# Transmembrane signalling in eukaryotes

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# Transmembrane signalling in eukaryotes: a comparison between higher and lower eukaryotes

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*Abbreviations:* C, catalytic subunit PKA; EGF, epidermal growth factor; FGF, fibroblast growth factor; GRK, G-protein coupled receptor kinase; $InsP_3$, inositol 1,4,5-trisphosphate; LAR, leucocyte common antigen-related; LCA, leucocyte common antigen; MAP kinase, mitogen-activated protein kinase; PDE, phosphodiesterase; PDGF, platelet-derived growth factor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; $PLA_2$, phospholipase $A_2$; PLC, phospholipase C; PLD, phospholipase D; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate; PTP, protein tyrosine phosphatase; R, regulatory subunit PKA; SH domain, *src* homology domain; TCR, T cell receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

## Introduction

All living organisms react to signals from their environment such as light, temperature, sound and chemical substances in order to survive and adapt to their surroundings. In multicellular organisms communication between cells is necessary to regulate growth and differentiation [3]. Neighbouring cells can communicate with each other by direct cell-cell contact through plasma-membrane-bound signalling molecules or through the formation of gap junctions [176]. Cells are able to communicate with other cells some distance away through secretion of chemical signals. The secreted signals can be soluble molecules such as most hormones and neurotransmitters; they bind to receptors on the cell surface of target cells. Hydrophobic signal molecules such as the steroid and thyroid hormones are able to pass the lipid bilayer of the plasma membrane and bind to specific proteins inside the cell [76]. The complex of hydrophobic hormone bound to its receptor protein is directly able to influence gene transcription by binding to specific DNA sequences [221].

This review discusses some of the mechanisms by which cells communicate with their surroundings through extracellular signals which bind to cell surface receptors. The receptors are able to transduce signals across the plasma membrane by activating intracellularly located proteins of the transmembrane signal transduction pathway. Many pathways were originally characterized in lower eukaryotes and were later shown to be present in higher organisms. Genetic analysis demonstrates that many components have been conserved during evolution and are shared by mammals and other vertebrates, invertebrates and microorganisms.

## Cell surface receptors

The receptors are classified into different families based on structural similarities and modes of transduction. Transmitter-gated ion channels,

such as the nicotinic acetylcholine, glycine and $\gamma$-aminobutyrate (GABA) receptors, are heteropentameric proteins surrounding a membrane pore. These receptors contain intrinsic channelling activity to allow the passage of ions across the cell membrane when activated by ligand binding [250, 100, 161]. The surface receptors that will be discussed here directly modulate the activity of proteins inside the cell upon binding of their ligand. G-protein-linked receptors have a putative structure containing seven transmembrane-spanning domains (Fig. 1A). Intracellularly the receptor couples to an intermediate protein to regulate an enzyme or ion channel. Within the family of catalytic receptors binding of ligand and generation of the intracellular signal are functions of one molecule. These receptors contain a single membrane-spanning domain. The catalytic receptors operate directly as enzymes through an intracellularly located domain with guanylyl, cyclase, tyrosine kinase or tyrosine phosphatase activity (Fig. 1B–D).

*G-protein-coupled receptors*

Proteins belonging to the G-protein-coupled receptor superfamily share two properties. First, binding of ligand to the receptor induces the activation of a heterotrimeric guanine nucleotide-binding protein, or G-protein. Through G-proteins these receptors are linked to intracellular effector enzymes such as adenylyl cyclase, phospholipase C, cGMP-dependent phosphodiesterase, and some ion channels. Second, the deduced amino acid sequences of cloned G-protein-coupled receptor genes predict a common

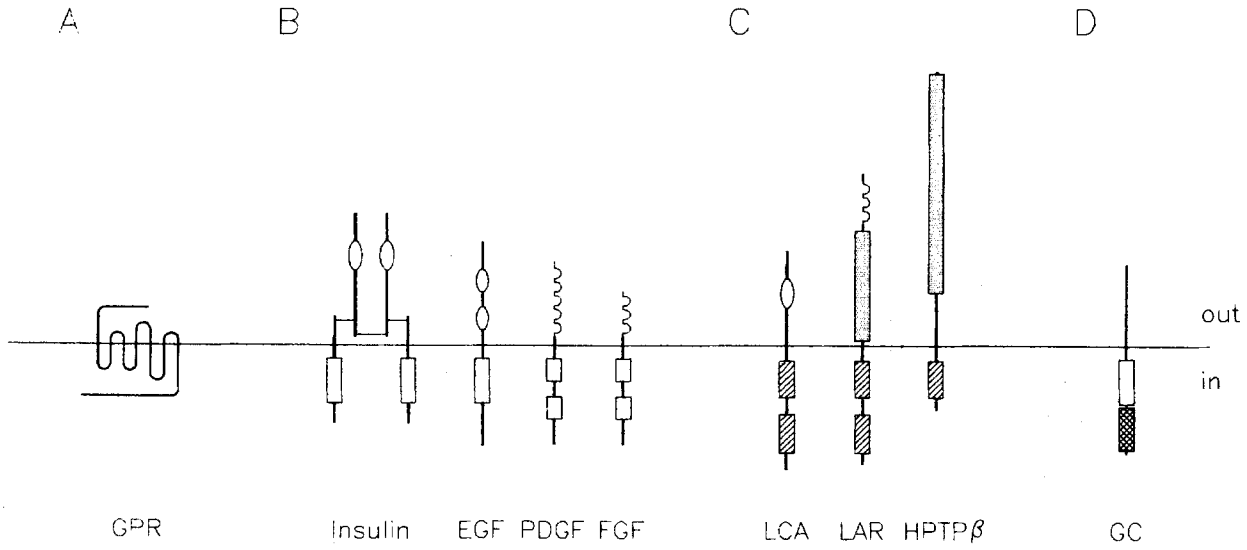


*Fig. 1.* Structure of cell surface receptor proteins. In the plasma membrane surface receptors the amino terminus is extracellular, whereas the carboxy terminus is cytoplasmic. Conserved regions: *open oval*, cystein rich domain; *open box*, protein tyrosine kinase catalytic domain; *half circle*, immunoglobuline-like region; *diagonally striped box*, protein tyrosine phosphatase domain; *filled box*, fibronectin type III repeat; *hatched box*, guanylyl cyclase catalytic domain. A. G-protein-linked receptors (GPR) contain seven hydrophobic stretches which are proposed to be embedded in the plasma membrane. The length of the extracellular domain varies great in the different receptors belonging to this family. B. Protein tyrosine kinase receptors. The insulin receptor and growth factor receptor subclasses PDGF (platelet-derived growth factor), EGF (epidermal grow factor) and FGF (fibroblast growth factor) have an intracellulary located protein tyrosine kinase domain. The kinase domains of the PDGF and EGF receptors contain an insertion sequence. C. Protein tyrosine phosphatase receptors. Structures of the leucocyte common antigen (LCA) receptor, LCA-related receptor (LAR), and human protein tyrosine phosphatase $\beta$ (HPTP$\beta$) receptor. HPTP$\beta$ receptor contains a single protein tyrosine phosphatase domain. D. Guanyly cyclase (GC) receptors contain an intracellularly located guanylyl cyclase catalytic domain and a protein tyrosine kinase-like domain.

structure consisting of seven hydrophobic transmembrane-spanning α helices (Fig. 1A). This structure is based on hydropathy plots, and is predicted to be similar to that of bacteriorhodopsin (which however does not bind a G-protein), identified by electron crystallography [112]. In the seven-membrane-spanning structure model the amino terminus is extracellular, the carboxyl terminus cytoplasmic, with the helices arranged counterclockwise in the membrane. Based on structural similarities and amino acid sequence homology G-protein-coupled receptors can be divided into different groups.

A homogeneous group is formed by G-protein-linked receptors in mammalian cells that are activated by small peptide ligands. This group consists of receptors for a family of related brain-gut peptides, such as secretin and vasoactive intensinal peptide, and receptors for calcium-regulating peptide hormones, such as calcitonin and parathyroid hormone [256]. These receptors show a high degree of similarity to each other in the hydrophobic membrane-spanning regions, but are not homologous to other G-protein-linked receptors (less than 12%). They contain a large (ca. 130 amino acids) amino-terminal domain with conserved cysteine residues.

Recently, a $Ca^{2+}$-sensing receptor gene from bovine and human parathyroid containing seven putative membrane-spanning domains was cloned [30]. The receptor is thought to regulate calcium homeostasis by sensing extracellular $Ca^{2+}$ levels [230]. It shares similarity (30%) with a separate group of G-protein-linked receptors, the metabotropic glutamate receptors [204]. The sequences of both receptor types predict a very large putative extracellular domain (over 600 amino acids) containing conserved cysteine residues and a hydrophobic segment in the amino terminus. The $Ca^{2+}$-sensing receptor shows clusters of acidic amino acid residues possibly involved in calcium binding. The presence of other extracellular ion-sensing receptors, for example for $K^+$ and $PO_4^{3-}$, is assumed [30].

The main group of G-protein-linked receptors in mammalian cells bind diverse ligands: glycoproteins such as thyroid-stimulating and follicle-stimulating hormones, small organic molecules such as adrenaline and acetylcholine, hydrophobic compounds such as the cannabinoids, and the chromophore retinal. The putative extracellular amino-terminal domains of the receptors vary greatly in length. The receptors share a limited number of conserved amino acid sequences, mainly in the putative membrane-spanning regions [233]. Olfactory receptors were cloned using a polymerase chain reaction (PCR) strategy based on the assumption that the receptors would contain conserved transmembrane amino acid sequences [32]. A gustatory receptor, expressed in taste buds, is similar to the olfactory receptors (56% amino acid identity) and may be a taste receptor [1]. Through examination of features common to G-protein-linked receptors, regions for interaction between receptor and ligand, and receptor and G-protein are predicted [reviewed in 243]. A region with less sequence identity among this group of diverse receptors is the proposed third cytoplasmic loop between the fifth and sixth transmembrane regions; this region is thought to be involved in the interaction of the receptor with specific G-proteins. However, for rhodopsin a small loop is predicted, but it is capable of activating a variety of G-proteins. The membrane-spanning regions are proposed to form a ligand-binding pocket in the α- and β-adrenergic receptors, muscarinic acetylcholine receptors, and the receptors involved in vision, the opsins [166, 206, 207]. In the photoreceptor system, retinal is proposed to be surrounded by the transmembrane domains of the visual light pigment rhodopsin and activated when photons are absorbed. The three colour pigment receptor molecules expressed in cones are proposed to be arranged in a similar way. Visual pigments of the fruit fly *Drosophila* photoreceptor cells, R1–R8, were shown to share sequence homology with mammalian rhodopsin [217, 322]. Mammalian and *Drosophila* opsin genes are thus proposed to derive from a common ancestor.

The proposed topology of seven hydrophobic membrane-spanning domains in receptors linked to G-proteins is found in microorganisms. Conserved receptors are cAMP receptors of *Dictyos-*

*telium discoideum* and pheromone receptors of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The cellular slime mould *Dictyostelium discoideum* is an ideal system for studying signal transduction processes [63, 306]. Growth and cellular differentiation are entirely separated processes. The differentiation programme is induced by starvation during which cells communicate with each other by means of chemotactic signals. The four cAMP receptors involved in chemotaxis and development are expressed at distinct stages of *Dictyostelium* development [143, 129, 244]. In the budding yeast *S. cerevisiae* and the fission yeast *S. pombe* mating involves the fusion of two opposite cell types to produce a diploid cell [119, 312]. Each partner produces a peptide pheromone that is detected by surface receptors of the opposite type (Fig. 2). In *S. cerevisiae* the **a**- and α-mating factors are produced by **a** and α cell types, respectively. The **a** cell type produces a unique receptor protein encoded by the *STE2* gene, whereas the α cell type produces a receptor protein encoded by the *STE3* gene. Although the protein sequences encoded by *STE2* and *STE3* are predicted to have the same structure characteristic of the G-protein-coupled receptors, amino acid similarity between the two receptors is absent [33, 205, 105]. In *S. pombe* the two mating types are called $h^+$ and $h^-$; the pheromones secreted by these cells are called P-factor and M-factor, respectively. The deduced amino acid sequences of the *mam2* gene encoding the receptor for P-factor and the *map3* gene encoding the receptor for M-factor are very different [142, 287]. However, the Mam2 receptor of *S. pombe* shows significant homology (26% amino acid identity) with the α-factor receptor Ste2 of *S. cerevisiae*, and the Map3 receptor is homologous to the a-factor receptor Ste3. In *S. pombe* the mating factors are not only involved in the initiation of mating, as in *S. cerevisiae* but also in meiosis.

### *Receptor protein tyrosine kinases*

Receptor tyrosine kinases contain an extracellular amino-terminal ligand-binding domain, a single putative membrane-spanning domain and intracellularly, a tyrosine kinase domain. The receptors belonging to this family can be divided

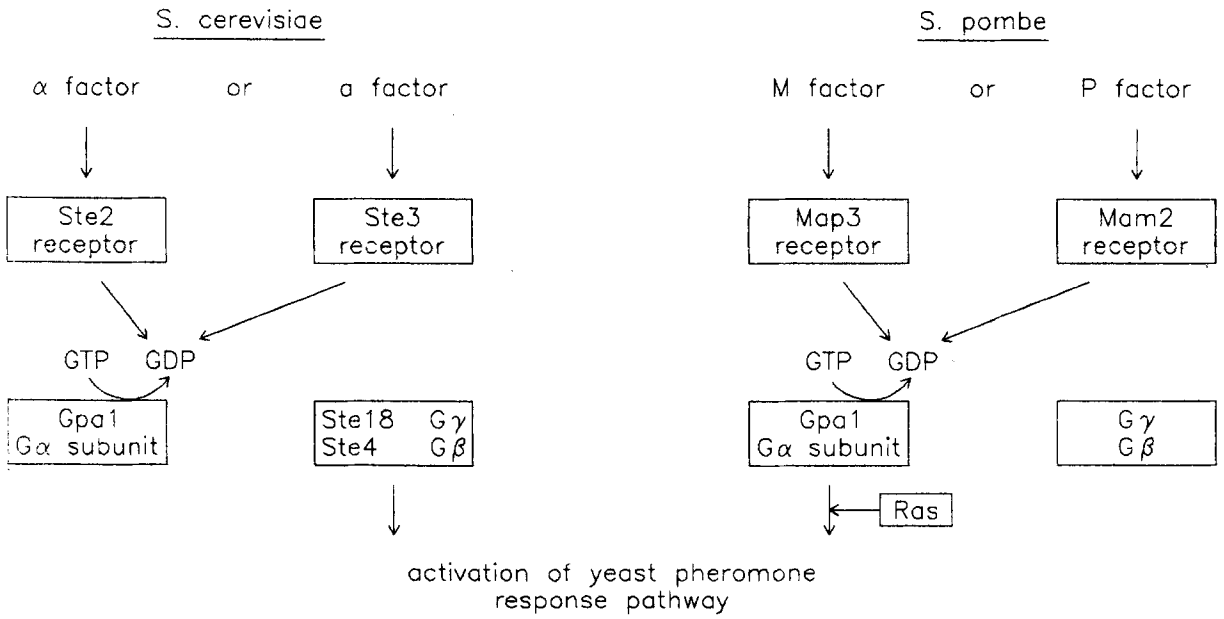


*Fig. 2.* Activation of yeast signal transduction pathways by heterotrimeric G-proteins. In *Saccharomyces cerevisiae* βγ subunits of G-proteins transduce the pheromone signal, whereas in *Schizosaccharomyces pombe* an α subunit and a Ras protein are involved.

into four subclasses based on sequence similarity and structural characteristics [302]: the epidermal growth factor (EGF) receptor, the insulin receptor, the platelet-derived growth factor (PDGF) and the fibroblast growth factor (FGF) receptor subfamilies (Fig. 1B). The EGF and insulin receptors share homologous cysteine repeats in their extracellular domains. The extracellular domains of PDGF receptors have an immunoglobulin-like structure. Members of the FGF receptor subclass contain three immunoglobulin-like sequence repeats, similar to the five repeats in the PDGF receptor. Binding of ligand to the extracellular domain induces receptor dimerization, which in turn is thought to induce the activation of the intracellular tyrosine kinase domain [reviewed in 246]. The monomeric ligand EGF, and the dimeric PDGF mediate dimerization of neighbouring receptors [111, 99]. The insulin receptor is a dimer, which is stabilized upon binding of its ligand. Activation of receptor tyrosine kinases induces cells to proliferate and differentiate in the case of the growth factor receptors, or to turn on metabolic pathways in the case of the insulin receptor [247].

The tyrosine kinase domain is the most conserved region between all receptor tyrosine kinase molecules. It contains the consensus sequence for ATP binding, resembling the catalytic subunit of cAMP-dependent protein kinases. Mutations in the ATP binding site result in the absence of protein tyrosine kinase activity and abolish all signal transduction processes [see references in 302]. The kinase domains of the PDGF and FGF receptor classes contain an insertion sequence of hydrophilic amino acid residues, dividing the kinase domain in half. The kinase insertion sequence is probably not involved in kinase activity, but is thought to play a role in receptor interactions with substrate proteins [138].

Autophosphorylation of the receptor plays an important role in both enzyme activation and interaction with the substrate protein [147]. The activated receptors transduce extracellular signals not only by phosphorylation, but also by direct protein-protein interactions. Many protein substrates of growth factor receptor tyrosine kinases contain the *src* homology domains SH2, a motif originally found in the cytoplasmic tyrosine kinases related to c-Src. The SH2 domains bind to autophosphorylated tyrosine residues on the receptors [reviewed in 233]. In the PDGF and EGF receptors phosphorylation sites are found in the non-catalytic regions of the cytoplasmic domains. Substrate proteins include phospholipase C$\gamma$1, the phosphotyrosine phosphatase Syp, the GTPase-activating protein of Ras (GAP), and Gbr2 (growth factor receptor-bound protein 2), an adaptor protein involved in Ras signalling [139, 140, 184, 179, 4, 197]. The receptor tyrosine kinases are thus able to couple to many, diverse intracellular signalling pathways, each of the substrate proteins binding at specific phosphorylation sites within the receptor [38, 78, 267]. The activated insulin receptor phosphorylates an SH2-docking protein, named IRS1, on multiple tyrosine residues [280]. This intermediate protein is able to bind the SH2 domains of Grb2, Syp, and phosphatidylinositol 3-kinase [280, 223].

The receptor tyrosine kinase homologues identified in nonmammals control cell fate determination during development. In *Drosophila* the Sevenless receptor is required for photoreceptor cell R7 development [103], and the EGF receptor homologue DER affects the correct dorsoventral patterning of the eggshell and the embryo [232]. The structure of the torso receptor of *Drosophila* is reminiscent of the mammalian PDGF receptor, and involved in determination of terminal cell fates [268]. The Let-23 receptor tyrosine kinase from the nematode *Caenorhabditis elegans* is structurally similar to the mammalian EGF receptor, and is required for vulval induction [7]. The developmental pathways activated by receptor tyrosine kinases in invertebrates have been investigated genetically, and shown to involve signalling through Ras proteins (see below).

### *Receptor protein tyrosine phosphatases*

The overall putative structure of receptor tyrosine phosphatases resembles that of receptor tyrosine

kinases, with an extracellular amino-terminal domain, a single membrane-spanning domain, and intracellulary the catalytic domain [87]. The different receptor forms share conserved catalytic domains with the soluble protein tyrosine phosphatases; there is no similarity with serine/threonine phosphatases. After the first, soluble, protein tyrosine phosphatase, named PTP-1B, was purified from human placenta, part of its sequence was recognized to be similar to the tandem C-terminal homologous domains of the leucocyte common antigen (LCA), CD45 [41, 42]. CD45 is expressed as a major surface component on nucleated hematopoietic cells and contains high intrinsic phosphatase activity [300]. Its putative structure includes a cysteine-rich extracellular domain, a hydrophobic region that has the characteristics of a membrane-spanning structure and two intracellular tyrosine phosphatase domains (Fig. 1C). Only one tyrosine phosphatase domain contained *in vitro* enzymatic activity when expressed in *Escherichia coli* whereas the other, carboxyl-terminal domain, although showing no detectable catalytic activity, influenced substrate specificity [276]. CD45 is involved in regulating T-cell receptor (TCR) signalling, as CD45-deficient T-cells failed to proliferate or produce cytokines after induction with antigen [228, 309]. CD45 is proposed to link the TCR to intracellular signalling pathways such as phosphatidylinositol hydrolysis and calcium mobilization, by dephosphorylating a putative negative regulatory site of cytosolic protein tyrosine kinases [151, 152]. In particular, CD45 is proposed to activate the Lck and Fyn tyrosine kinases [198, 260]. As ligands for receptor tyrosine phosphatases are undefined, a chimaeric tyrosine phosphatase receptor was constructed, in which the extracellular and transmembrane domains of CD45 were replaced with those of the EGF receptor [62]. This chimaeric receptor was able to restore TCR signalling in CD45-deficient cells. Interestingly, addition of EGF ligand to these cells abolished all TCR mediated signalling, indicating that *in vivo* CD45 tyrosine phosphatase activity may be constitutively functional and negatively regulated by ligand binding.

Members of the family of receptor protein tyrosine phosphatases contain structural diverse amino-terminal domains (Fig. 1C). The extracellular domain of CD45 is heavily glycosylated and contains a cystein-rich region. The leucocyte common antigen-related (LAR) receptor, isolated by cross-hybridization to an LCA cDNA probe, is expressed on cells of epithelial origin [274]. The amino-terminal region of LAR and its *Drosophila* homologues DLAR and DPTP, contain immunoglobulin- and fibronectin type III-like domains, similar to domains found in neural cell adhesion molecules [275]. Thus, it is possible that LAR, DLAR and DPTP are cell adhesion receptors. Other receptor tyrosine phosphatases, mammalian HPTP$\alpha$ and HPTP$\varepsilon$, contain very short external segments [153]. HPTP$\beta$ and *Drosophila* DPTP10D contain multiple fibronectin type III domains and are unique in that they have only one of the conserved intracellular tyrosine phosphatase domains [153, 295]. Mammalian HPTP$\zeta$ contains a domain with homology to the enzyme carbonic anhydrase besides fibronectin type III repeats [12].

Apart from CD45 function, a definitive role for receptor protein tyrosine phosphatases in cellular signalling has not been established yet. The *in vivo* function of non-receptor tyrosine phosphatases has been established during recent years through genetic studies in lower eukaryotic organisms. Non-receptor protein tyrosine phosphatases appear to play a role in the development of *Dictyostelium* and *Drosophila*, and in cell-cycle regulation in *Schizosaccharomyces pombe*. The fission yeast *S. pombe* contains three tyrosine phosphatases, pyp1, pyp2 and pyp3, which play a role in the timing of the onset of mitosis through regulation of the cell-cycle cdc2 protein kinase [98]. Pyp1 and pyp2 phosphatases negatively regulate mitosis; deletion of both genes is lethal, whereas overexpression of these proteins results in a delay in mitotic onset [194, 216]. Deletion of the *pyp3* gene has no effect, indicating that it does not have an essential role in the cell cycle, but overproduction advances the onset of mitosis [193]. The differences in function of these phosphatases could be reflected in their structure; pyp1 and

pyp2 contain a long amino-terminal extension preceding the catalytic domain, which is lacking in pyp3. *In vitro*, pyp3 was shown to directly activate cdc2 by removing an inhibitory phosphate of the kinase [reviewed in 158]. Pyp1 and pyp2 do not interact directly with cdc2, but positively regulate the activity of kinases involved in inhibiting cdc2 through phosphorylation.

In *Dictyostelium* the PTP1 tyrosine phosphatase is involved in regulating development [118]. Gene disruptants formed normal fruiting bodies, but the developmental process was accelerated. Cells overproducing PTP1 showed a delayed development, which was not completed to form normal fruiting bodies. In *Drosophila*, the *corckscrew* gene encodes a putative non-receptor tyrosine phosphatase containing two adjacent SH2 domains in its amino-terminal non-catalytic region [226]. It is a positive transducer of the signal generated by the receptor tyrosine kinase torso, possibly by binding to the activated torso receptor tyrosine kinase [227]. Corckscrew mammalian homologues are PTP1C, PTP1D, SH-PTP1 and SH-PTP2 in man, and Syp in mouse [258, 307, 89, 81]. Syp and PTP1D were shown to be regulated by binding to EGF and PDGF receptors through their SH2 domains. Binding of the phosphatases did not dephosphorylate the receptors, but the interaction with the activated receptor tyrosine kinases phosphorylates and thereby activates the phosphatases.

### *Guanylyl cyclases*

Cyclic GMP is an ubiquitous mediator regulating cGMP-dependent ion channels, phosphodiesterases and protein kinases, involved in processes as diverse as renal and intestinal ion transport, maintenance of blood pressure, and light response in photoreceptor cells [125, 277]. Soluble and membrane-bound guanylyl cyclase enzymes have been cloned. The ligands that activate membrane-bound guanylyl cyclases are peptides [reviewed in 93]. In mammals guanylyl cyclase type A [45, 178] is activated by the atrial and brain natriuretic peptides ANP and BNP, guanylyl cyclase type B [40, 253] by type C natriuretic peptide, and guanylyl cyclase type C [254] is activated by enterotoxin of *E. coli* and by an endogenous peptide from the gut named guanylin. Guanylyl cyclase in sea urchin spermatozoa is stimulated by the peptides resact and speract secreted by sea urchin eggs [294, 266]. These peptides influence sperm motility. The ligand for the mammalian receptor cyclase, human retinal guanylyl cyclase, is as yet unidentified [263]. This cyclase could be responsible for the resynthesis of cGMP after phototransduction. Recoverin, a calcium sensor in vision, was initially proposed to be a soluble activator of retinal guanylyl cyclase [70, 159]. However, further experiments demonstrated that it was not recoverin that stimulates photoreceptor guanylyl cyclase, but an at present unidentified factor [121]. Calcium-bound recoverin is proposed to be involved in prolonging the photoresponse, possibly by blocking phosphorylation of activated rhodopsin [137].

Guanylyl cyclase receptors are predicted to be single membrane spanning proteins, analogous to receptor tyrosine kinases (Fig. 1D) [321]. The extracellular amino terminus is proposed to bind ligand. The cyclase catalytic domain is located at the carboxy terminus. Guanylyl cyclase receptors contain an intracellular domain homologous to protein kinases. The position of the kinase domain in the protein appears to be similar to that in receptor tyrosine kinases, and many of the amino acids highly conserved among the catalytic regions of protein kinases are present. However, no protein kinase activity has been detected in the guanylyl cyclase receptors. Deletion of the kinase-like domain resulted in a constitutive activation of guanylyl cyclase, proposing a role for this domain in the regulation of the catalytic domain of guanylyl cyclase receptors [44, 150]. The guanylyl cyclase catalytic domain shows a high degree of conservation with each of the catalytic domains of adenylyl cyclases. Dimerization of two cyclase catalytic domains is proposed to be required for catalytic activity, analogous to dimerization of receptor tyrosine kinases [93].

The soluble forms of guanylyl cyclase are not stimulated directly by hormones, but contain a

prosthetic heme group and are regulated by nitric oxide, presumably through binding of nitric oxide to the heme group [reviewed in 248]. Mammalian soluble guanylyl cyclase isoforms are heterodimers with each subunit containing a cyclase catalytic domain similar to that of the membrane-bound form [see references in 46, 97]. Transfection experiments suggest that expression of both subunits is required for catalytic activity [202, 108, 109]. Unlike the membrane-bound forms, the soluble guanylyl cyclase do not contain a region homologous to protein kinases. A head-specific soluble guanylyl cyclase has been cloned from *Drosophila* [320]. Activation of this cyclase is thought to be involved in opening of the cGMP-dependent light-activated channel in invertebrate photoreceptor cells. Guanylyl cyclase in the protozoans *Paramecium* and *Tetrahymena* is tightly associated with the cytoskeleton and plays a role in cell motility. The genes have not yet been identified. The $Ca^{2+}$ flux across the ciliary membranes of *Paramecium* and *Tetrahymena* stimulates guanylyl cyclase through direct binding with a $Ca^{2+}$/calmodulin complex [144, 251, 252]. In these organisms an increase in intracellular $Ca^{2+}$ stimulates enzyme activity in the cilia. In *Dictyostelium*, guanylyl cyclase is activated by extracellular cAMP. The enzyme activity appears to be controlled through a cAMP surface receptor and G-protein pathway [127]. Like human retinal guanylyl cyclase, the *Dictyostelium* enzyme is inhibited by $Ca^{2+}$ ions [303]. Cloning of the gene will have to reveal to which class *Dictyostelium* guanylyl cyclase belongs.

## G-proteins

G-proteins are a superfamily of proteins that bind and hydrolyze GTP. They include proteins involved in protein synthesis, such as the elongation factor Tu (EF-Tu), and two classes of proteins that transduce signals; large heterotrimeric G-proteins and small proteins such as the proto-oncogene Ras [reviewed in 28]. Heterotrimeric G-proteins consist of $\alpha$, $\beta$ and $\gamma$ subunits. Activated by binding of GTP, the protein dissociates; the $\beta$ and $\gamma$ subunits form a tightly associated complex, free from the $\alpha$ GTP-bound subunit. The crystal structures of proteins representative of each G-protein family have been reported [16, 210, 218]. Although bacterial EF-Tu, Ras (or p21, the products of the *ras* oncogenes) and $G_{t\alpha}$ (the $\alpha$-subunit of the hetrotrimeric G-protein transducin) share less than 20% amino acid sequence identity, their GTPase domains demonstrate the same overall structure. In the GTPase core, five sequence regions conserved in all GTPases are found in loops that bind GTP and GDP. G-proteins function as molecular switches which cycle between the GTP-bound active form and the GDP-bound inactive form. The interconversion between the active and inactive state occurs by GTP hydrolysis, and from the inactive to the active state by nucleotide exchange. The interconversion of Ras from the GDP to the GTP bound state and vice versa is regulated by accessory proteins [reviewed in 24]. Guanine-nucleotide exchange factors (GEFs) mediate the replacement of GDP with GTP. Through the association of a GEF with the G-protein, GDP dissociates from the complex at an increased rate. Subsequently, the 'empty' Ras/GEF complex binds GTP, leading to the dissociation of GEF and activation of the Ras G-protein. GTPase-activating proteins (GAPs) accelerate the G-protein's intrinsic GTPase activity. In heterotrimeric G-proteins the situation is different: G-protein $\alpha$ subunits contain both intrinsic GEF and GAP activities. The 3-dimensional structure of $G_{t\alpha}$ predicts a unique $\alpha$-helical domain, inserted in the GTPase core domain [210]. Together these domains surround the guanine nucleotide. The $\alpha$-helical domain in the GTPase core has been proposed to be involved in GDP release as well as acting as a built-in GAP for $G_{\alpha}$. In heterotrimeric G-proteins the bound GDP is inaccessible, unlike the situation in other G-proteins which bind the nucleotide in a partially exposed surface cleft. Activation of an appropriate G-protein-linked receptor is proposed to stimulate opening of the closed structure of $G_{\alpha}$ to allow exchange of GDP for GTP.

*Heterotrimeric G-proteins*

Binding of GTP to a heterotrimeric G-protein results in dissociation and the formation of an active $\alpha$ subunit and/or active $\beta\gamma$ subunit complex. The regions of the G$\alpha$-protein that interact with $\beta\gamma$ subunits, receptors and effectors have been reviewed recently by Conklin and Bourne [55]. A single receptor can activate multiple G-protein molecules thereby amplifying the external signal. Some $\alpha$ subunits possess specific residues that can be covalently modifided by bacterial toxins through ADP-ribosylation of the $\alpha$ subunit. Modification by pertussis toxin uncouples receptors from G-proteins thereby inhibiting signalling, whereas cholera toxin constitutively activates the G-protein by inhibiting GTPase activity [310, 305].

Amino acid sequence comparisons of the $\alpha$ subunits present in mammalian cells reveal four subfamilies after which the heterotrimeric G-protein is named: $G_s$, $G_i$, $G_q$ and $G_{12}$ [272, 113]. As G-protein were first identified functionally, names were assigned in accordance with the role they performed; for instance, members of the $G_s$ family stimulated adenylyl cyclase, whereas $G_i$ inhibited this enzyme. Other $\alpha$ subunits were isolated by genetic techniques; cellular functions are not always known. The $\alpha$ subunit of the G-protein involved in odorant signalling, $G_{olf}$, belongs to the $G_s$ family [130]. It couples the odorant receptors with a distinct form of adenylyl cyclase. Structurally, the G-proteins involved in vertebrate vision, transducins ($G_t$), belong to the $G_i$ family. Activated transducin binds to one of the two inhibitory $\gamma$ subunits of a cGMP-dependent phosphodiesterase (PDE). The activated PDE increases the rate of cGMP hydrolysis which leads to closure of cGMP-gated cation channels in the plasma membrane [277]. $G_{t1}$ is activated by rhodopsin in retinal rods, while $G_{t2}$, expressed in cones, is activated by cone opsins [170]. Gustducin, a member of the $G_i$ family, is expressed specifically in taste buds and is presumably involved in taste sensing [188]. The function of $G_{\alpha o}$ also classified as a $G_i$ protein, is unclear; it could be involved in regulating various ion channels [113]. Members of the $G_q$ family stimulate a specific group of phosphoinositide-specific phospholipase C isoforms [292, 264, 22]. Functions for the $G_{12}$ family have not been identified yet.

Structurally, the G-protein $\alpha_s$, $\alpha_i$, $\alpha_q$ and $\alpha_o$ subunits from *Drosophila* show a clear relation to the classes found in mammals [reviewed in 122]. The *concertina* gene is most similar to $\alpha$ subunits of mammalian $G_{12}$, and is required for normal gastrulation [222]. The functions of the other G-proteins remain to be identified. *Drosophila* $\alpha_q$ is expressed only in photoreceptor cells, where it is proposed to play a role in the activation of phospholipase C in phototransduction. In the nematode *C. elegans* $G_\alpha$ subunits homologous to $\alpha_s$, $a_q$ and $\alpha_o$ were found, but also three unique G$\alpha$ proteins [175, 86, R.H.A. Plasterk, personal communication]. A cDNA cloned from *Xenopus laevis* oocytes is 89% identical to the $\alpha$ subunit of rat $G_o$ [213].

Eight $G_\alpha$ genes have been identified in *Dictyostelium* that are expressed at distinct stages of the developmental cycle [234, 101, 314]. Outside of the proposed guanine nucleotide-binding domains, they bear no specific homology to a mammalian subtype. Some are essential for chemotaxis and development. Deletion of $G_\alpha 2$, expressed at high levels during aggregation, resulted in cells which could not aggregate and in which all cAMP-receptor-mediated signalling was lost [155, 141]. $G_\alpha 4$ is essential for proper development and spore production [102]. Deletion of $G_\alpha 1$, expressed during vegetative growth and early development, did not result in a growth or developmental phenotype, suggesting it is not essential under normal conditions. It appears to play a role in cytokinesis, as cells overexpressing $G_\alpha 1$ during the growth phase yield very large, multinucleated cells [155].

The budding yeast *S. cerevisiae* contains two G-protein $\alpha$ subunit genes, *GPA1* and *GPA2* [200, 201]. These $\alpha$ subunits have an extra region of 80–100 amino acids inserted near the amino-terminus compared to mammalian $\alpha$ subunits. Disruption of the *GPA1* gene leads to cell cycle arrest in G1 phase [195, 66]. Gpa1 plays a nega-

tive role in mating signal transduction, as in the absense of Gpa1 the pheromone response pathway is constitutively activated. Disruption of *GPA2* did not cause any obvious phenotype [201]. In the fission yeast *S. pombe* also two $\alpha$ subunits have been identified. Disruption of *S. pombe gpa1* resulted in viable, but sterile cells, indicating it is required in the developmental pathway for mating and sporulation (Fig. 2) [211]. *S. pombe* Gpa2 is involved in the monitoring of nutrition [123]. Disruption of the *gpa2* gene resulted in smaller cells, which grew slower than the wild type. cAMP levels were only one-third of wild-type level, and *gpa2* null cells did not produce cAMP in response to glucose stimulation, coupling Gpa2 to adenylyl cyclase.

The $\beta$ and $\gamma$ subunits of G-proteins form a tightly associated $\beta\gamma$ complex. The amino-terminus of the $\beta$ subunit has been identified as an essential region by $\beta\gamma$ interaction, presumably through the formation of a structure called an $\alpha$-helical coiled coil with the $\gamma$ subunit [94]. The heterotrimer is associated with the plasma membrane, although none of the G-protein subunits contain potential membrane-spanning domains. Lipid modifications in the $\gamma$ subunits (prenylation) serve to anchor the subunits to the membrane. The $\beta\gamma$ subunits were first merely thought to be inhibitors of G-protein activity and nonspecific anchors. However, it is now evident that $\beta\gamma$ subunits are capable of activating effector proteins themselves [21]. In mammals $\beta\gamma$ subunits have been demonstrated to interact with some forms of adenylyl cyclase and phospholipase C, and stimulate opening of $K^+$ channels [36, 289, 177]. The activation of effector molecules can depend on the combined interaction of $\alpha$ and $\beta\gamma$ subunits, derived from two different G-proteins, as will later be discussed for activation of adenylyl cyclase.

In *Dictyostelium*, a single $\beta$ subunit is expressed during its entire life-cycle [171]. This suggests the $\beta$ subunit couples to all of the transiently expressed G-protein $\alpha$ subunits. Deletion of this gene does not influence growth, but inhibits chemotaxis and further development.

In the budding yeast *S. cerevisiae* $\beta\gamma$ subunits are involved in responses to mating factors (Fig. 2). Mutations in the *STE4* or *STE18* genes, encoding the $\beta$ and $\gamma$ subunits respectively, suppress the lethality of mutations in the *GPA1* GENE [311]. By $\beta\gamma$ subunits of *S. cerevisiae* participate in the activation of a mitogen-activated protein (MAP) kinase cascade, linking receptor activation to intracellular phosphorylation. In *S. pombe* however, interaction between the Gpa1 $\alpha$ subunit of a heterotrimeric G-protein and a Ras homologue is required for activation of a MAP-kinase cascade (discussed further in Ras signalling).

*Ras signalling pathways*

The superfamily of Ras-related G-proteins consists of small, monomeric proteins. Based on their structural and functional homology they are divided into four subfamilies [106]. Two subgroups containing Ras- and Rho-like proteins control extracellular signalling pathways. Ras-like proteins play a role in the control of normal and transformed cell growth and differentiation. The Rho-like proteins control signal pathways involved in the organization of the actin cytoskeleton. The Rab- and ARF-like (ADP-ribosylation factor) members of the Ras-related superfamily are involved in intracellular vesicle transport [25, 271, 257]. Recently, ARF was shown to activate phospholipase D [31, 54]. Therefore, assembly of coat proteins and regulation of the phospholipid content of membranes could both the mediated by ARF [131].

*Ras* genes have been identified in virtually all eukaryotic organisms examined. Post-translational modifications are necessary to localize Ras to the plasma membrane. Ras proteins act as key signal-transducing elements, coupling receptor-activated pathways to a cascade of protein kinases which regulate the activity of nuclear transcription factors. Biochemical studies in animal cells and genetic studies in lower eukaryotes have led to the identification of proteins involved in the activation of Ras by receptor tyrosine kinases. In addition to SH2 domains, many proteins involved in transducing signals contain a related conserved

*src* homology domain, SH3 [147]. The number of SH2 and SH3 domains differs and some proteins only contain SH2 or SH3 domains. Adaptor proteins containing one SH2 and two SH3 domains regulate the specificity of protein-protein interactions from the activated receptor to the nucleotide exchange factor. In mammalian cells the adaptor protein Grb2 binds, via its SH3 domains, to a nucleotide exchange factor for Ras, named mSos [179, 43]. After cell stimulation the Grb2/mSos complex binds to phosphorylated receptor tyrosine kinases via the SH2 domain of Grb2. The result is translocation of mSos to the plasma membrane where it is now able to activate Ras (Fig. 3). Similar pathways for Ras activation were identified in *Drosophila* and *C. elegans* [reviewed in 247]. Sem-5 is the *C. elegans* homologue of Grb2 in the Let-23 tyrosine kinase pathway involved in vulval induction [51]. In *Drosophila* the adaptor protein Drk binds to the activated *Sevenless* receptor tyrosine kinases [265, 214]. Mammalian Sos is named after the *Drosophila* Son of sevenless (Sos) guanine nucleotide exchange factor [26].

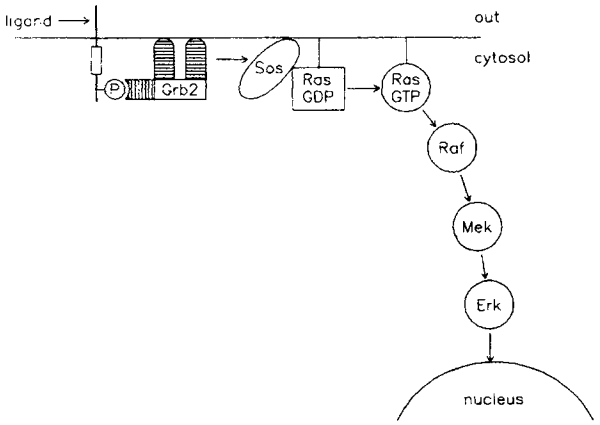


*Fig. 3.* Activation of Ras by protein tyrosine kinase growth factor receptors. Upon ligand activation, a growth factor receptor autophosphorylates tyrosine residues in its cytoplasmic domain. The adaptor protein Grb2 binds to a specific phosphorylated tyrosine in the receptor via its SH2 domain. The SH3 domains of Grb2 bind to a proline-rich, carboxyl-terminal region in Sos and target the complex to the plasma membrane, where Sos activates Ras by stimulating exchange of GDP for GTP. Ras activation leads to activation of nuclear transcription factors through the Raf, Mek and Erk protein kinases.

Downstream targets of Ras in mammalian cells are the mitogen-activated protein (MAP) kinases, Erk1 and 2 [27, 110]. MAP kinases were shown to be activated in response to growth factors, and are postulated to include nuclear transcription factors such as Fos and Jun as substrates [225, 96]. The MAP kinase cascade consists of MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK or MEK) and MAP kinase (Table 1). MAP kinases become activated only when both tyrosine and threonine residues are phosphorylated on a specific TEY sequence in the protein [2, 208]. This phosphorylation is catalyzed in mammals by Mek, a unique kinase with dual specificity for both tyrosine and serine/threonine [259, 56, 255]. In the Ras signalling pathway Mek is activated by phosphorylation on serine and threonine residues by Raf, a mammalian MAPKKK [61, 157]. The direct effector of Ras in this pathway remains to be identified, it is proposed to be Raf (Fig. 3). Besides Ras, protein kinase C was shown to activate Raf (see 'Phospholipid-dependent protein kinases').

A similar protein kinase cascade is activated during pheromone-induced mating in yeast cells (Table 1) [reviewed in 74]. In *S. cerevisiae* $\beta\gamma$ subunits of G-proteins participate in the activation of MAP kinases instead of Ras. The $\beta\gamma$ subunits activate, probably directly, the protein kinase Ste20 [163]. Ste20 mediates activation of a MAPKKK, Ste11, which in turn activates Ste7, the yeast homologue of mammalian Mek1. Activation of the *S. cerevisiae* MAP kinases Fus3 and Kss1 required tyrosine and threonine phosphorylation, which is achieved through Ste7 activity [75]. In the fission yeast *S. pombe* the pathway is activated by the Gpa1 $\alpha$ subunit, in combination

*Table 1.* Components of the MAP kinase cascade. The MAP kinase cascade is conserved from yeasts to vertebrates (see text for abbreviations).

| | Mammals | *S. cerevisiae* | *S. pombe* |
|---|---|---|---|
| Activator | Ras/PKC | $G\beta\gamma$ + Ste20 | $G\alpha$ + Ras |
| MAPKKK | Raf | Stell | Byr2 |
| MAPKK | Mek | Ste7 | Byr1 |
| MAPK | Erk1, 2 | Fus3, Kss1 | Spk1 |

with a Ras homologue, instead of $\beta\gamma$ subunits [211]. The protein kinases Byr2, Byr1 and Spk1 of *S. pombe* are structurally related to Ste11, Ste7 and Fus3/Kss1, respectively (Table 1). A mammalian homologue of Ste11/Byr2 called Mekk has been identified, which is able to catalyse the phosphorylation of Mek [160]. Therefore it is possible that heterotrimeric G-proteins also stimulate the MAP kinase pathway in mammals.

*Dictyostelium* contains at least two *Ras* genes encoding proteins homologous to mammalian Ras, expressed at different stages of development [237, 240]. *DdrasG* is expressed during growth and early development, *Ddras* during multicellular development. *Dictyostelium* cells overexpressing a mutant Ddras protein, Ddras-Thr$^{12}$, show an abnormal development, forming multiple tips during culmination [238]. The phospholipid turnover in these cell is increased which is due to increased phosphatidylinositol kinase activity, leading to increased levels of inostiol 1,4,5-trisphosphate in a compartment with a high metabolic turnover [304].

*S. cerevisiae* contains two closely related *RAS* genes [57, 231]. Although neither are essential genes by themselves, deletion of both genes is lethal, indicating that some Ras function is required for cell growth [133, 290]. The activity of Ras is regulated by yeast proteins encoded by CDC25 [35, 29], a GDP/GTP exchange protein homologous in its carboxyl-terminal domain to Sos, and *IRA1* AND *IRA2*, encoding GTPase-activating proteins [286]. Ras in *S. cerevisiae* was shown to activate adenylyl cyclase [297]. *S. pombe* has a single *ras* gene which encodes a protein similar in size to the mammalian Ras proteins, and does not contain the large inserts found in the C-terminal regions of *S. cerevisiae* Ras proteins [90].

## Effectors catalysing the formation of second messengers

Upon stimulation of cell surface receptors, the activity of second messenger-generating enzymes, adenylyl cyclase and PLC, is regulated. Adenylyl cyclase catalyses the formation of cAMP, a ubiquitous intracellular second messenger in many cell types. cAMP plays an important role in signal transduction through its activation of cAMP-dependent protein kinase and regulation of ion channel functions [3]. It is firmly established that many hormones and neurotransmitters stimulate the hydrolysis of PtdIns(4,5)$P_2$ to form the second messenger Ins(1,4,5)$P_3$ and diacylglycerol by activating phosphoinositide-specificic phospholipase C enzymes [19]. Diacylglycerol is also generated through hydrolysis of other phospholipids, mainly phosphatidylcholine (PC) [173]. Hormone-induced breakdown of PC can be achieved through activation of a variety of phospholipases, namely PC-specific PLC, phospholipase D (PLD) and phospholipase $A_2$ ($PLA_2$) [reviewed in 52]. Activation of cytosolic $PLA_2$ is involved in generation of arachidonic acid which is further metabolized to eicosanoids; its role in intracellular signalling is still unclear. PC hydrolysis by PLC yields diacylglycerol, while hydrolysis by PLD yields phosphatidic acid (PA) which can be converted to diacylglycerol by PA phosphohydrolase. Activation of phospholipase-induced PC breakdown can be achieved by different mechanisms: by G-proteins, by protein kinase C (PKC), by $Ca^{2+}$, and by growth factor receptors [reviewed in 77]. No sequences for PLD enzymes have been reported yet. A sequence for cytosolic $PLA_2$ from mammalian cells reveals a calcium regulatory domain present in some isoforms of PKC (see 'Phospholipid-dependent protein kinases') [50].

### *Adenylyl cyclase*

In mammalian cells six distinct membrane bound adenylyl cyclase enzymes have been identified and their genes cloned [154, 80, 11, 92, 135]. All share considerable sequence homology and based on hydropathy plots a similar topology has been predicted. The proposed structure of the mammalian membrane-bound forms consists of twelve transmembrane-spanning regions, divided in two domains of six spans each (Fig. 4A; [154]). It

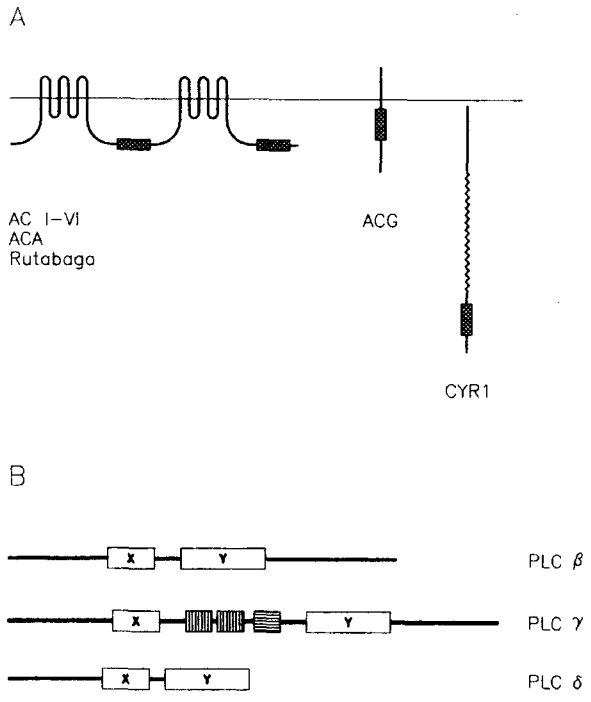


*Fig. 4.* Effector enzymes adenylyl cyclase and phospholipase C. Conserved regions: *hatched box*, adenylyl cyclase catalytic domain; *zig zag line*, repeated domain similar to domain in human glycoproteins; *X* and *Y box*, proposed catalytic domains in PLC; *vertically striped box*, SH2 domain; *horizontally striped box*, SH3 domain. A. Mammalian adenylyl cyclase types I to VI (AC I–VI), *Dictyostelium* adenylyl cyclase expressed during aggregation (ACA) and *Drosophila* Rutabaga contain two domains with six hydrophobic stretches each, which are proposed to traverse the plasma membrane. *Dictyostelium* adenylyl cyclase expressed during germination (ACG) contains a single membrane-spanning domain. Yeast adenylyl cyclase (CYR1) does not contain potential membrane-spanning domains. B. Phosphoinositide-specific phospholipase C enzymes PLC $\beta$, $\gamma$ and $\delta$ contain two conserved domains, X and Y. In PLC $\gamma$ regulatory SH domains are located between domains X and Y.

resembles the structure of various plasma membrane channels and transporters, although no evidence for channel activity has been found in adenylyl cyclase. The catalytic core consists of two large cytoplasmic domains; one located between the two membrane-spanning clusters, the other at the carboxyl end of the enzym. These domains within the protein sequence are similar to one another, and to the catalytic domains of guanylyl cyclases. The mammalian adenylyl cyclase subtypes are all activated by $G_s\alpha$ subunits, but differ in their response to regulation by $Ca^{2+}$/calmodulin and $\beta\gamma$ subunits of heterotrimeric G proteins [reviewed in 48]. Type I and III adenylyl cyclase are stimulated by $Ca^{2+}$/calmodulin. Type I can be stimulated directly by $Ca^{2+}$ and calmodulin, or indirectly by stimulation of muscarinic receptors that mediate mobilization of intracellular $Ca^{2+}$. Type III, the adenylyl cyclase found in olfactory neurons [11], requires activation of the enzyme by G-protein coupled receptors before stimulation by the $Ca^{2+}$/calmodulin complex can occur [47]. The $\alpha_s$ and $\alpha_i$ subunits of G proteins were initially classified as stimulating or inhibiting adenylyl cyclase, respectively. Now it is clear that $\beta\gamma$ subunits can differentially regulate adenylyl cyclase activity [289]. In the presence of activated $G_{s\alpha}$, addition of $\beta\gamma$ subunits inhibited type I adenylyl cyclase activity, enhanced stimulation of type II and IV adenylyl cyclase activity, but did not affect the activity other types [288]. *In vivo* the $\beta\gamma$ subunits of $G_i$ proteins were proposed to mediate these effects [79]. Thus, this appears to represent a mechanism for cross-talk between signalling pathways.

The *Drosophila* adenylyl cyclase *rutabaga* gene is most similar to the mammalian type I enzyme in sequence and $Ca^{2+}$/calmodulin responsiveness [169]. In flies this adenylyl cyclase is involved in learning and memory processes; it is expressed in the mushroom bodies of the fly brain. Expression of *rutabaga* in mammalian cells shows its activity is regulated by endogenous G proteins and calmodulin [169].

In *Dictyostelium* the adenylyl cyclase expressed during aggregation, ACA, is responsible for receptor and G protein-regulated adenylyl cyclase activity [229]. The proposed structure resembles that of mammalian membrane-bound adenylyl cyclases. Cells with a disrupted *aca* gene fail to aggregate, but are not affected in growth or chemotaxis. The proposed topology of a second adenylyl cyclase, ACG, expressed during germination, consists of a large extracellular domain connected to a single transmembrane-spanning domain and one cytoplasmic catalytic domain at the carboxyl terminus (see Fig. 4A). Although this

structure resembles that of membrane-bound guanylyl cyclases, ACG was shown to contain adenylyl and not guanylyl cyclase activity [229]. ACG expressed in cells with a disrupted *aca* gene secrete cAMP constitutively and cAMP production is not regulated by surface receptors.

*S. cerevisiae* adenylyl cyclase, encoded by the *CYR1* gene, is required for cell growth [186]. It resembles the above discussed adenylyl cyclases only in its carboxyl terminal catalytic domain, and lacks potential transmembrane-spanning domains (see Fig. 4A; [134]). CYR1 contains multiple copies of a 23 amino acid repeating unit similar to a repeat found in human glycoproteins. Budding yeast adenylyl cyclase is not regulated by heterotrimeric G proteins, but stimulated by Ras proteins. *S. pombe* adenylyl cyclase is similar to *CYR1* of the budding yeast [317]. Its structure predicts the 23-amino acid repeats and a carboxyl-terminal adenylyl cyclase domain, but *S. pombe* adenylyl cyclase misses an amino-terminal segment present in *S. cerevisiae* adenylyl cyclase. The Gpa2 $\alpha$ subunit of a heterotrimeric G-protein regulates cAMP production in *S. pombe* as cells defective Gpa2 fail to produce cAMP in response to glucose stimulation [123].

*Phosphoinositide-specific phospholipase C*

The enzyme phospholipase C (PLC) generates two second messengers upon phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) hydrolysis: membrane-bound diacylglycerol and water-soluble inositol 1,4,5-trisphosphate ($InsP_3$). Through its activation of PKC, diacylglycerol is involved in regulating protein phosphorylation, while $InsP_3$ regulates intracellular $Ca^{2+}$ [209, 273, 19]. Calcium, contained within intracellular stores, is released to the cytosol when $InsP_3$ binds to its receptor (see below).

The mammalian phospholipase C enzyme family specific for hydrolysing polyphosphatidylinositols is divided into three classes based upon sequence conservation, each class containing isoforms [see references in 53, 239]. All groups contain two conserved amino acid domains, named X and Y, which are thought to form the catalytic site (Fig. 4B). PLC-$\beta$ isoforms contain a large carboxyl-terminal domain after the conserved Y region which is involved in PLC activation by G-protein $\alpha$ subunits [219, 315]. PLC-$\gamma$ isoforms contain the *src* homology domains SH2 and SH3 located in between the X and Y domains [279, 270]. This region is not essential for $PtdInsP_2$ hydrolysing activity [73], but the SH2 domains target PLC to tyrosine phosphorylated sequences in growth factor receptors, as described previously. The SH3 domain is involved in targeting the enzyme to cytoskeletal components [13]. PLC-$\delta$ does not contain a large carboxyl-terminal sequence, nor the SH domains. The amino-terminal domain of PLC-$\delta$ was shown to form part of a $PtdInsP_2$-binding site proposed to bind the enzyme to the membrane surface during hydrolysis [236, 49]. On the protein level the existence of a PLC-$\alpha$ isoform has been implicated. However, a sequence claimed to encode PLC-$\alpha$ [15], with no sequence similarity to known PLC isoforms, was in fact shown to be protein disulphide isomerase [269]. The PLC-$\alpha$ proteins, with a low molecular weight, could be proteolytic fragments derived from other PLCs.

The activity of the three PLC classes are regulated differently, although all depend on the presence of $Ca^{2+}$. The PLC-$\gamma$ family is activated by tyrosine phosphorylation, as discussed above. In a reconstitution system with purified proteins, m1 muscarinic receptor and G-proteins of the $G_q$ family were sufficient to stimulate PLC-$\beta_1$ [17]. Furthermore, with this system it was also shown that addition of PLC-$\beta_1$ stimulated hydrolysis of $G_q$-bound GTP. Thus, this PLC isoform serves as a GAP for the G-protein that mediates its activation [18]. Another $\alpha$ subunit of the $G_q$ family, $G_{\alpha 16}$, was the most efficient in activating PLC-$\beta_2$ [164]. G-protein $\beta\gamma$ subunits increased PLC-$\beta_1$, -$\beta_2$ and -$\beta_3$ activity two-, four- and eight-fold, respectively [39, 220]. In PLC-$\beta_2$ regions for $\beta\gamma$ interaction were shown to be located in the amino-terminal region of the PLC protein, and are thus separate from G$\alpha$-activating regions [316]. PLC-$\delta_1$ activity was increased two-fold after stimulation with $\beta\gamma$ subunits [220]. In

permeabilized HL60 cells, G-protein-stimulated PLC activity depended on the presence of a cytosolic compound identified as phosphatidylinositol transfer protein, involved in transporting the lipid [293].

In Swiss 3T3 cells, diacylglycerol levels in the nucleus increased and $PtdInsP_2$ levels decreased upon stimulation with insulin-like growth factor 1 [67]. PLC activity in the nucleus has been reported, as have other enzymes involved in the inositide cycle [68, 69, 185]. However, immunologically only the PLC-$\beta$, isoform could be detected in the nucleus. Therefore, the regulation of PLC in the nucleus remains unclear, as PLC-$\beta$ has been reported to be regulated by G-proteins, not by growth factor receptors.

Activation of PLC activity has proven to be not essential for induction of DNA synthesis in mammalian cell lines [114, 196]. The role of PLC in several invertebrates and microorganisms has well been studied. In *Drosophila* PLC is implicated to be involved in phototransduction [23, 249]. No receptor potential A (*norpA*) mutants fail to respond to light stimulation. These mutants contain no PLC activity in the head region. The *norpA* gene encodes a protein with similarity to bovine retinal PLC-$\beta$ [82]. A second PLC in *Drosophila*, also of the $\beta$ type, is expressed in the central nervous system [262]. *Xenopus* contains a PLC protein with 64% identity to the mammalian PLC-$\beta_3$ subtype [181]. Injection of oocytes with PLC antisense oligonucleotides significantly reduced receptor stimulated $Cl^-$ currents. The microorganisms *S. cerevisiae* and *Dictyostelium* contain a PLC-$\delta$ like sequence [318, 71]. Deletion of PLC in yeast resulted in viable cells which however were retarded in cell growth when growing conditions were not optimal [88, 224]. In *Dictyostelium* deletion of the DdPLC gene resulted in cells containing no measurable PLC activity [72]. Surprisingly, the cells were able to grow and develop. Analysis of the $InsP_3$ concentration revealed that levels in cells with a deleted *plc* gene were only slightly lower (20%) than in wild-type cells. As there are no indications for other PLCs, alternative pathways for synthesizing $InsP_3$ have to be considered.

**Proteins activated by second messengers**

Cytosolic protein kinases are divided into different families, based on their primary structure and functional studies. We will discuss two types regulated by second messengers: protein kinase C and cyclic nucleotide-dependent protein kinases. The number of identified genes belonging to the protein kinase family has risen dramatically during the past years [120]. Sequence alignments have identified a conserved catalytic core of about 260 residues shared by all protein kinases [107]. For many, only the deduced protein sequences have been reported, with no known protein function yet. The structure of the catalytic domain is derived from the crystal structure of cAMP-dependent protein kinase [145]. It consists of a bilobed structure with an ATP-binding site on the smaller lobe, and a substrate-binding site on the larger lobe. Transfer of $\gamma$-phosphate of ATP from the protein kinase to a serine, threonine or tyrosine residue of the substrate is considered to alter the conformation, and thus the activity, of the substrate protein.

Several ion channels are activated by second messengers. The cGMP-gated cation channel of rod photoreceptor cells and the cAMP-gated channel from olfactory neurons contain a carboxyl-terminal cyclic-nucleotide-binding region, similar to the tandem repeat in cGMP-dependent protein kinase and cAMP-dependent protein kinase, respectively [136, 65]. The ryanodine receptor and the $InsP_3$ receptor are intracellular calcium channels regulated by $Ca^{2+}$ and $InsP_3$. The $InsP_3$ receptor is conserved as genes encoding similar proteins have been found in mammals, *Xenopus* and *Drosophila*.

*Cyclic nucleotide-dependent protein kinases*

The intracellular second messengers cGMP and cAMP regulate physiological functions by activation of specific family of serine/threonine protein kinases, the cyclic nucleotide-dependent protein kinases. The cAMP-dependent protein kinase (PKA) plays a role in the expression of a large

number of genes and has been shown to regulate the function of ion channels and the activity of metabolic enzymes. The cGMP-dependent protein kinase (PKG) is involved in platelet aggregation and the relaxation of hormonally contracted smooth muscle. Through phosphorylation of calcium channels, PKG regulates cytosolic calcium levels, although the mechanism by which PKG induces a reduction in the calcium concentration is still unresolved [189]. Although they bind different cyclic nucleotides, PKA and PKG show considerable similarity in amino acid sequence, especially in their catalytic domains (70% homology), and their cyclic nucleotide-binding domains. Each of the cyclic nucleotide-binding domains of PKA and PKG shows similarity in amino acid sequence (20%) to the cAMP-binding domain of the catabolite gene activator protein (CAP) from *E. coli* [187].

In mammalian cells, inactive PKA is a tetramer, consisting of two regulatory (R) subunits and two catalytic (C) subunits ($R_2C_2$). In the absence of cAMP, the R subunit functions to inhibit the activity of the C subunit. The C subunit is proposed to recognize the R subunit in a manner comparable to the recognition of protein substrates, each R subunit binding to one C subunit at its catalytic site. Upon cAMP binding, the holoenzyme dissociates into $R_2$ dimers and active monomeric C subunits. Three different mammalian genes for the C subunit of PKA have been identified, $C_\alpha$, $C_\beta$ and $C_\gamma$, which are 80% similar to each other [14]. The conserved protein kinase catalytic domain comprises most of the PKA C subunit, from amino acid residue 40 to 300 (see Fig. 5A; total length ca. 350 amino acids). Two different types of mammalian R subunits, $R_I$ and $R_{II}$, have been found [296, 284]. The R subunit is composed of an amino-terminal dimerization domain, a pseudosubstrate domain which is able to bind the protein kinase inhibitor PKI [146] and is involved in interaction with the C subunit, and at the carboxy terminus two tandem cAMP-binding domains (Fig. 5A).

Three *S. cerevisiae* catalytic subunit genes with predicted structural and amino acid sequence similarity to mammalian PKA catalytic subunits have been identified, *TPK1*, *TPK2* and *TPK3* [299]. Gene disruption experiments have demonstrated that the proteins have overlapping functions, as the presence of at least one C subunit is sufficient for normal growth. Haploid spores lacking all three genes are able to germinate, but grow extremely slowly [299]. *S. cerevisiae* contains one gene for a PKA regulatory subunit encoded by the *BCY1* gene homologous to mammalian isoforms [298, 37]. *BCY1* expressed in *E. coli* was shown to bind 2 mol of cAMP per mole of R monomer [128].

In contrast to mammalian and yeast PKAs, the *Dictyostelium* holoenzyme is a dimer (RC) [58]. The R subunit of *dictyostelium* PKA does not contain the amino-terminal domain suggested to be required for dimerization (see Fig. 5A; [199]). The pseudosubstrate domain is conserved, and *Dictyostelium* R subunits can form holoenzymes with mammalian C subunits [167]. Two potential cAMP-binding sites were found in the R subunit sequence, which was surprising since binding experiments indicated the presence of only one cAMP-binding site [64, 59]. The carboxyl-terminal cAMP-binding domain is predicted to bind cAMP with low affinity [199]. *Dictyostelium* contains one catalytic subunit gene *pkaC* [34, 183] which encodes a protein that is twice as large as mammalian PKA C subunits [6]. Cells in which the *pkaC* gene has been disrupted do not aggregate, while cells overexpressing PKA C are accelerated in their development [183, 5].

Comparison of the predicted structures for PKA and PKG enzymes suggest that early in eukaryotic evolution the fusion of separate genes for a regulatory and a catalytic domain resulted in the formation of a chimaeric PKG gene [285]. Purified bovine PKG was shown to exist as a homodimer, consisting of two identical subunits [172, 313]. Amino acid sequence comparison of PKG genes from mammalian cells and *Drosophila* predict a polypeptide containing an N-terminal dimerization domain, two cGMP-binding domains and the catalytic domain (see Fig. 5A; [132, 116]). Dimerization probably occurs via a hydrophobic leucine/isoleucine-zipper motif, which is not found in the dimerization domain of

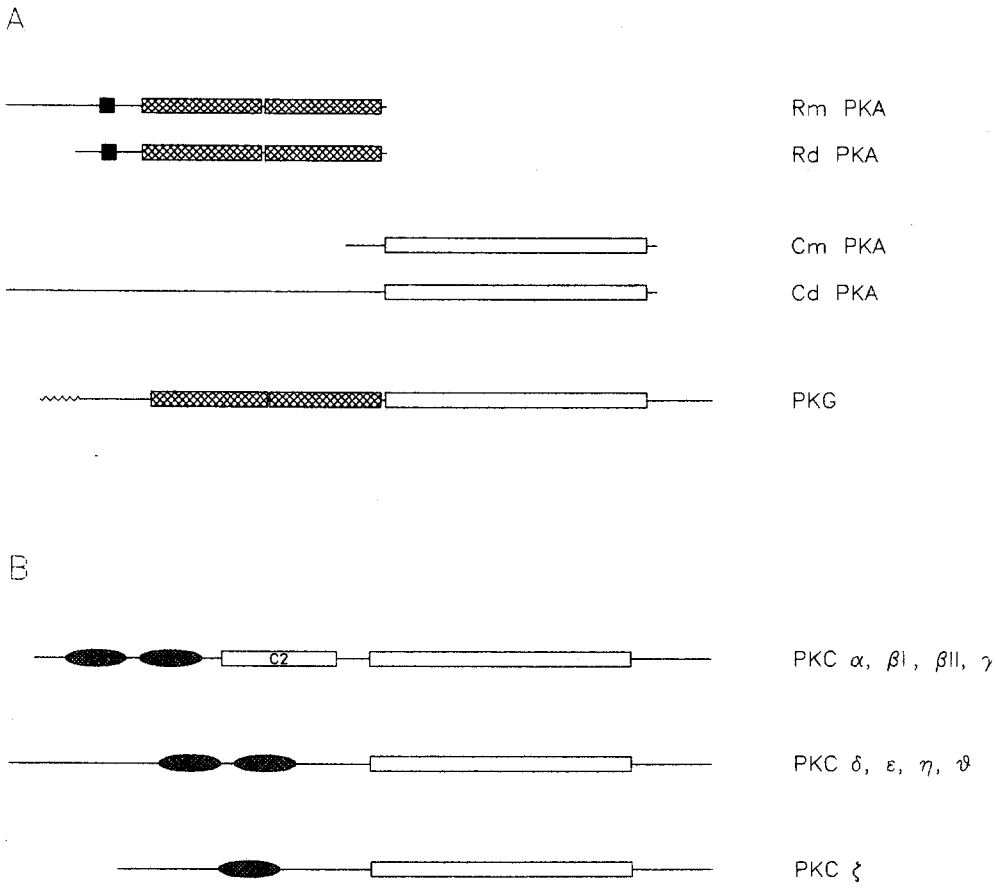


*Fig. 5.* Comparison of structures of cyclic nucleotide activated protein kinases and protein kinase C. Conserved regions: *open box*, protein kinase catalytic domain; *closed box*, pseudosubstrate domain; *hatched box*, cyclic nucleotide binding domain; *zig zag line*, leucine/ isoleucine-zipper motif; *filled oval*, zink-finger-like domain; *C2*, $Ca^{2+}$-activated domain. A. The regulatory subunit of *Dictyostelium* (Rd) cAMP-dependent protein kinase (PKA) misses an amino-terminal dimerization domain present in mammalian R (Rm) subunits. Compared to the mammalian catalytic (Cm) subunit of PKA, the catalytic subunit of *Dictyostelium* (Cd) contains an amino-terminal extension. The regulatory subunits of PKA contain a pseudosubstrate domain for binding to the C subunit. In cGMP-dependent protein kinase (PKG) the cyclic nucleotide-binding and catalytic domains are located in the same molecule. B. Protein kinase C (PKC) isoforms α, βI, βII and γ contain a calcium regulatory sequence, missing in the other isoforms. PKC ζ has only one zinc-finger-like domain.

mammalian PKAs [10]. Mammalian cells contain two distinct classes of PKG genes that encode different PKG proteins, showing homology in the catalytic and cGMP-binding domains, but no homology in the amino terminus [301].

PKG enzymes purified from the lower eukaryotes *Paramecium*, *Tetrahymena* and *Dictyostelium* are found in the monomeric form [reviewed in 116]. No sequences for these PKG enzymes have been reported yet. The presence of monomeric cGMP and cAMP kinases in some unicellular organisms suggests that dimerization of the kinases occurred later in evolution.

### *Phospholipid-dependent protein kinases*

Protein kinase C (PKC) was originally characterized as a phospholipid-diacylglycerol-$Ca^{2+}$-dependent protein kinase [reviewed in 209]. Its assumed role in tumorigenesis arrises through the fact that PKC proteins serve as receptors for

phorbol ester tumour promoters. Tumour-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) directly activate PKC *in vitro* and are used as analogues of the physiological PKC activator, diacylglycerol. Diacylglycerol produced by hydrolysis of phospholipids by phosphoinositide-specific PLC is involved in transient activation of PKC [173]. However, sustained activation of PKC is necessary for cell proliferation and differentiation [8]. Diacylglycerol produced from phosphatidylcholine breakdown mediated by other signal-activated phospholipases, notably phospholipase D, is proposed to be involved in the long-term activation of PKC [77].

Molecular cloning and biochemical analysis have revealed the presence of twelve PKC isoforms in mammalian tissues at present [see references in 209, 9, 60]. All require the presence of phospholipid for activation, but differ in their need for calcium and diacylglycerol. The conventional isoforms, PKC-$\alpha$, -$\beta$I, -$\beta$II and -$\gamma$, are activated by $Ca^{2+}$ and DAG. The amino-terminal half of PKC proteins contain the regulatory domain consisting of a tandem repeat of a zinc-finger-like sequence and, in the contential PKC isoforms, a second conserved domain (C2) (see Fig. 5B). The regulatory domain also contains a strech of basic amino acids which appears to be an inhibitory pseudosubstrate domain [17]. The carboxyl-terminal region is the catalytic domain, consisting of the ATP-binding site and sequences similar to other protein kinases. The novel class of PKCs, PKC-$\delta$, -$\varepsilon$, -$\eta$ and -$\theta$, contain the cysteine-rich zinc-finger-like sequences, but not the C2 domain (Fig. 5B). Novel PKC isoforms are activated by DAG and the tumour-promoting phorbol esters, but are insensitive to $Ca^{2+}$, proposing a role for the C2 domain in $Ca^{2+}$ activation [148, 212]. Regulation of the atypical PKC $\zeta$ has not yet been fully established, but this isoform is insensitive to $Ca^{2+}$, DAG and phorbol esters [175, 9]. The amino-terminal domain of PKC-$\zeta$ contains only a single zinc-finger-like sequence [25]. PKC-$\zeta$ has been shown to be activated by the phospholipids PS and phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) [203]. Three new PKC isoforms have been cloned, but their sequences have not been reported yet.

Phorbol ester binding to PKC appears to be directly involved in down-regulating PKC activity as conventional and novel PKCs were degraded after prolonged treatment with phorbol ester, while PKC-$\zeta$ protein levels remained unchanged [308, 174]. The presence of multiple PKC isotypes has led to the suggestion that they perform specific functions within the cell. PKC-$\gamma$ and -$\varepsilon$ expression is restricted to the central nervous tissues, PKC-$\eta$ to the lung and skin, while other PKCs are expressed in many different cell types.

Phorbol ester stimulation of cells results in the increased transcription of many genes and phosphorylation of a variety of nuclear proteins. PKC-$\alpha$ was shown to directly phosphorylate and activate the Raf kinase [149]. Raf-1 is involved in activating nuclear transcription factors through MEK and MAP kinase, as discussed previously. Thus PKC provides an alternative route besides *ras* to activate oncogene class transcription factors. Upon stimulation, translocation of specific PKC isozymes to the nucleus was demonstrated in different cell lines; translocation was shown to coincide with an increase in nuclear PKC activity [67, 115, 162]. Translocation of PKC to the nucleus is suggested to be a second, direct way for PKC involvement in regulation of nuclear proteins.

In the nematode *Caenorhabditis elegans* strains were isolated which were resistant to the phorbol ester tumour promotor TPA. These strains were mutated in the *tpa-1* gene. The predicted *tpa-1* protein contains the kinase catalytic domain and the two zinc-finger-like structures, but lacks the C2 domain in its amino-terminal region [282]. *Drosophila* contains three PKC genes [241, 245]. Two are transcribed predominantly in brain tissue, one encoding a conventional, the other a novel PKC-like protein. The third PKC gene is expressed specifically in photoreceptor cells [245]. Interestingly, it contains the potential calcium regulatory site and is proposed to function in *Drosophila* phototransduction adaptation.

A PKC-like enzyme activity was demonstrated

to be present in the micro-organism *Dictyostelium* [180]. The myosin heavy-chain kinase of *Dictyostelium* is a member of the PKC family [235]. In the regulatory domain it contains the basic pseudosubstrate domain and the two cystein-rich sequences. In the C2 region it is only 10% similar to the putative $Ca^{2+}$ regulatory domain. A gene, *PKC1*, encoding a PKC enzyme closely related to mammalian conventional PKC has been isolated from *S. cerevisiae*. PKC1 is implicated to play a role in the cell division cycle [169]. Deletion of *PKC1* resulted in recessive lethality. The nonviable spores do germinate but are blocked in further growth.

*Inositol 1,4,5-trisphosphate receptors*

As mentioned above, $InsP_3$ produced by phospholipase C-mediated hydrolysis of $PtdInsP_2$ is able to release calcium from intracellular stores. $InsP_3$ binds to specific receptors that are coupled to calcium channels located in the endoplasmic reticulum (see Fig. 6; [190, 242]). Reconstitution of purified $InsP_3$-binding protein into liposomes showed that calcium was conducted as a function of ligand binding, suggesting that the same molecule mediates both $InsP_3$ binding and the release of $Ca^{2+}$ [83, 182]. Cloning of the $InsP_3$ receptor from mammalian cerebellum revealed a primary structure containing several putative membrane-spanning domains and a striking homology with another intracellular calcium channel, the ryanodine receptor of the sarcoplasmic reticulum [91, 190, 283].

The $InsP_3$ receptor and the ryanodine receptor show the greatest stretches of amino acid identity in their carboxyl termini, which contain the putative membrane-spanning domains. Their tertiary structures are predicted to be similar as both receptor types are homotetrameric proteins, in which the carboxyl terminus of each receptor subunit is suggested to form a part of the calcium channel [95]. The $InsP_3$ receptor contains an amino-terminal cytoplasmic domain with an $InsP_3$-binding site (see Fig. 6; [191]). $InsP_3$ receptor sequences isolated from mammalian cells indicate the presence of distinct receptor subtypes [278]. Expression of the amino-terminal region of two different receptor types from rat brain demonstrated similar specificity for $InsP_3$, but different affinities [278]. Binding of $InsP_3$ results in a conformational change of the receptor, which is proposed to result in opening of the channel. The receptor is proposed to traverse the endoplasmic reticulum membrane an even number of times, although the precise number remains to be determined. The region between the ligand-binding domain and the calcium channel is proposed to be involved in regulation of the $InsP_3$ receptor. This region contains putative PKA phosphorylation sites. PKA-induced phosphorylation of the $InsP_3$ receptor did not influence $InsP_3$ binding to the receptor, but decreased $InsP_3$-induced calcium release [281]. Autophosphorylation could also play a role as the receptor was shown to function as a protein kinase, phosphorylating both exogenous substrates and the receptor itself [85]. ATP promotes $InsP_3$-induced calcium release, and ATP-binding sites are predicted in the regulatory domain of $InsP_3$ receptors [84, 182].

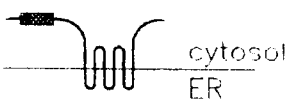


*Fig. 6.* Structure of the $InsP_3$ receptor. The $InsP_3$ receptor is an intracellular calcium channel responsible for mobilizing stored calcium. Four subunits combined form a functional ion channel. The exact number of membrane-spanning domains remains to be determined; here six are proposed as in a number of other ion channels. The amino-terminal $InsP_3$-binding domain (*hatched box*) and carboxyl terminus are located in the cytosol.

In *Drosophila* and *Xenopus* $InsP_3$ receptors homologous to those in mammals were identified. The *Drosophila* $InsP_3$ receptor shares 57% amino acid sequence identity with its mouse homologue [319]. The *Drosophila* $InsP_3$ receptor sequence however does not indicate it is a substrate for PKA. mRNA localization and $InsP_3$ binding to membrane preparations show that the receptor is expressed highly in the leg and thorax region and, interestingly, in the retina and antenna. As $InsP_3$ is thought to act as a second messenger in *Drosophila* vision, the $InsP_3$ receptor described here is

proposed to play a role in depolarization of photoreceptor cells through $InsP_3$-induced calcium release [319]. The *Xenopus* $InsP_3$ receptor shows 90% amino acid sequence identity with its mouse homologue. It contains ATP-binding sites and PKA phosphorylation sites in between the ligand-binding amino terminus and calcium channel domain in the carboxyl terminus [156]. *Xenopus* egg activation, measured by $InsP_3$-responsive cortical contraction, was inhibited in eggs microinjected with $InsP_3$ receptor antisense oligonucleotides. Immunocytochemical localization experiments suggest a role for the $InsP_3$ receptor in the formation and propagation of $Ca^{2+}$ waves during fertilization [156].

The $InsP_3$-induced calcium release measured in some studies indicates co-operative binding of $InsP_3$ to each of the four binding sites of the tetrameric receptor, while other studies indicate that binding of one $InsP_3$ molecule is sufficient to open the channel [reviewed in 291]. Calcium is not released gradually in response to increased $InsP_3$ concentration, but a fixed proportion is released (quantal release), with the remainder becoming accessible at higher $InsP_3$ concentrations [261]. Besides $InsP_3$, $Ca^{2+}$ itself also modulates channel opening [124]. Increased concentrations of cytosolic calcium first stimulate its own release, which is then followed by an inhibitory effect [192]. Thus calcium is capable of both positive and negative feedback regulation of the $InsP_3$ receptor, and may be involved in regulation of calcium oscillations and waves in the cell [reviewed in 20].

## Discussion and conclusions

Comparisons between higher and lower eukaryotes show that many processes involved in signal transduction have been evolutionary conserved. Pathways for activation of intracellular proteins through heterotrimeric G-proteins and Ras proteins are found in micro-organisms, invertebrates and vertebrates. Stimulation of tyrosine kinase pathways results in phosphorylation of proteins and the formation of active protein complexes; these reactions occur probably in all eukaryotes. Some proteins such as G-proteins, adenylyl cyclase and phospholipase C are remarkably well conserved in diverse organisms, whereas only the overall structure is conserved in other proteins, as is the case for the seven transmembrane segments in G-protein-linked surface receptors.

Although many proteins involved in transducing signals are present in virtually all eukaryotes, their connection in pathways may form specialized systems. Divergent pathways exist, as is the case for photoreceptor activation and activation of protein kinase cascades through Ras and heterotrimeric G-proteins. In mammalian rod cells, rhodopsin activates a specialized G protein, $G_t$, which transduces the signal to PDE in order to regulate cGMP levels. However, in *Drosophila*, rhodopsin appears to activate PLC. The signal is most likely transduced by $G_q$, which also mediates activation of PLC in mammalian systems. A second example of divergence is Ras. Ras proteins in mammalian cells are stimulated via receptor tyrosine kinases and activate a MAP kinase cascade. A Ras protein together with the $\alpha$ subunit of a heterotrimeric G-protein is involved in activating the MAP kinase pathway in *S. pombe*. In the yeast *S. cerevisiae* Ras has a completely different role as an activator of adenylyl cyclase; the MAP kinase cascade is activated by G-protein $\beta\gamma$ subunits.

Cellular effects are often the result of the interaction of multiple signal transduction pathways (cross-talk). G-proteins are able to regulate some forms of adenylyl cyclase and phospholipase C through both $\alpha$ and $\beta\gamma$ subunits. These subunits can be derived from different G-proteins, activated through independent surface receptors. Thus the effect of one ligand can be either enhanced or down regulated by the effect of another. Furthermore, PLC can be activated by two independent pathways, one coupled to tyrosine kinase-linked receptors, the other to heterotrimeric G-proteins. On the other hand, the MAP kinase activation pathway serves as a convergence point at which multiple signalling pathways meet. One of the proteins involved, Mek, can be activated by different protein kinases, Raf and

Mek kinase. These in turn are activated by multiple proteins, including Ras, PKC and possibly heterotrimeric G-proteins.

Feedback regulation of proteins plays an important role in controlling signal transduction and signal termination. G-protein-linked receptors demonstrate diminished responsiveness to their ligand by an uncoupling process during which the receptor is phosphorylated. This can be achieved by second-messenger-activated kinases such as PKA and PKC, or by the recently characterized G-protein coupled receptor kinases (GRKs) [126, 165]. $\beta$-Adrenergic receptor kinase activity is enhanced by $\beta\gamma$ subunits [104]. Hereby G-protein $\beta\gamma$ subunits negatively regulate the receptors' ability to activate G-proteins. Negative feedback regulation by phosphorylation of receptors by PKA appears to be a major mechanism for desensitization of adenylyl cyclase.

The mechanisms of signal transduction in plants are just beginning to be elucidated. It will be interesting to learn which pathways that are conserved between mammals and eukaryotic micro-organisms, are also present in plants. Knowledge of unique plant signal transduction cascades will be especially informative to discriminate between the general principles of sensory transduction and those used to provide specialized cells.

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