strongly substantiates that LPA is indeed able to exert multiple actions in its target cells via these GPCRs. It has been shown that LPA specifically activates the *Saccharomyces* pheromone response MAPK pathway in yeast cells functionally expressing the human putative LPA receptor Edg-2 (VzG-1) [40]. In addition, heterologous expression of mouse VZG-1 in neuronal and non-neuronal cells is both necessary and sufficient in mediating multiple effects of LPA [41].

4. Protein kinases

4.1. Phosphorylation: a type of protein modifications

One of the common scenarios in signal transduction is the modification of proteins leading to the alteration of their properties (e.g. activity). There are several types of modifications. They create new forms of amino acid residues and can thus expand the repertoire of chemistry that proteins are able to perform.

Isoprenylation (mostly farnesylation and geranylgeranylation), myristoyl and palmitoyl lipidation and methylation are often important for correct protein localization [63–67]. In several cases, glycosylation modulates the activity of a protein. The reversible linkage of *N*-acetylglucosamine to serines and threonines is also thought to be important for the assembly of protein complexes and to act as an antagonist of phosphorylation: glycosylation and phosphorylation may be competing for the same sites on many proteins [68,69].

Phosphorylation is an extremely important type of modification. When a molecule is phosphorylated, the γ -phosphate of ATP is transferred onto it by a kinase. Phosphorylation of a protein can either activate or inhibit it. The AP-1 (Fos/Jun) transcription factor cJun contains phosphorylation sites at its N-terminus and near the downstream DNA binding region. N-terminal phosphorylation (by MAPK family members) is involved in cJun activation, whereas phosphorylation near the DNA binding site (by GSK-3) silences cJun [70]. Phosphorylation of tyrosine residues can also create highly selective docking sites for SH2 or other PY binding domain containing proteins [3]. The presence of protein phosphatases allows switching between active and inactive states of proteins and their complexes: they dephosphorylate their targets by removing phosphate modifications. Although many phosphatases have a wide range of substrates, specific protein phosphatases have also been described recently (see below).

4.2. Conserved protein kinase catalytic domains: some mechanistic aspects

Enzymes belonging to the superfamily of protein kinases are related by virtue of their kinase catalytic

domains [71]. In *S. cerevisiae* (for which the complete genome has been sequenced) alone, there are 113 conventional protein kinase genes. It is estimated that there are more than a thousand human protein kinases [72]. For many years, the kinase proteins have been divided into two classes: kinases that phosphorylate serine and/or threonine residues and kinases that phosphorylate tyrosine amino acids. The enzymes were grouped into families and subfamilies, solely on the basis of sequence alignments. After the elucidation of several crystal structures of protein kinases in the active and/or inactive state, they are now also structurally and mechanistically analyzed and compared [73]. Although serine/threonine and tyrosine kinases differ in the residues to which they transfer phosphate, their catalytic domains appear to have many common features and a basically identical mode of action [74].

Histidine kinases were originally identified in prokaryotes and are very distinct from the superfamily of conventional protein kinases described in this section. They auto-phosphorylate on histidine residues and are involved in the phosphorylation of aspartate amino acids in their targets. The eukaryotic histidine kinases include the *Saccharomyces* Sln1p, *Arabidopsis* ETR1 and CKII, and *Dictyostelium* DokA, DhkA and DhkB proteins [75,76]. Although the mammalian mitochondrial branched-chain α -ketoacid dehydrogenase and pyruvate dehydrogenase kinases phosphorylate their target proteins on serines, they structurally belong to the histidine kinases as well [77].

The kinase catalytic domain of the members of the superfamily of conventional protein kinases consists of 250–300 amino acids. This domain is further divided into twelve smaller subdomains (indicated by Roman numerals; Fig. 2A). The subdomains are very rarely interrupted by large amino acid insertions and contain characteristic patterns of conserved residues. The overall kinase domain folds into a two-lobed structure. The smaller N-terminal lobe (subdomains I–IV) is primarily involved in anchoring and orienting ATP. The larger C-terminal lobe (subdomains VIa–XI) is largely responsible for binding the substrate and initiating phosphotransfer. The subdomain V residues span the two lobes. The deep cleft between the two lobes is the site of catalysis. There is flexibility between the two lobes in response to substrate binding, via a so-called ‘in-

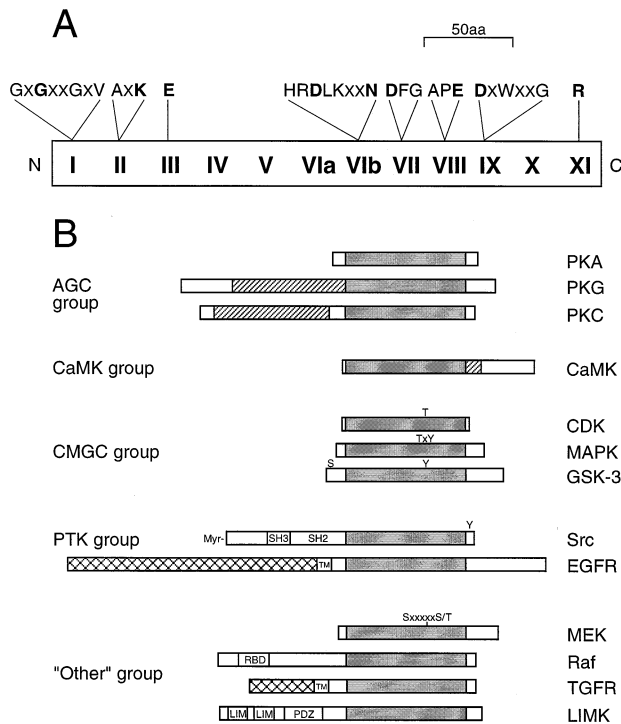


Fig. 2. (A) Typical 300 amino acid protein serine/threonine kinase catalytic domain. The 12 conserved subdomains are indicated by Roman numerals. Consensus sequences found in the subdomains are shown with invariant or nearly invariant residues in bold. In conventional protein tyrosine kinases, the Kxx part of the motif in subdomain VIb is replaced by RAA or AAR. (B) Schematic diagram of some major families of protein kinases. The kinase catalytic domains are shaded. Where applicable, monomeric forms of the proteins are shown. The AGC group includes the cyclic nucleotide-dependent kinase (PKA, PKG) and PKC families. For PKA, the catalytic subunit is shown. The cGMP binding domain of PKG and the regulatory domain of PKC are diagonally striped. The autoregulatory domain (which is able to bind Ca^{2+} /calmodulin) of CaMK is also diagonally striped. The CMGC group contains the CDK, MAPK/ERK and GSK-3 families. The regulatory serine (S), threonine (T) and tyrosine (Y) amino acids are indicated. The conventional protein tyrosine kinase (PTK) group includes the Src family and the EGFR RTK family. The regulatory tyrosine (Y) residue of Src and its SH2 and SH3 domains are indicated. The N-terminal myristoyl (Myr) modification is required for its membrane attachment. The RTKs contain ligand binding extracellular domains (double diagonally striped) and transmembrane (TM) domains. Among the group of 'other' kinases are the MEK/Ste7p, Raf and activin/TGF- β receptor (TGFR) families and the LIM kinases. The regulatory SxxxxxS/T motif of the MEK/Ste7p proteins and the Ras binding domain (RBD) of the Raf proteins are indicated. The activin/TGF- β receptors have ligand binding extracellular domains (double diagonally striped) and transmembrane (TM) domains. Finally, the LIM and PDZ domains of the LIM kinases are shown. For further details and references, see main text.

duced fit mechanism': the transition from open (inactive) to closed (active) conformations proceeds by a defined route [73].

Subdomain I contains the consensus motif GxGxxGxV, in which the second glycine (G) is invariant. This region can act as a flexible clamp, that anchors the non-transferred phosphates of ATP. Subdomain II includes an invariant lysine, which is essential for maximal kinase activity. This lysine is almost immediately preceded by an alanine residue (AxK motif). This region importantly contributes to the anchoring and orienting of ATP. The invariant lysine residue is localized by interaction with the nearly invariant glutamate (E) amino acid in subdomain III. The subdomains IV and V contain no invariant or nearly invariant residues.

Subdomain VIa seems to act mainly as a support structure. Subdomain VIb includes invariant aspartate and asparagine amino acids within a HRDLKxxN or corresponding motif (the sequence mentioned here represents the consensus for serine/threonine kinases: see below). The loop containing the invariant aspartate (D) has been named the catalytic loop, since this residue is the most likely candidate for being the catalytic base. The lysine (K) in the loop contacts the ATP γ -phosphate and may help facilitate phosphotransfer. The side chain of the invariant asparagine (N) and the invariant aspartate (D) amino acid of the highly conserved DFG triplet in subdomain VII are involved in binding ATP chelating metal. Subdomain VIII includes the APE motif; this subdomain folds into a loop, which faces the catalytic cleft. The nearly invariant glutamate (E) forms an ion pair with an arginine (R) in subdomain XI, thereby stabilizing the C-terminal lobe.

Subdomain IX contains a nearly invariant aspartate (D) residue within a DxWxxG consensus motif. This aspartate amino acid stabilizes the catalytic loop. Subdomain X is poorly conserved. In the MAPK family member ERK2, conformational changes in the so-called 'MAPK insertion region' (at the C-terminus of subdomain X) are thought to have a regulatory role [78]. As already mentioned, subdomain XI assists in stabilization of the large kinase domain lobe, through interaction with subdomain VIII (for consensus motifs, see Fig. 2A) [71,73].

The region between the DFG and APE motifs (subdomains VII and VIII, respectively) has been

defined as the activation segment. The residues, that lie in the central part of this segment, are often well-conserved among the members of individual protein kinase families. This is, for instance, the case for the TxY motif of the MAPK family: this is the site on which the family members are phosphorylated by their kinase regulators [78,79]. In the conformation of inactive kinases, interactions between the DFG region and the N-terminal lobe keep the lobes open. Modification of the segment between the DFG and APE motifs is thought to trigger the correct disposition of the two kinase domain lobes for activation. The interaction with the substrate also depends upon a defined conformation of the activation segment. There is thus a crucial role for this segment in substrate recognition [73]. Obviously, the serine/threonine or tyrosine residue undergoing phosphorylation and the surrounding kinase and substrate residues influence the catalytic event as well. Especially for serine/threonine protein kinases, many substrate sequence elements, which direct efficient phosphorylation, have been identified. These are often referred to as specificity determinants, which have been reviewed extensively by Pinna and Ruzzene [80]. Moreover, it is well possible that the three-dimensional structure of the substrate protein is at least as important in many instances.

Phylogenetic trees, derived from an alignment of catalytic domains, served as the basis for a classification of protein kinases by Hanks and Hunter [71]. They have divided the superfamily of eukaryotic protein kinases into five groups. It turned out that the members of each group not only show similarities in the catalytic domain, but also in other characteristics: they are often similar in overall structural topology (Fig. 2B) and frequently display analogous modes of regulation and substrate specificities. We will only present a brief overview of these groups of protein kinases; links to more detailed information can, for instance, be found in the Protein Kinase Resource on the World Wide Web (http://www.sdsc.edu/Kinases/pk_home.html) [81].

4.3. *The AGC group of protein kinases*

The AGC group serine/threonine protein kinases tend to be basic amino acid-directed enzymes

[71,80]. Many of these kinases are activated upon the release of second messengers. The AGC group includes the cyclic nucleotide regulated protein kinase (PKA, PKG) family, the PKC family (see below), the ‘RAC’ (PKB/Akt) family, the GPCR kinase family and the ribosomal S6 protein kinase family. The cAMP activated mammalian PKA enzymes are tetramers composed of two regulatory (cAMP binding) and two catalytic (kinase) subunits [82]. GPCR kinases such as the β -adrenergic receptor kinase are only active when a ligand induced conformational change of the substrate receptor has occurred [42]. The PIP₃-dependent p70 S6 protein kinase (S6K) is stimulated by means of serine/threonine phosphorylation by upstream kinases; S6K can in turn phosphorylate the 40S ribosomal protein S6. Only in 1997, it has been demonstrated that S6K is an *in vivo* and *in vitro* substrate for the recently identified enzyme PDK1 (which also phosphorylates PKB/Akt). In a more recent paper, Weng et al., however, suggested that *in vivo* S6K activity could be more closely related to another phosphorylation event [6,83–86].

4.4. *The CaMK group of protein kinases*

The CaMK group serine/threonine protein kinases tend to be basic amino acid-directed as well [71,80]. Regulation via second messenger pathways is also common for this group. The CaMK group includes the Ca²⁺/calmodulin-dependent kinase (CaMK) and SNF1/AMPK families. Ca²⁺ is mainly responsible for the regulation of CaMKs. It can bind to the so-called ‘EF-hands’ of calmodulin, which in turn regulates CaMK activity by direct interaction (Fig. 2B) [52,87]. The myosin light chain kinases and the plant calcium-dependent protein kinases (which contain an intrinsic calmodulin-like domain) belong to the family of CaMKs too [88,89]. The SNF1/AMPK family contains the yeast and plant SNF1 and vertebrate AMPK homologs. The latter are activated by elevated AMP levels. The SNF1/AMPK proteins are thought to function in cellular responses to environmental changes, by regulating key metabolic pathways [90]. An additional important member of the CaMK group is MAPKAP2 (MAPK activated protein kinase 2).

4.5. The CMGC group of protein kinases

The CMGC group serine/threonine kinases are not regulated by second messengers, but act further downstream protein kinase cascades. These kinases commonly exhibit regulatory phosphorylation sites in the activation segment (Fig. 2B). The CMGC group includes the CDK (cyclin-dependent kinase) family, the MAPK/ERK family, the GSK-3 family and the Clk (Cdc-like kinase) family. The members of the CDK and MAPK/ERK families are proline-directed, while kinases belonging to the GSK-3 family are acidophilic [80]. The CDKs are involved in the control of cell cycle progression in eukaryotes. They are the catalytic subunits in complexes with cyclin regulatory subunits. Phosphorylation of a conserved threonine residue is universally required for CDK activation [91,92]. Almost all members of the MAPK/ERK family have a TxY activation motif in the region between the subdomain VII DFG and subdomain VIII APE consensus sequences. The MAPK family and its subfamilies will be discussed in more detail below. The GSK-3 family contains the GSK-3 and casein kinase II isoforms. Phosphorylation of a specific tyrosine residue is a requisite for activation of GSK-3 [93]. The mammalian, *Drosophila*, plant and *Dictyostelium* GSK-3 homologs have all been implicated to have an important role in development [88,94]. The multifunctional enzyme casein kinase II (CK2) is required for viability and cell cycle progression. The constitutively active holoenzyme consists of two catalytic (α and/or α') and two regulatory (β) subunits. CK2 phosphorylates more than 160 substrates, among which are several transcription factors, the tumor suppressor protein p53 and the HIV-1 Rev gene product. The regulation of CK2 is just beginning to be elucidated. Different domains of the β subunits are involved in positive and negative control of the catalytic subunits. An acidic cluster in the N-terminal region of the β subunit is implicated in down-regulation, which can be counteracted by polybasic peptides, such as histones [95–97]. HIV-1 Rev (which contains an arginine-rich cluster) has recently been shown to be a potent *in vitro* activator of CK2 as well [98].

4.6. The conventional protein tyrosine kinase group

The group of the conventional protein tyrosine kinases (PTKs) differs from all the other groups in that the members exclusively phosphorylate tyrosine residues. PTKs are often involved in the transduction of growth and differentiation signals in metazoans. The most important sequence differences between the serine/threonine and conventional tyrosine kinases lie within the catalytic loop and the activation segment. A sequence according to the HRDLKxxN motif in the catalytic loop (subdomain VIb) indicates serine/threonine specificity; HRDLRAAN and HRDLAARN are the consensus sequences indicative of conventional non-receptor PTKs and RTKs, respectively. In the activation segment, there is a conserved threonine in serine/threonine kinases; in PTKs, the equivalent residue is a proline. This could create a more open site for the larger tyrosine substrate residue to be accommodated [73,74].

Genes for hundreds of mammalian PTKs have been cloned [99]. Nine non-receptor PTK and thirteen RTK families have been defined; several additional PTKs do not appear to belong to any of these families [71,100]. Among the non-receptor PTKs are the Src, Csk, JAK and FAK families (see previous sections). Src and other kinases of its family have an SH2 and an SH3 domain adjacent to their catalytic domain. The kinase activity of Src is repressed when a C-terminal tyrosine residue (Fig. 2B) is phosphorylated by Csk (C-terminal Src kinase); this creates a binding site for the Src SH2 domain. An intramolecular PY–SH2 interaction locks the kinase in its inactive conformation. Src can be activated by destabilization of this PY–SH2 interaction: this can be achieved by dephosphorylation of the C-terminus or binding of the SH2 domain to extramolecular sites [3,100].

4.7. The group of 'other' protein kinases

The protein kinases falling outside the four major groups are combined in the group of 'other' kinases. The individual members within defined families of this 'other' group are clearly related to one another; it is, however, difficult to group these families into a larger category. The group of 'other' kinases includes the MEK/Ste7p family, the MEKK/Ste11p family,

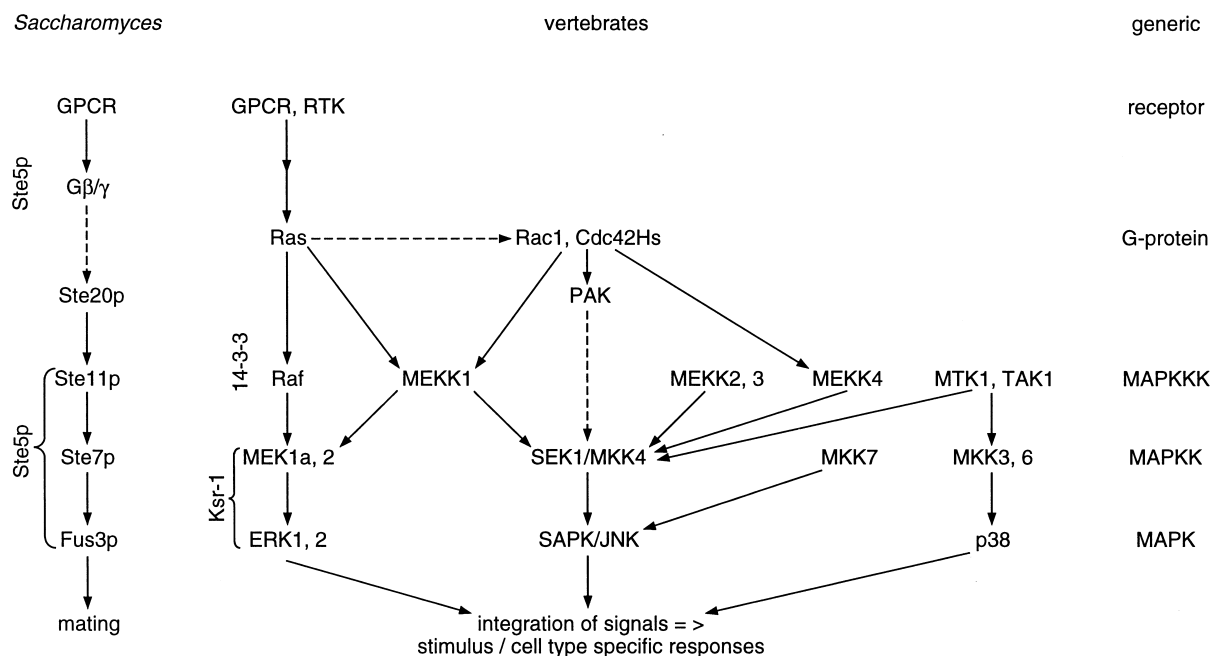


Fig. 3. Schematic diagram of some major MAPK family pathways in *Saccharomyces* (left) and vertebrates (middle). Generic names of the transduction components are indicated on the right. Dotted arrows represent interactions, which have not been fully elucidated. The double arrow represents multiple steps. For abbreviations, details and references, see main text.

the PAK/Ste20p family, the Raf family, the activin/TGF- β receptor family, the flowering plant receptor kinase family, the casein kinase I family and the LIM kinases.

The members of the first four families function in the MAPK family protein kinase cascades (Fig. 3). The mammalian MEK1a (MAPK/ERK kinase 1a) and *Saccharomyces* Ste7p isoforms are activated by means of phosphorylation on conserved serine/threonine residues by Raf-1 and Ste11p, respectively (Fig. 2B) [101]. MAPK kinases, like MEK1a, are able to phosphorylate both threonine and tyrosine residues on MAPKs by themselves; MAPKKs are therefore called dual specificity kinases [102]. These dual specificity kinases have a lysine (K) within the catalytic loop motif in subdomain VIb, which is otherwise indicative of serine/threonine specificity. Mammalian MEKKs (MEK kinases) activate the MAPK family SAPKs or JNKs (stress-activated protein kinases or cJun N-terminal kinases) via the dual specificity kinase SEK1 (SAPK/ERK kinase 1) [79]. MEKK1 also binds the small G-protein Ras and kinase-inactive MEKK1 inhibits ERK activation in response to EGF. The PAKs (p21-activated kinases) are activated by interaction with the small Rho family G-

proteins Rac1 and Cdc42Hs. PAKs are able to mediate the activation of SAPKs, independent of MEKKs [103]. The *Saccharomyces* Ste20p protein phosphorylates Ste11p in vitro [104,105]. The Raf family kinases (which include the *Arabidopsis* CTR1 protein) have a large N-terminal section, which is involved in regulation of the kinase activity and interaction with other proteins. Mammalian Raf-1 can directly bind Ras and Rap1a at its Ras binding domain (Fig. 2B). Ras-GTP is involved in the translocation of Raf-1 to the plasma membrane, which is the first step required for full Raf activation [106,107]. Ras-GTP is assumed to displace half of a so-called '14-3-3 dimer' from the N-terminus of inactive Raf-1. After phosphorylation of Raf-1, the 14-3-3 dimer is then proposed to stabilize the active conformation of Raf-1 by binding to newly phosphorylated sites [108].

Many members of the flowering plant (putative) receptor kinase family have been identified recently. The physiological functions of most of these kinases are presently unknown. This does not apply to the *Brassica* S-receptor kinase: its activity is required for self-incompatibility [88,109]. The casein kinase I (CK1) isozymes are a family of constitutively active

monomeric enzymes, which are phosphate-directed. CK1 is an exceptional protein kinase, in that its primary structure contains neither the APE motif in subdomain VIII, nor the interacting arginine (R) in subdomain XI (Fig. 2A). Its three-dimensional folding is, however, still very similar to that of other protein kinases [80,110]. CK1 has been implicated in the pathogenesis of Alzheimer's disease, through hyperphosphorylation of the τ protein (which is the major component of the abnormal paired helical filaments); it also phosphorylates a proteolytic fragment of the disease associated protein presenilin-2 in vitro and in vivo [111,112]. In lower eukaryotes, individual CK1 isoforms are involved in the regulation of repair pathways, cell proliferation and morphogenesis [113,114].

When Hanks and Hunter [71] defined their classification of protein kinases, the first LIM kinases (LIMKs) were just being discovered. They contain two N-terminal LIM domains, an internal PDZ domain (see below) and a C-terminal protein kinase domain (Fig. 2B) with an unusual catalytic region (subdomain VIb: HRDLNSHN); a similar catalytic region (HRDLTSKN) is found in the human and rat testis-specific protein kinase TESK1. These kinases are most related to members of the activin/TGF- β receptor family. Their catalytic activity is towards serine/threonine residues [115,116]. LIM domains contain a cysteine-rich motif that was first identified in the products of the *C. elegans lin-11*, the rat *ISL1* and the *C. elegans mec-3* genes; the term LIM is a combination of the first letters of the gene names. LIM domains are defined by a conserved pattern of cysteine, histidine or alternative metal-coordinating residues; they coordinate two zinc ions each. There is ample evidence that these domains mediate intra- or intermolecular protein-protein interactions. Although the similarity to zinc fingers might suggest so, there is no evidence for DNA binding by LIM domains [117–119].

Human, rat, mouse and chicken LIMKs have been found. They possess unique structural features when compared to known protein kinases. Therefore, LIMKs have already been thought to play a specific role in previously uncharacterized signaling pathways, since they were first identified. Human LIMK1 tightly interacts with PKC- γ and - ζ ; the LIM2 domain is critical for the binding between

LIMK1 and PKC- γ [120]. LIMK1 may negatively regulate its catalytic function by interaction between LIM and kinase catalytic domains; LIMK1 has also been demonstrated to associate with LIMK2 [121]. LIMK1 has recently been shown to be involved in the accumulation of actin filaments: LIMK1 is activated by Rac1 to phosphorylate and inactivate cofilin, which, in its active state, promotes disassembly of actin filaments [122,123]. Our laboratory has cloned a cDNA encoding a structural homolog of the LIMKs from *D. discoideum* (P.W. Schenk et al., unpublished results), which is the first non-vertebrate LIMK to be identified.

4.8. Protein kinase C

Protein kinase C (PKC) has diverse functions in growth, differentiation and the control of membrane processes. The PKC family includes 11 individual isoforms. All PKC isoforms consist of a regulatory and a catalytic domain (Fig. 2B). There are four conserved (C1–C4) and five variable (V1–V5) regions. C1 and C2 regions are situated in the regulatory domain; the C3 and C4 regions are contained within the catalytic domain. The regulatory domain contains a pseudo-substrate site, which is involved in blocking the kinase. The association between the pseudo-substrate site and the C4 region results in an inactive PKC conformation [124,125]. The C1 region has one or two cysteine-rich domains, which form zinc finger structures. These structures bind activating compounds, such as the lipid DAG (diacylglycerol) and the phorbol ester PMA (phorbol 12-myristate 13-acetate, also named TPA, for 12-*O*-tetradecanoyl phorbol 13-acetate) [126]. The C2 region is involved in the binding of Ca²⁺: its canonical form is absent in Ca²⁺-independent PKC isoforms [127]. This region also contains a so-called 'pseudo-anchoring site'. This site is involved in the regulation of PKC binding to receptors for activated C-kinase (RACKs). RACKs are localized in the particulate fraction and bind to the C2 region of activated PKC. These components are important in regulating PKC function: the inhibition of RACK-PKC interactions disrupts PKC activation [128]. The pseudo-anchoring site binds to the RACK binding site, which contributes to the formation of an inactive PKC conformation [129]. Several anchoring proteins,

which assist in keeping PKCs inactive (RICKs, for receptors for inactive C-kinase), are also thought to play their parts. PKC's isozyme specificity seems to be partly mediated by association of each isoform with specific RICKs and RACKs [130]. PKC's C3 and C4 regions comprise the 12 conserved kinase subdomains: the C3 region contains the ATP binding site, while the C4 region is responsible for substrate binding [125].

The presence of DAG causes a striking and selective increase in PKC's affinity for membranes, which is accompanied by activation and pseudo-substrate release. Membrane translocation is mediated by DAG binding to the C1 domain and phosphatidyl-serine binding to the C2 domain. For conventional PKCs, the affinity for acidic lipids is increased by Ca^{2+} [125]. Exploration of the time course of PKC's activation state under specific cell stimulation is a prerequisite for the clarification of its physiological roles. Very few studies have, however, succeeded in the direct monitoring of its kinase activity in intact cells. A method, that has therefore been used frequently, is measuring the signal-dependent change in the intracellular state of the protein. Most importantly, this involves redistribution of PKC, which is often from the cytosol to the plasma membrane [131].

Signals that stimulate GPCRs, RTKs or non-receptor PTKs can cause DAG production. PKC has been shown to be rapidly activated by a transient rise in DAG levels, resulting from PLC stimulation. There is also a sustained increase in DAG and PKC activity, which is thought to be the result of (among others) phosphatidyl-choline hydrolysis by phospholipase D [124,125,132]. Other lipid metabolites, such as PIP_3 , can activate PKC as well. Several PKC isotypes are activated independently in a redundant manner through the PLC and the PI3K pathway, which generate DAG and PIP_3 , respectively. PKC- ϵ activated via the PLC pathway translocates to the cytoskeletal fraction, whereas that activated via PI3K does not. This indicates a functional difference between PKC activated through different pathways [131].

Different PKC isozymes are also believed to have distinct biological functions. On the basis of structural elements and activational characteristics, the PKC family has been divided into three subfamilies.

PKC was originally identified as a phospholipid- and calcium-dependent protein kinase [133]. PKC- α , - β I, - β II and - γ are the members of the so-called 'classical' or 'conventional PKC' (cPKC) subfamily. These kinases have two zinc fingers in the C1 region and are phosphatidyl-serine-, DAG- and Ca^{2+} -dependent. They have a molecular mass of 77–78 kDa [124]. For several years, it has been established that PKC- α directly phosphorylates and activates the serine/threonine kinase Raf-1 in vitro and in vivo [134]. It is, however, still unclear how this phosphorylation contributes to Raf activation. Although dominant negative Ras expression can not block Raf-1 activation by signals that stimulate PKC, this activation was recently reported to depend on the formation of Ras-GTP-Raf-1 complexes [135]. PKC- α , - β I and - γ specifically inactivate GSK-3 β by phosphorylation; this leads to derepression of the cJun transcription factor [136]. The subfamily of non-classical or novel PKCs (nPKCs) includes the δ , ϵ , η and θ isoforms. Their activity is only dependent on phosphatidyl-serine and DAG; it is independent of Ca^{2+} . The nPKCs have a molecular mass of approximately 80 kDa [124]. Like PKC- α , PKC- δ and - ϵ have been suggested to activate the MEK-ERK pathway via Raf [137,138]. PKC- θ has recently been shown to synergize with the Ca^{2+} -dependent phosphatase calcineurin to stimulate JNK1 via Rac1 [139]. The related sequence PKC- μ /protein kinase D is also activated by DAG in the absence of Ca^{2+} . The 115-kDa PKC- μ protein contains an insert of 74 amino acids between the two zinc fingers of the C1 region, which is associated with inefficient phorbol ester binding and apparently constitutive in vitro kinase activity. It has two unique N-terminal hydrophobic domains and lacks a typical pseudo-substrate site. Taken together, its characteristics place PKC- μ somewhere between the novel and the atypical PKCs [140]. The remaining PKC isoforms (ζ and ι/λ) are placed in the subfamily of the atypical PKCs (aPKCs). The aPKCs have a molecular mass of approximately 67 kDa. They lack the canonical C2 region and one of the cysteine-rich domains in the C1 region. This results in DAG- and Ca^{2+} -independent PKCs [124,132]. PKC- ζ and ι/λ can be activated by PIP_3 [141,142]. A direct interaction between PKC- ζ and Ras is required for the stimulation of ERKs by angiotensin II in vascular smooth muscle cells [143].

Finally, both TPA-sensitive and -insensitive PKCs play a role in the regulation of gene expression by so-called 'TPA response elements' [142,144].

4.9. Mitogen-activated protein kinases

The mitogen-activated protein kinases (MAPKs) form a family of well-conserved serine/threonine kinases, with a molecular mass of 38–55 kDa [79]. Members of the MAPK family have a central role in a wide variety of protein kinase cascades. These cascades are found in all eukaryotic organisms and consist of a three-kinase module that includes a MAPK, a MAPK kinase (MAPKK) and a MAPK kinase kinase (MAPKKK): the MAPK is activated by a MAPKK, which is in turn activated by a MAPKKK (Fig. 3). Stimulation of MAPKs often results in transcriptional activation [145]. MAPKs are grouped on the basis of sequence similarity, mechanism of upstream regulation and sensitivity to activation by different MAPKKs. Until recently, the MAPK family was just subdivided into three subfamilies: the ERK (extracellular signal regulated kinase), the SAPK/JNK (stress-activated protein kinase/cJun N-terminal kinase) and the p38 subfamily. The most studied subfamily is that including ERK1 (42 kDa) and ERK2 (44 kDa). These are involved in cascades consisting of Raf, MEK1a/MEK2 and ERK1/ERK2 isoforms. Raf is in turn activated by a dynamic combination of phosphorylation (by PKC and/or other protein kinases) and interactions with Ras-GTP and 14-3-3 proteins [108]. The Raf–MEK–MAPK/ERK pathway has effects in non-proliferating cells, but mitogenic signals especially stimulate the pathway; proliferation can be blocked by inhibiting it. The SAPKs/JNKs and the p38 subfamily kinases mediate responses to cellular stress. During the last few years, genes encoding novel MAPKs, such as ERK5 and ERK6, have been cloned from vertebrates. ERK5, ERK6 and the previously identified kinase ERK3 are probably not regulated like the members of the three subfamilies mentioned. This suggests the existence of more MAPK pathways in metazoans than was originally realized [146]. ERK5 was recently shown to mediate a Ras-dependent, Raf-independent pathway activating the transcription factor cMyc (which is also a substrate for ERK1 and ERK2) [147].

In *S. cerevisiae*, there are six MAPKs. Five of these have been associated with biological responses to specific stimuli. Inappropriate cross-talk is, for instance, precluded by Fus3p. This MAPK channels G β / γ -dependent signals from the pheromone ligand through the mating pathway and prevents the signal from activating filamentation differentiation via the Kss1p MAPK [148]. The yeast probably uses Ste5p as a scaffold for the Ste11p–Ste7p–Fus3p cascade complex [149]. Interestingly, functional binding between G β and the LIM domain-like region of Ste5p has recently also been demonstrated to be required for the activation of Ste11p (Fig. 3) [150].

The vertebrate Ras–Raf–MEK–MAPK/ERK pathway can be switched on by extracellular signal molecules binding to either RTKs or GPCRs. One activation mechanism involving GPCRs is mediated by G-protein β / γ subunits stimulating Ras-GTP formation [57,151]. The GPCR ligand angiotensin II can, for instance, also stimulate ERK via a putative Ras- and Raf-independent, PKC-dependent pathway [152]. The members of the ERK subfamily have a TEY motif in the activation segment between the subdomain VII DFG and subdomain VIII APE consensus sequences. MEK1a and MEK2 are able to phosphorylate ERK1 and ERK2 on both threonine and tyrosine in this TEY motif. The activating phosphorylation of these ERKs by these MEKs is highly specific and requires the native form of MAPK [79,102]. The Ksr-1 (kinase suppressor of Ras 1) protein has been proposed to serve as a scaffold for MEK and ERK [153]. The dual phosphorylation on TEY seems to be the main requirement for translocation of ERK1 and ERK2 to the nucleus; catalytic MAPK activity is not required. Moreover, phosphorylation induces ERK2 dimerization; this is also necessary for its nuclear localization [154]. Interestingly, unlike the other MAPK family members, ERK3 is constitutively nuclear [155].

Because of their broad range of substrate recognition, activated ERK1 and ERK2 can phosphorylate a large number of proteins. Among the substrates of these ERKs are transcription factors and other nuclear proteins (such as the ternary complex factor Elk-1 and cMyc). In fibroblasts, ERK1 and ERK2 promote entry into the cell cycle, at least in part, by positively regulating the transcription of *cyclin D1* [156]. Other substrates for these ERKs are upstream

proteins of the MAPK cascade (such as the EGF receptor and Raf-1); it is possible that phosphorylation by ERK serves as a feedback mechanism for the upstream components that finally activate it. Yet another group of substrates is formed by downstream protein kinases, such as p90^{rsk} (RSK, for ribosomal S6 kinase) and MAPKAP2. Finally, an important group of ERK1 and ERK2 substrates consists of cytoskeletal elements (such as MAP-1 and MAP-2). About half of the ERKs in activated cells are bound to the cytoskeleton. Also, a basal ERK activity is required for the maintenance of cell–matrix interaction in preference to cell–cell contacts. These findings support the idea that ERKs are involved in cytoskeletal reorganization [102,157,158].

Mitogens and many types of stress (e.g. cycloheximide, UV irradiation and heat shock) can stimulate SAPK/JNK subfamily kinases. A defining property of SAPKs/JNKs is that, instead of the motif TEY on ERKs, they contain the sequence TPY; this must also be phosphorylated on both threonine and tyrosine for activation [79]. SAPK/JNK subfamily kinases are activated by SEK1 (also named MKK4, for MAPK kinase 4) and the recently identified MAPK kinase 7 (MKK7); SEK1 can in turn be phosphorylated and activated by MEKKs and other kinases (Fig. 3) [103,146,159]. The transcription factors cJun, Elk-1 and ATF-2 serve as physiological substrates for the SAPKs; SAPK phosphorylation promotes transactivation [105]. ERK and SAPK/JNK often have opposite roles in the regulation of apoptosis. In many cell systems (such as PC12 cells), ERK is thought to be protective and SAPK/JNK to be facilitative; in other systems (such as B-cells), this is believed to be the other way round [160,161].

p38 is a homolog of the *Saccharomyces* Hog1p (high osmolarity glycerol response 1) protein. It is defined by the sequence TGY (instead of TEY or TPY) in the activation segment. p38 and the members of the ERK and SAPK/JNK subfamilies can all be stimulated by hyperosmolarity stress [79]. p38 is phosphorylated by MKK3 and MKK6. MAPKs of the SAPK/JNK and p38 cascades are, for instance, activated by MTK1 (MAP three kinase 1) and TAK1 (TGF- β activated kinase 1) (Fig. 3) [146,162,163]. Among the substrates of p38 is MAPKAP2, which in turn phosphorylates the heat shock protein Hsp25/Hsp27; this confirms the role of the p38 cascade in

responding to stress [79]. In fibroblasts, p38 antagonizes ERK1 and ERK2 by inhibiting the transcription of *cyclin D1* [156]. Recently, p38 was shown to act as a component of the spindle assembly checkpoint in somatic cell cycles [164]. In PC12 cells, p38 activation corresponds to the induction of apoptosis; in primary myocardial cells, its activation is thought to protect from apoptosis [165]. Taken together, the effects of ERK, SAPK/JNK and p38 modulation appear to be largely dependent on the context in which these components act. Nowadays, the ability of cells to either survive or undergo apoptosis is thought to be dictated by a critical balance between several MAPK pathways. In a recent paper, Berra et al. also postulated a regulating role for the PI3K-protein kinase B/Akt survival module [166].

Dephosphorylation of MAPKs by specific phosphatases plays a critical role in their inactivation. An emerging family of dual specificity phosphatases dephosphorylate both threonine and tyrosine residues in the TxY motif. These phosphatases include CL100/MKP-1, PAC1, MKP-2 to MKP-5 and M3-6. M3-6 and MKP-3 appear to be exceptional, in that they seem to be able to discriminate between different MAPKs. The M3-6 phosphatase displays specific inactivation of SAPK/JNK and p38 subfamily members. MKP-3, on the other hand, selectively inactivates ERK subfamily members. MKP-3 can be activated by specific binding of ERK2 to its non-catalytic N-terminus; this modulation is independent of protein kinase activity [167–169].

5. Looking ahead

Signal transduction comprises the transmission of extracellular signals to intracellular responses. Originally, the functions of signal transduction components were mostly identified by simple enzyme–substrate and gene expression analyses. From current evidence, it is clear that phosphorylation and dephosphorylation by kinases and phosphatases play very important roles during signal transmission processes. As more and more information has become available, it is now, however, also evident that, in living cells, complete dynamic networks of components rather than separate cascades exist. At the same time, an adequate level of signaling specificity has

to be maintained. The latter is often essential to the physiological roles of cells. In other words: cross-talk is definitely present, but, in many cases, has to be minimized in order to obtain the right response at the right time and place.

Of course, a considerable amount of specificity is obtained by direct recognition of protein substrate sites by kinases [73,80]. In addition, phylogenetic analyses (such as that performed by Hanks and Hunter [71]), the elucidation of ‘active’ crystal structures (such as those of PKA and ERK2 [73,78]), two-hybrid screens [170], co-immunoprecipitation experiments (such as those performed by Brady-Kalnay et al. [13]) and overlay assays have demonstrated the presence of an ever-growing number of interactions and interaction motifs for many signal transduction components. Examples of these motifs are SH2 and other PY binding domains, proline-rich sequence binding SH3 domains (such as that present in Src), so-called ‘pleckstrin homology domains’ (such as those in Sos and IRS-1) [3], LIM domains and so-called ‘PDZ’ (for PSD-95/Dlg/ZO-1 homology) domains [171].

In the first place, several interactions between signal transduction and cytoskeletal components (which may assure correct localization) have been described. In many other cases we mentioned above, transmission molecules, which are often modified themselves, constitute docking sites or scaffolds for other components or complexes. Yet another nice example is provided by the *Drosophila* InaD protein, which basically consists of five PDZ domains. InaD serves as a scaffold to assemble different components of a phototransduction cascade. It is believed that its PDZ domains function as key elements in the organization of transduction complexes in vivo [171]. Other mechanisms to obtain higher specificity do, of course, also exist. The pathway-dedicated yeast MAPK Fus3p is, for instance, thought to impart specificity by simply channeling a particular signal through the right pathway; by doing so, it prevents the signal from activating another [148].

Also, simple ligand–receptor interactions are often not enough to activate underlying cascades. The receptor-like protein tyrosine phosphatase- β is, for example, assumed to be activated by lateral mobility of specific ligands: this induces formation of monomers from inactive dimers [172]. Receptor clustering (such

as that of integrins [33,34]) can be essential as well. In general, a combination of sensitivity and wide dynamic range of response is obtained if a cell has both clusters and single receptors on its surface, especially if the propagation of the signal can adapt to external conditions [173].

Much of the current knowledge has been obtained using in vitro approaches and transfected cell lines. Taken together, the reflections above lead to the notion that an adequate picture of signal transduction by, for example, protein kinases, has to be obtained from living cells, which have been manipulated as little as possible. The use of model organisms, such as *Saccharomyces* and *Dictyostelium*, can be of great value: they potentially provide true in vivo situations. Modern imaging techniques, like those employing green fluorescent protein [174] and its derivatives (which are shifted in their excitation and emission spectra), will also prove very valuable when analyzing localization and interactions of proteins. The combined use of such systems and methods will greatly contribute to a better understanding of how signal transduction components work in the dynamic environment of intact cells.

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